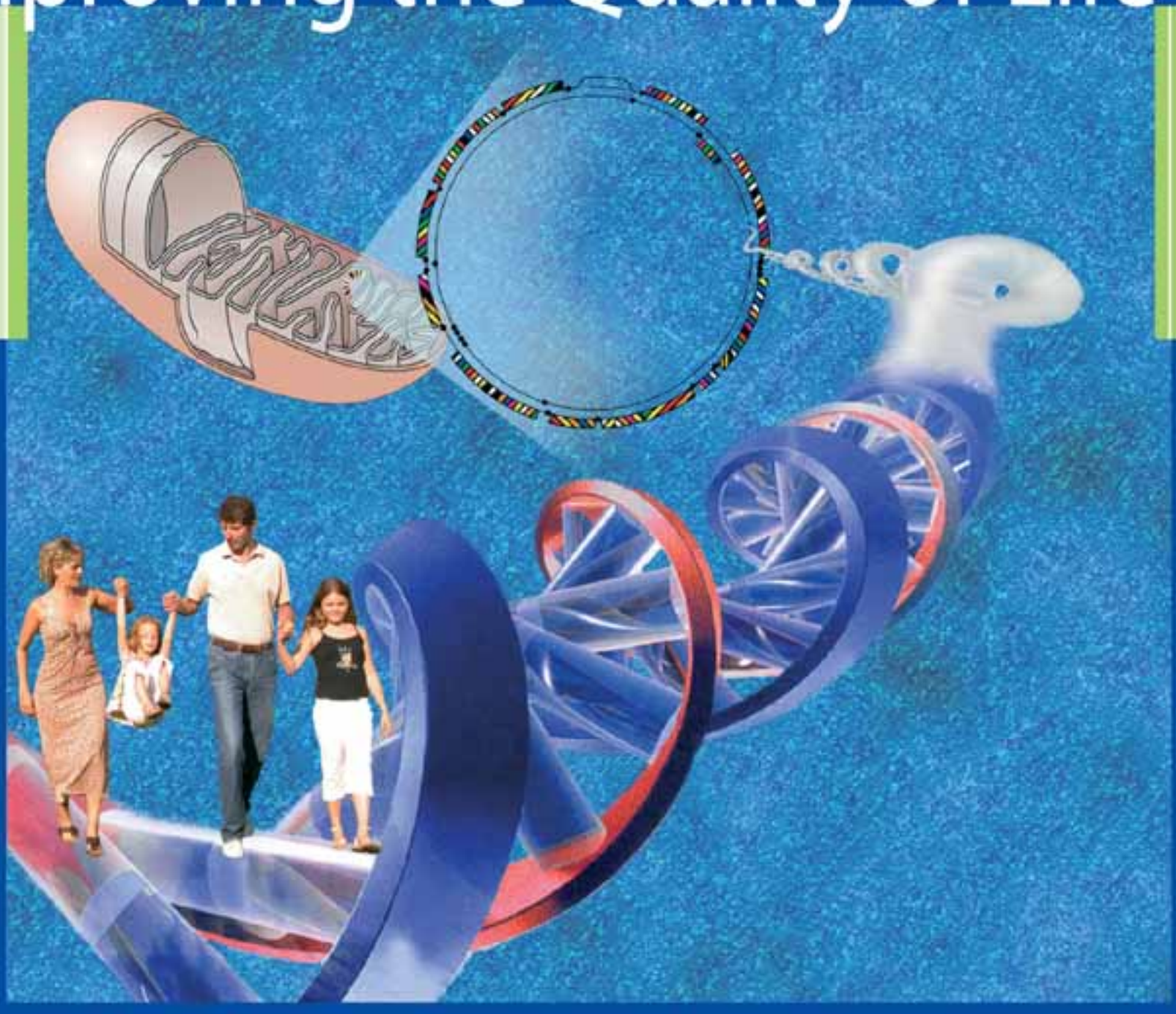
Vilberto Stocchi • Pierpaolo De Feo
David A. Hood *(Eds)*

Role of Physical Exercise in Preventing Disease and Improving the Quality of Life



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Preface

During the last few decades a significant body of scientific evidence has shown the importance of physical activity in preventing modern-day chronic diseases such as obesity, metabolic syndrome, type II diabetes mellitus, cardiovascular diseases, hypertension, tumors, osteoporosis, etc. In particular, regular aerobic exercise plays an important role in modifying human skeletal muscle, causing a significant number of molecular modifications. At present, we still do not have a complete understanding of the many metabolic changes that affect human muscle tissue and other tissues in people who exercise regularly.

It is important to consider that a healthy individual's muscle tissue represents more than 40% of his or her total body mass, and represents about 90% of the insulin-sensitive tissues in lean individuals. Skeletal muscle shows significant plasticity even in the elderly, which must be taken into account when studying the molecular modifications induced by physical exercise because of the significant mass of muscle tissue that may be involved and the fact that, even in the elderly, the skeletal muscle cell modifies its structure and function in response to physical exercise.

Though we do not fully understand the metabolic changes brought about by physical activity, we do know that aerobic exercise is able to promote the expression of a significant number of nuclear and mitochondrial genes responsible for mitochondrial biogenesis, an important metabolic process which increases aerobic capacity. This new steady-state condition of the skeletal muscle cell contributes to an individual's overall health, playing a role in the prevention of modern-day chronic diseases and improving the quality of life.

During the next 20–30 years the mean life expectancy will continue to increase, and it is likely that we will see a parallel increase in modern-day chronic diseases resulting in a growing, unsustainable economic burden on the healthcare systems of both developed, and developing countries.

Hence, it is imperative that governments promote research and prevention programs to effect changes in the lifestyle of the general population, from children to the elderly. This will help to effectively counterbalance this large-scale and alarming trend which represents one of the biggest challenges facing all countries in the new millennium.

July, 2007

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List of Abbreviations

8OH-dG	8-hydroxy 2-deoxyguanosine
AD	Alzheimer's disease
ADA	American Diabetes Association
adPEO	autosomal dominant progressive external ophthalmoplegia
AICAR	5-aminoimidazole-4-carboxamide riboside
AIF	apoptosis inducing factor
AMPK	adenosine monophosphate protein kinase
ANT	adenine nucleotide translocase
ANT1	muscle-heart specific isoform of mitochondrial adeninenucleotide translocator
Apaf-1	apoptosis-protease activating factor 1
Ape/Ref-1	apurinic-apyrimidinic endonuclease/redox effector factor
ATF2	activating transcription factor 2
ATP	adenosine triphosphate
BAT	brown adipose tissue
BIA	bioimpedance analysis methods
BM	Bethlem myopathy
BMI	body mass index
BP	bodily pain
CAD	coronary artery disease
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
CARD	caspase-recruitment domain
Cat	catalase
CBP	CREB-binding protein
CI	confidence interval
CICR	Ca^{2+}-induced Ca^{2+} release
COX	cytochrome c oxidase activity
CR	caloric restriction
CRE	cAMP response element
CREB	cAMP response element binding protein
CS	citrate synthase

CSA	cyclosporin A
Cyp D	cyclophilin D
CyP-D	cyclophilin D
DA	dopaminergic
DIABLO	direct inhibitor of apoptosis-binding protein with a low isoelectric point
DISC	death inducing signaling complex
DLW	doubly labeled water
Drp1	dynamin-related protein 1
ECM	extracellular matrix
EE	energy expenditure
EndoG	endonuclease G
ERRα	estrogen-related receptor α
GH	general health
GLUT 4	glucose transporter 4
GPx	glutathione peroxidase
GRACILE	growth retardation, aminoaciduria, cholestasis, iron overload lactic acidosis, and early death
GST	glutathione transferase
HCR	high capacity runners
HDAC	histone deacetylase
HR	heptad repeat domain
HRQL	health-related quality of life
HtrA2	high temperature requirement protein A2
IAP	inhibitor of apoptosis protein
IMF	intermyofibrillar
IMS	intermembrane space
KSS	Kearns-Sayre syndrome
LCR	low capacity runners
LENS	European Laboratory of Non Linear Spectroscopy
LHON	Leber's hereditary optic neuropathy
M	myosin
MAC	mitochondrial apoptosis-induced channel
MEF2	myocyte enhancer factor 2
MELAS	mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes
MERRF	myoclonic epilepsy with ragged-red fibers
MET	metabolic equivalent
mETC	electron transport chain
Mfn	mitofusin
MH	mental health
MHC	myosin heavy chain
MLC	myosin light chain

MNGIE	mitochondrial neurogastrointestinal encephalomyopathy syndrome
MOMP	mitochondrial outer membrane permeabilization
mPTP	mitochondrial permeability transition pore
mtDNA	mitochondrial DNA
NARP	neurogenic muscle weakness, ataxia and retinitis
NCX	sodium (Na^+) - Ca^{2+} exchanger
nDNA	nuclear DNA
NRF-1	nuclear respiratory factor 1
NRF-1	nuclear respiratory factor 1
NRF-2	nuclear respiratory factor 2
OT	optical trap
OXPHOS	oxidative phosphorylation
PAD	physical activity duration
PARL	presenilin-associated rhomboid like
PBR	peripheral benzodiazepine receptor
PD	Parkinson's disease
PEO	progressive external ophthalmoplegia
PF	physical functioning
PGC-1α	PPARγ coactivator-1α
PGC-1α-/-	PGC-1α knockout
PGWBI	psychological general well-being inventory
Pi	phosphate
PI3K-Akt	phosphatidylinositol 3′-kinase-Akt
PKC	protein kinase C
PRMT1	PGC-1α by arginine methyltransferase 1
PRO	patients-reported-outcome
Prx	peroxiredoxins
PTP	permeability transition pore
RE	emotional role limitation
RNS	reactive nitrogen species
ROS	reactive oxygen species
RP	physical role limitation
RRFs	ragged red-like fibers
RyR	ryanodine receptor
SDH	succinate dehydrogenase
SDS-PAGE	polyacrylamide gel electrophoresis
SERCA2	SR Ca^{2+}-ATPase
SF	social functioning
SF-36	short form 36 items health survey
SOD	superoxide dismutase
SR	sarcoplasmic reticulum
SRC-1	steroid receptor coactivator-1

SS	subsarcolemmal
SURF1	surfeit gene
TBW	total body water
TCA	tricarboxylic acid
Tfam	transcription factor A
TIM	transporters of the inner membrane
TNFα	tumor necrosis factor α
TOM	transporters of the outer membrane
TP	thymidine phosphorylase
UCMD	Ullrich congenital muscular dystrophy
VDAC	voltage dependent anion channel
Vo	velocity
VT	vitality
WHO	World Health Organization
Wmax	maximum power output
WT	wild-type

SECTION I

Metabolic Modifications and Physical Exercise

Chapter 1

Cellular and Molecular Mechanisms of Skeletal Muscle Plasticity

Monica Canepari and Roberto Bottinelli

Introduction

Skeletal muscles are used for a wide variety of motor tasks ranging from maintaining posture to whistling, from jumping to breathing, from running at ~40Km/h for 10s (100 meters) to running at half the speed for ~2h (i.e., the marathon, 42,195Km). The capacity to accomplish such variable motor tasks relies on the very fine motor control performed by the nervous system and on the very large functional heterogeneity and plasticity of skeletal muscles. The nervous system can finely tune the performance of a given muscle adapting its power output to the motor task on a very short time base, milliseconds/seconds (phasic control). Skeletal muscles can adapt their contractile properties to the requirements of their predominant motor tasks by changing their structure on a long time base, weeks-months (tonic control). Skeletal muscles are known to differ regarding a variety of aspects, among which the most relevant are their power output and their energy metabolism. Several reviews have recently dealt in detail with most aspects of skeletal muscle plasticity [1-3]. This chapter will consider the variability in the parameters at the basis of power generation (force, velocity, ATP consumption). It is not meant to be a review on such a wide topic, but it will tell the story of how the understanding of the mechanisms underlying the heterogeneity and plasticity of skeletal muscle power output has been developing in our laboratory and of how our experimental approach has been updated to further our knowledge of the mechanisms from the cellular level to the molecular level. In so doing, it will be shown that macroscopic phenomena, such as the large difference in the running speed between elite sprinters and marathon runners, are linked through a complex cascade of mechanisms to very fine differences in the rate of release of ADP among the molecular motors which propel muscle contraction, namely the myosin isoforms.

V. Stocchi (ed), *Role of Physical Exercise in Preventing Disease and Improving the Quality of Life*

Quantitative and Qualitative Mechanisms of Skeletal Muscle Plasticity

It is now clear two major mechanisms of skeletal muscle heterogeneity and plasticity exist: a quantitative mechanism based on a change in muscle mass and a qualitative mechanism independent from muscle mass.

Both mechanisms have been known for quite a while. It is straightforward to understand that muscles can change their performance through a "quantitative mechanism" just by looking at elite sprinters and marathon runners, two athletes able to run at very different speeds for very different times. It is easy to note that skeletal muscles of the sprinters are much larger than those of the marathon runners. As larger muscles can develop more force and therefore more power, the higher velocity of the sprinters could be simply explained on the basis of a larger muscle mass.

The question of whether the "quality" of skeletal muscles can be different from muscle to muscle and can change in response to neuromuscular activity or to other stimuli is more complex. To put it another way, does a given amount of skeletal muscle mass of a marathon runner have the same contractile properties as an equal muscle mass of a sprinter? A now classic paper published by Close more than 40 years ago [4] definitely answered no to such a question and indicated that a "qualitative mechanism" of muscle plasticity does exist. Figure 1 shows the relation between the load applied (x axis) and the velocity of shortening (y axis), i.e., the force-velocity relationship, of the soleus and EDL muscles of rats at different ages. It can be seen that EDL can shorten at much a higher speed than soleus against the same relative load and that maximum shortening velocity (the intercept of the force-velocity relationship with the y axis) is much higher in EDL than in soleus (Fig. 1b-d). As the load at each velocity is expressed as a percentage of the isometric force of the muscle (relative load), differences in force, based on variations in muscle mass, cannot determine the differences in velocity observed. Some qualitative mechanisms must, therefore, exist which make the EDL muscle intrinsically faster than the soleus muscle. Moreover, as the difference between EDL and soleus is not present at birth (Fig. 1a), and develops later in life (Fig. 1b-d), the figure is also a good example of the plasticity of skeletal muscles, i.e., of the capacity to vary contractile properties by adjusting them to the variable motor requirements.

Like most research groups, we have devoted our work to the understanding mostly of the qualitative mechanism of skeletal muscle plasticity.

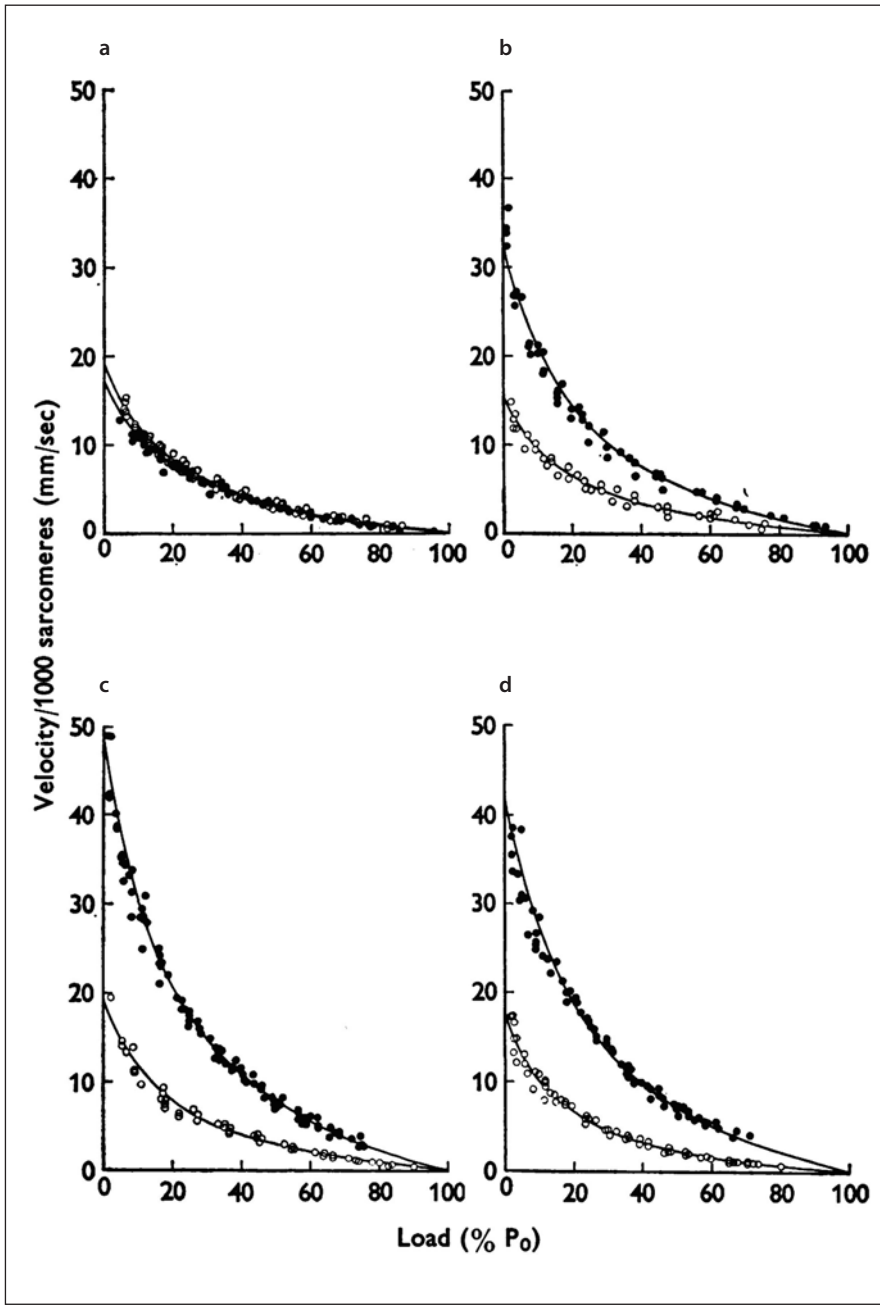

Fig. 1 Relationship between load (x axis) and shortening velocity (y axis) during isotonic contractions of EDL (•) and SOL (◦) muscles of the rat showing the large differences in shortening velocity at the same relative load. Muscles from new-born (**a**), 10-day-old (**b**), 35-day-old (**c**), and 100-day-old (**d**) animals. Velocity is expressed per 1000 sarcomeres and load as the percentage of maximum isometric force (relative load). The figure indicates that EDL muscles are much faster than soleus muscles against the same relative load. Reproduced with permission [4]

Myosin Isoforms

The understanding of the mechanisms of skeletal muscle heterogeneity and plasticity progressed very slowly from the pioneering work by Close [4] up to the end of the 1980s, when several works and especially those coming from Pette's and Schiaffino's laboratories uncovered the large molecular heterogeneity of the basic contractile unit of striated muscle, the sarcomere [3, 5-7]. They showed that myosin, the protein that was known to bind to actin, to split ATP, and to go through the conformational change at the basis of force generation and shortening, existed in several isoforms. Isoforms can be defined as proteins which have very similar, but not identical, amino acid sequences, are coded by different genes, and are interchangeable, i.e., one isoform can take the place of another isoform in the sarcomere. It is now well known that most myofibrillar proteins exist in a number of isoforms [3]. As regards myosin, both myosin heavy chain (MHC) and myosin light chain (MLC) isoforms were found to exist. Although considerable work has been performed on the MLC role [8-12], the attention mostly focused on the MHC isoforms as MHCs are the portion of the molecule that attaches to actin and split ATP, and they are mostly responsible for power generation. It was shown that in the adult skeletal muscle of small mammals four MHC

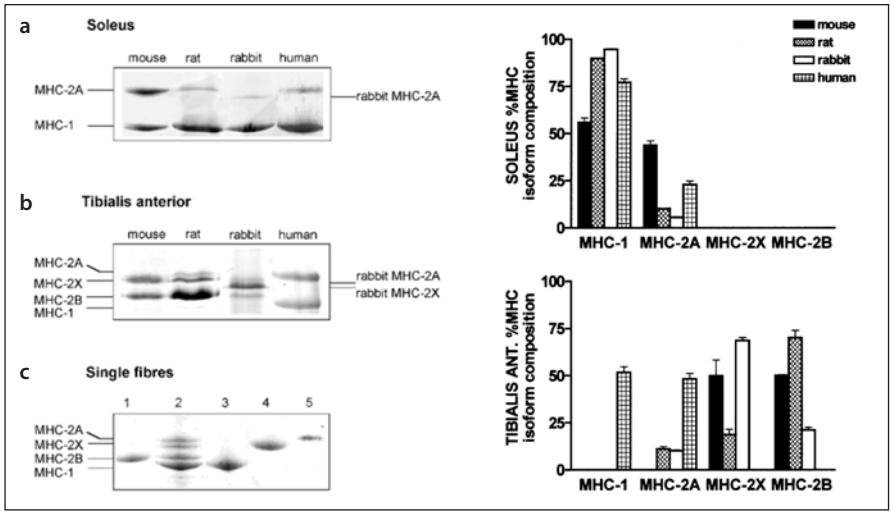

Fig. 2 Mammalian myosin heavy chain (MHC) isoforms separated by polyacrylamide gel electrophoresis (SDS-PAGE). MHC isoforms content in soleus (**a**), tibialis anterior (**b**), and single muscle fibers (**c**) of mouse, rat, rabbit, and man. (**c**) Lane 1, rat pure fast 2B fiber; lane 3, rabbit pure slow fiber; lane 4, mouse pure fast 2X fiber; lane 5, mouse pure fast 2A fiber. Lane 2 shows a mixed rat muscle sample. The histograms on the right report the relative percentage (mean ± S.E.M.) of MHC isoforms of soleus and tibialis anterior muscles of four mammalian species. The figure indicates that: MHC isoforms can be separated by SDS-PAGE; skeletal muscles have variable MHC isoform distribution. Reproduced with permission from [13]

isoforms could be expressed: MHC-1 (or slow isoform) and MHC-2A, MHC-2X (also called 2D), and MHC-2B (or fast isoforms) (Fig. 2).

Interestingly, in humans, the gene for MHC-2B is present, but it is believed not to be expressed in adult muscles [14, 15].

Functional Properties of Myosin Isoforms: Fiber Types and their Contractile and Energetic Properties

Since the discovery of MHC isoforms, it was straightforward to assume that they were functionally different and that the functional heterogeneity among skeletal muscles depended on their differential expression. However, it took a considerable amount of work to definitely prove that this was actually the case. In this respect, the earliest demonstration came when contractile properties and MHC isoform content could be determined in the same muscle fiber, enabling a direct relationship between function and MHC isoform content to be established. For several reasons, skinned fibers turned out to be the specimen of choice in this kind of study. Skinned fibers are chemically or mechanically demembranated and can be maximally activated by exposure to solutions containing calcium (Fig. 3). Using skinned specimens, most contractile and energetic parameters of muscle fibers could be precisely determined, including force and specific force (or tension), rate of tension elevation, velocity of shortening, power, ATP consumption, and tension cost. At the end of the experiment, fiber segments could be stored for subsequent analysis of MHC isoform content. A high-resolution electrophoresis in

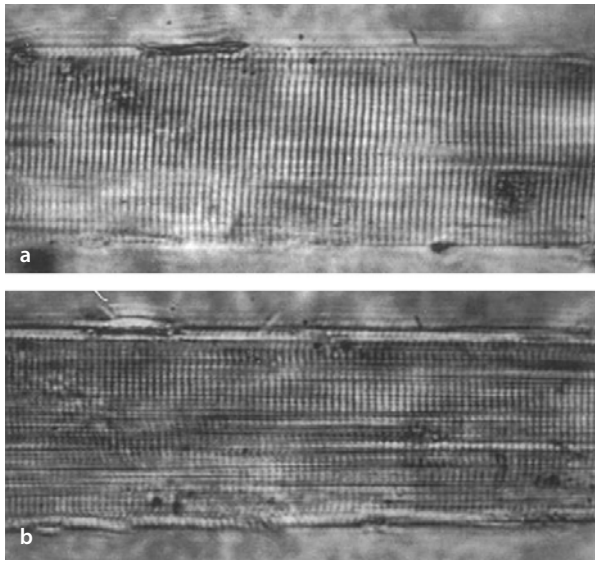

Fig. 3 Photomicrograms of a human skinned skeletal muscle fiber in relaxing (**a**) and activating (**b**) solution. Photomicrograms in panel B was taken at the end of 3 min maximal activation for force-velocity determination. Scale bar, 50mm. The figure shows that skinned fibers maintain an ordered striation pattern upon skinning and activation. Reproduced with permission from [16]

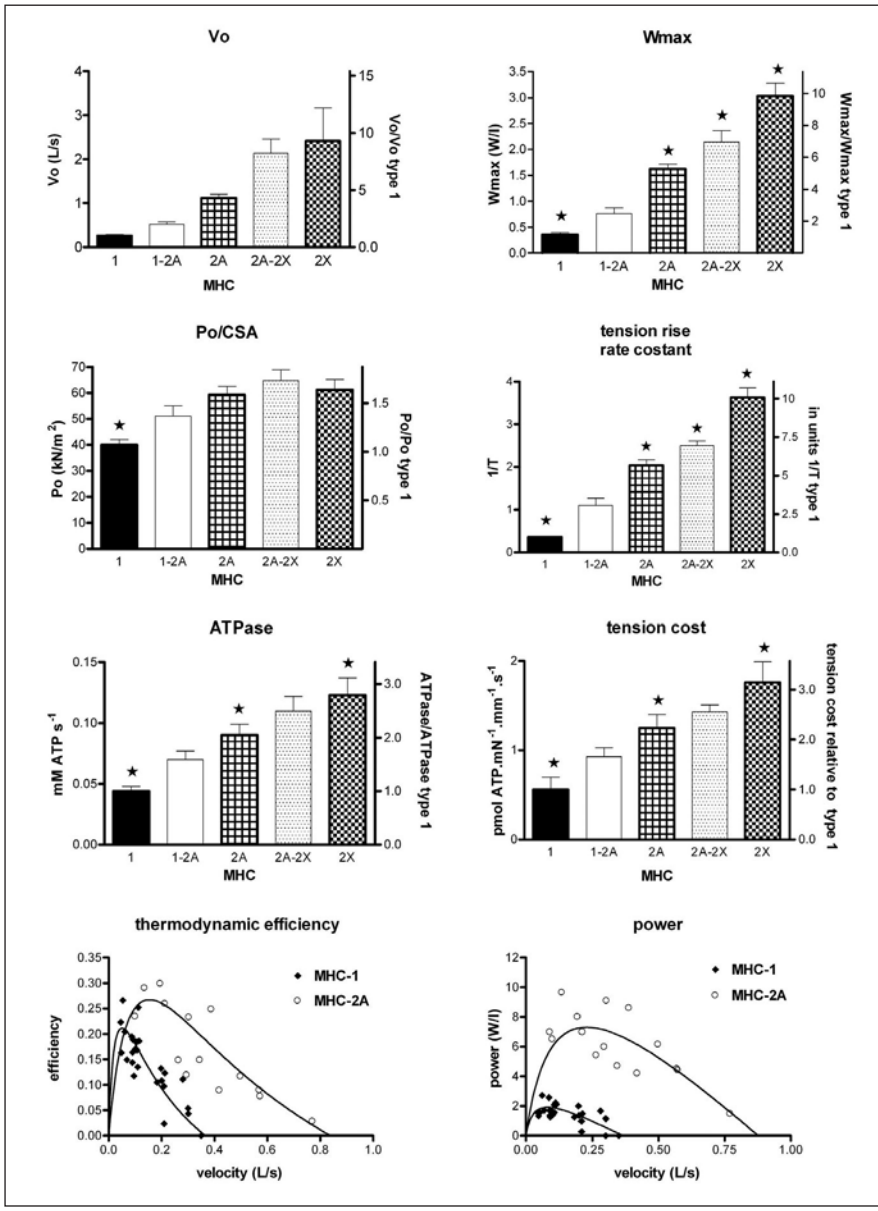

Fig. 4 Contractile and energetic properties of human muscle fibres containing different myosin heavy chain (MHC) isoforms. Bar graphs reporting the mean values of unloaded shortening velocity (Vo), maximum power (Wmax), specific force (Po/CSA), rate constant of tension rise (1/T), ATPase activity (ATPase) and tension cost. The relationship between thermodynamic efficiency and power output, and velocity of shortening of the same human fibres is reported in the lower part. Vo Wmax Po/CSA and 1/T data from [16]; ATPase and Tension cost data from [17]; Thermodynamic efficiency and Power data from [18]. Experimental conditions were: 12°C, 200 mM ionic strength, maximal activation (pCa 4.5), and optimal sarcomere length for force developing in all cases. CSA for Po/CSA was determined in relaxing solution, assuming a circular shape of the fibres without correction for swelling

denaturated conditions (polyacrylamide gel electrophoresis, SDS-PAGE) was developed to precisely assess MHC isoform content using as little as a few hundred microns of a fiber segment (Fig. 2c). Using SDS-PAGE, muscle fibers of small mammals could be grouped in seven types on the basis of their MHC isoform content. Four were pure types and were mostly predominant: type 1 or slow, containing MHC-1 isoform; and types 2A, 2X, and 2B (or fast types), containing MHC-2A, MHC-2X, and MHC-2B, respectively (Fig. 3). Three were hybrid types expressing two MHC isoforms: type 1-2A, type 2AX, and type 2XB.

As in humans, MHC-2B is not expressed [14, 15]; human muscles contain three (type 1, 2A, and 2X) and not four pure fiber types and two (type 1-2A and 2AX), and not three hybrid fibers types.

When muscle fibers were grouped on the basis of MHC isoform content, very large differences among groups were observed, with type 1 fibers being the slowest and type 2B fibers being the fastest fiber type. In humans, for example, fibers containing MHC-1 (type 1) had tenfold lower unloaded shortening velocity (Vo) [16], maximum power output (Wmax) [16], rate constant of tension rise (1/T), and threefold lower ATPase and tension cost than type 2X fibers [17], type 2A being intermediate (Fig. 4). As expected, hybrid fibers were intermediate between pure fiber types. Interestingly, two properties did not differentiate muscle fiber types as much as all the others. Specific force (Po/CSA) was only 30%-40% lower in slow fibers than in fast fibers, whereas no differences were observed among fast fibers [16]. Thermodynamic efficiency was found to be similar in slow and fast human fibers [18], and only slightly higher in slow than in fast rat fibers [19].

The above findings and a large body of information collected by other research groups in the same years definitely proved that myosin isoforms had very different functional properties.

Fiber Type Distribution and Contractile Properties of Skeletal Muscles

The finding that fiber types differed significantly in most contractile and energetic properties provided a simple basis for the functional heterogeneity of skeletal muscles. As in all mammals, skeletal muscles are mixed muscles expressing the different fibers types in variable proportions (Fig. 2a,b); it is expected that the higher the percent of fast fibers, the higher the shortening velocity of the muscles. The classic work by Thorstensson et al. [20], showing that velocity of knee extension was proportional to the percentage of fast fiber content of the contracting muscle, was the earliest demonstration that this was actually the case (Fig. 5). A similar conclusion can be drawn from the analysis of MHC isoform distribution in elite sprinters and marathon runners in comparison to young healthy controls [21] (Fig. 6). It

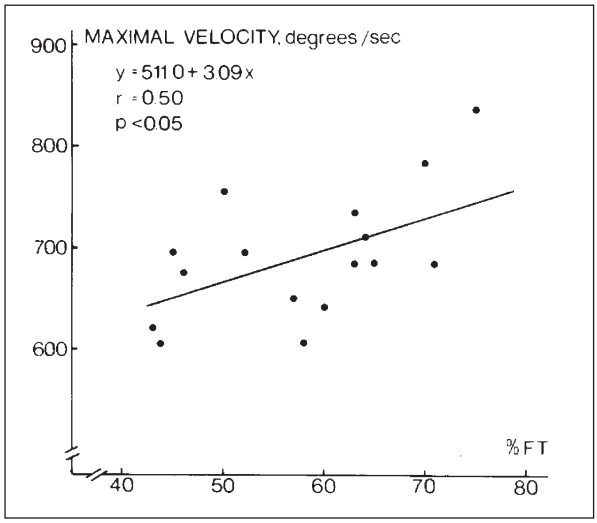

Fig. 5 The relationship between maximal velocity of knee extension versus percentage of fast fiber content of the contracting muscle. The regression line shows that the higher the fast fiber content the higher shortening velocity. Used with permission from [20]

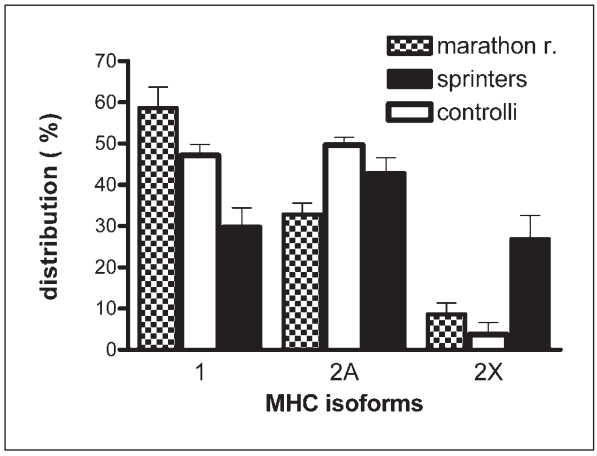

Fig. 6 The distribution of myosin heavy chain (MHC) isoforms in the quadriceps muscles of elite sprinters and marathon runners in comparison to young healthy controls. Data of marathon runners and sprinters are from [20]. Data of controls are from [16]

can be seen that MHC isoform composition is shifted towards the slow MHC-1 in marathon runners and towards the fastest MHC-2X isoform in sprinters in comparison to controls.

Our "story" so far clearly indicates that the qualitative mechanism of skeletal muscle heterogeneity and plasticity shown by Close [4] long ago is based on the expression of functionally different myosin isoforms within fiber types and on a differential distribution of fiber types in mammalian muscles. It is now well documented that the soleus muscle of the rat mostly contains type 1 fibers whereas the EDL muscle mostly contains fast fibers (2A, 2X, and 2B). It is not surprising that the EDL was found to be much faster than the soleus muscle.

Why do Myosin Isoforms have Different Functional Properties?

The understanding of the major role of MHC isoforms in generating the qualitative mechanism of muscle heterogeneity and plasticity was not the end of the story, but prompted a fundamental question: why is MHC-1 so much slower than fast MHC isoforms, i.e., which are the kinetic and molecular bases of myosin isoform diversity?

To understand the scientific issue that we were facing it is necessary to briefly summarize the chemomechanical cycle myosin goes through when interacting with actin (Fig. 7). When muscle is at rest (Fig. 7e), myosin is detached from actin and most molecules are in the state M.ADP.Pi, i.e., myosin (M) still carries the products of ATP hydrolysis, ADP and phosphate (Pi). The rate of product release from myosin is, in fact, low in the absence of actomyosin interaction. In the state M.ADP.Pi, myosin has a moderate affinity for

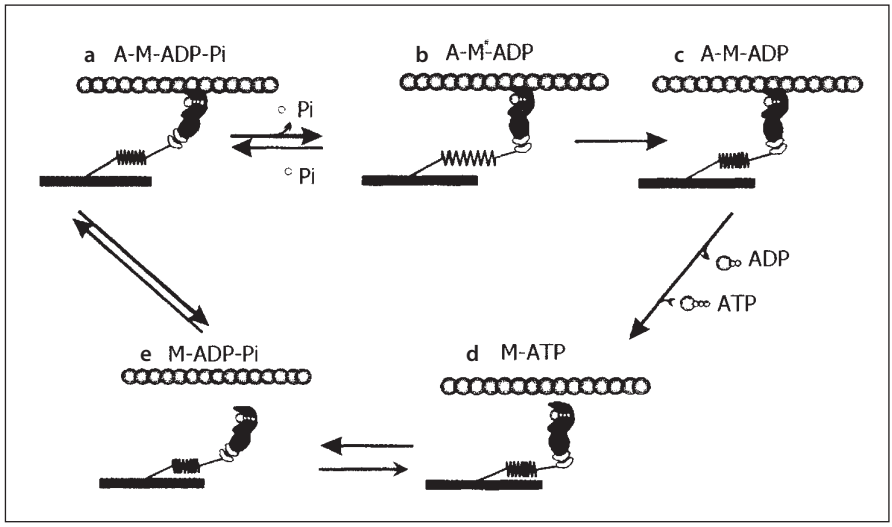

Fig. 7 A mechanochemical cross-bridge cycle for myosin. The ATPase cycle is shown as states (**a**-**e**) in cartoon and biochemical states (*A*, actin; *M*, myosin head). *ATP* binding to the myosin head (**c**) results in a rapid dissociation of the myosin head from actin (**d**). Following detachment from actin, the *ATP* is hydrolyzed to *ADP* and P_i (**e**). The hydrolysis is accompanied by a conformational change which represents the reversal of the power stroke. The affinity of M·ADP·P_i for actin is significantly higher than that of M·ATP. Therefore, if an actin site is within reach of the myosin head it will bind rapidly (**a**). When the myosin head binds to actin the interaction with actin can promote a change in conformation (the power stroke) which is accompanied by the dissociation of P_i (**a**, **b**). If the filaments carry an external load then the power stroke results in the distortion of an elastic element (**b**) (the strained A·M$^{\#}$·ADP state). While the myosin head carries a load and is elastically distorted, the dissociation of P_i is a reversible event and P_i can rebind to reverse the power stroke (and also back through intermediates **d** and **e**). If the external load is small, then the power stroke results in the relative sliding of the actin and myosin filaments (**c**). Following the sliding *ADP* is released very quickly to be replaced by *ATP* and the myosin head dissociates once more to complete the cycle. Reprinted with permission and modified from [22]

actin. Upon calcium release and muscle activation, myosin can therefore readily bind to actin and the complex A.M.ADP.Pi is formed (Fig. 7a). Myosin attachment to actin is very quickly followed by Pi release. The release of phosphate is strictly related to the conformational change in the myosin head that propels the actin filament towards the center of the sarcomere, although it is still debated which of the two events occurs earlier. The displacement of the actin filament (or step size) is generally thought to be related to the transition between the A.M.ADP.Pi (Fig. 7a) and A.M.ADP (Fig. 7c) states and to be as large as 5nm. The affinity of M.D for actin is very high and myosin remains strongly attached to actin in this state. The detachment of myosin from actin requires that ADP is released (A.M.ADP → A.M transition) and that ATP binds to myosin (A.M → A.M.ATP transition) (Fig. 7c, d). The complex M.ATP has a low affinity for actin and quickly detaches from it. Immediately after acto-myosin detachment, myosin splits ATP and uses the energy released by ATP to go through a reversal of the conformational change which occurred during acto-myosin attachment. Myosin enters the M.ADP.Pi state and is now ready to repeat the cycle as long as calcium concentration is high in the cytoplasm.

Therefore, during the sequence of events described above, which is generally called the cross-bridge cycle, myosin goes through two conformational changes. One conformational change, which occurs when myosin is detached from actin, stores the energy released by ATP. The other conformational change, which occurs during acto-myosin attachment and which propels actin filaments, is the release of the energy stored in the molecule during the first conformational change.

In the cross-bridge cycle, myosin propels actin filaments the distance δ (or step size) during the time t_{on}, i.e., the time it remains attached to actin. The velocity of sliding of the actin filament, and therefore the velocity of shortening of a sarcomere, muscle fiber, or muscle, is therefore equal to δ/t_{on} (i.e., distance travelled/time required). The question as to why MHC-1 is slower than MHC-2B can be therefore be expanded to the following question: is MHC-1 slower than MHC-2B because its step size is smaller, because it remains attached to actin longer, or for both reasons?

To address the latter question, skinned muscle fibers could not be the specimen of choice any longer. In a muscle fiber an extremely large number of myosin molecules work randomly and asynchronously in an ensemble. Assessing the size of the displacement determined by a single molecule and for how long a molecule remains attached with the resolution required to compare different myosin isoforms is very complex, even using intact frog fibers, which maintain much higher sarcomere uniformity during contraction [23, 24] than skinned fibers. We reasoned we had mainly two independent and complementary ways to study mammalian skeletal myosin isoforms in this respect. We could study the kinetics of the acto-myosin cycle applying biochemical assays on pure myosin isoforms interacting with actin in solution. We could

also directly study a single myosin molecule interacting with an actin filament by an optical trap (OT) set-up. We decided to follow both approaches. A great deal of work was required to refine the techniques, but in the long run both approaches proved successful and provided consistent results.

Slow Myosins Spend More Time Attached to Actin than Fast Myosins

To study acto-myosin kinetics of different skeletal myosin isoforms in solution we could take advantage of the experience coming from existing biochemical assays used to study myosin from bulk skeletal muscles [25, 26]. However, we had to overcome at least two major problems that had been preventing the analysis of pure skeletal myosin isoforms until that time.

First of all, we had to obtain a sufficient amount of pure myosin isoforms to be loaded in biochemical assays. As mammalian skeletal muscles are mixed muscles, extraction of myosin from bulk muscle invariably gives a mixture of two-three-four isoforms. We reasoned that single muscle fibers which mostly contain a single myosin isoform were a store of pure isoforms to extract. We therefore developed an approach which enabled characterization of single muscle fibers according to their MHC isoform content and then extracted myosin from them, thereby obtaining micrograms of pure myosin of known type [27].

Second, we had to use biochemical assays which could provide reliable measurements of micrograms of myosin. In the meantime, Mike Geeves (University of Canterbury, UK) had been developing a new flash-photolysis light scattering apparatus which enabled to study acto-myosin kinetics in solution on such small amounts of myosin. The flash photolysis takes advantage of well-known properties of acto-myosin in solution. In the absence of ATP, actin (A) and myosin (M) form a stable A.M, or rigor complex. The A.M complexes in solution scatter the light much more than A detached and M. Acto-myosin dissociation can therefore be studied following, with very high time resolution, the decrease in light scattering occurring when ATP is released in a solution containing A.M and A and M are formed. The flash photolysis enabled also to assess the affinity of the acto-myosin complex for ADP. From the later parameter, it was possible to estimate the rate of ADP release from acto-myosin. The experimental approach and the analysis of the data are rather complex and have been described in detail by Weiss et al. [28, 29]. Figure 8 shows the experimental data and summarizes the procedure used.

Collaborating with Mike Geeves, we succeeded in extracting sufficient amounts of the four myosin isoforms of the rat, 1, 2A, 2X, and 2B, from single muscle fibers and studying their acto-myosin kinetics in solution [29]. We focused on two rates affecting the time of attachment [25, 26]: (i) the rate of ADP release from acto-myosin and (ii) the rate of acto-myosin dissociation induced

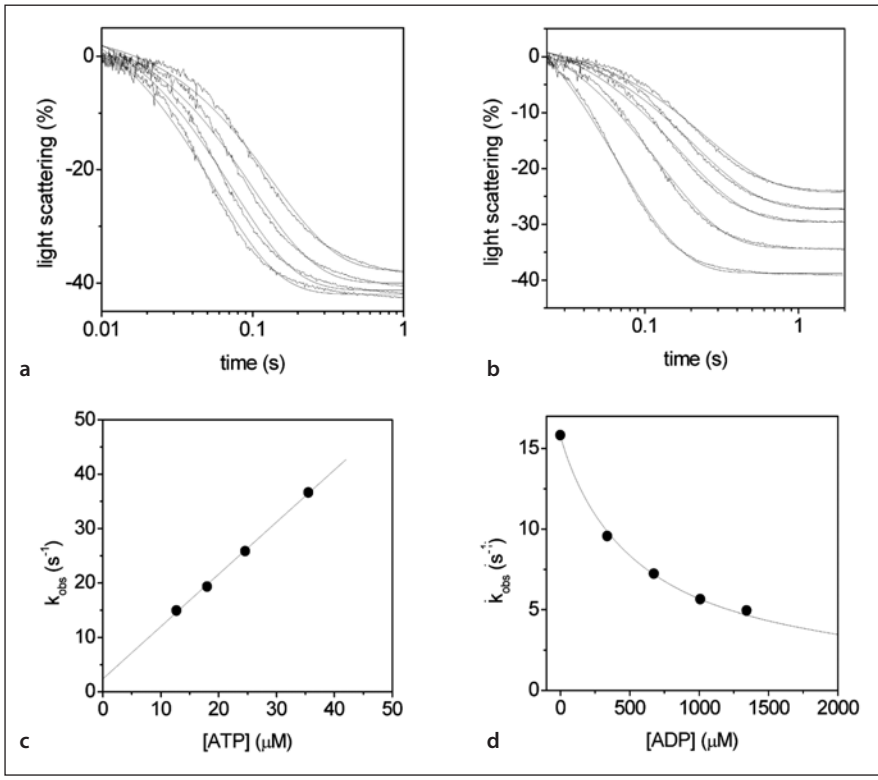

Fig. 8 Experimental traces of flash photolysis experiments used to compare the kinetics of acto-myosin interaction of slow and fast myosin isoforms. (**a**, **c**) ATP-induced dissociation of myosin and actin. (**a**) Light-scattering signals from a 20-ml sample containing 0.5mM actin, ~0.15mM myosin, 0.5mM caged ATP in a multiple flash experiment. The decrease in light-scattering was described by a single exponential and the best fit to a single exponential is shown superimposed. (**c**) The observed rate constant Kobs of the reaction is plotted versus the amount of ATP release. The slope of a linear fit of the data gives the second order rate constant of ATP-induced dissociation of myosin and actin (K_1k_{+2}). (**b**, **d**) Determination of ADP affinity for actomyosin. (**b**) Light-scattering signals from a 20-μl sample containing 1μM actin, ~0.75μM myosin, 1mM caged ATP in a multiple flash experiment in the presence of ADP in variable concentrations. The best fit to a single exponential decay of the light-scattering decrease is shown superimposed. (**d**) The plot shows the ADP dependence of the observed rate constant Kobs of the reaction. The data were fitted to a model of scheme 1* and gave values of the dissociation constant for ADP binding to actomyosin (K_{AD}). The rate of ADP release (k_{-AD}) was determined from K_{AD} as described by Weiss et al. [29]. Courtesy of M. Geeves and S. Weiss

by ATP (Fig. 7c-d). We did not consider the rate of Pi release (Fig. 7a, b) as it has been previously shown to be very fast and not to significantly affect the time of attachment and the overall rate of the cross-bridge cycle [25, 26].

We found that that both the rate of ADP release from acto-myosin and the rate of acto-myosin dissociation induced by ATP were slower in myosin 1 than in the fast isoforms and increased in the order myosin 1 → 2A → 2X → 2B

*Scheme 1

$$\text{Kobs} = \frac{K_1\, k_{+2}\, [\text{ATP}]}{1 + \frac{[\text{ADP}]}{K_{AD}}}$$

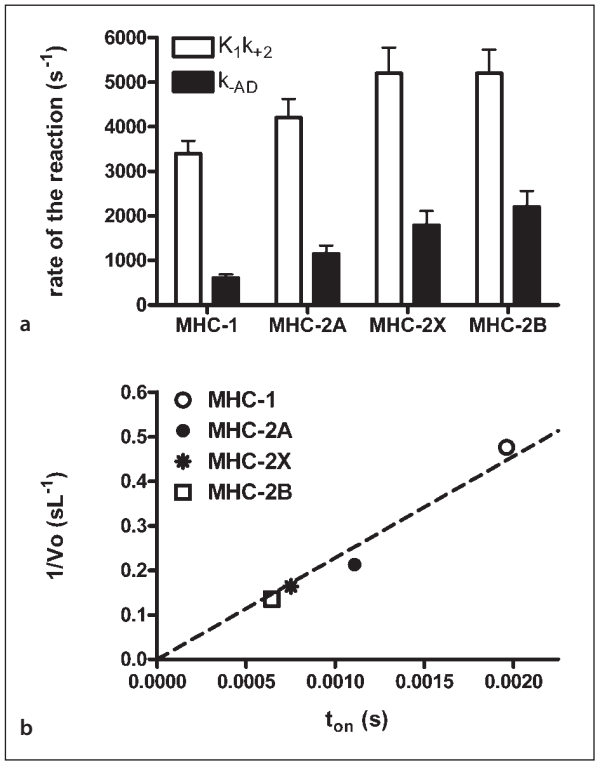

Fig. 9 The parameters determining the time spent by myosin attached to actin measured by the flash photolysis. **a** Rate of ADP release from acto-myosin (k-AD) and rate of acto-myosin dissociation induced by ATP (K1k+2). **b** Relationship between the time spent by myosin in the attachment state (ton) and the unloaded shortening velocity (Vo) of the muscle fiber types from which a given myosin isoform was extracted. Ton was determined using the formula: ton = 1/k-AD + 1/K1.k+2. **b** Shows that the higher Vo of single fibers the shorter ton, i.e., slow myosin spend more time attached to actin than fast myosins. Data from [29]

(Fig. 9a from [29]). From the two rates it is possible to determine the time spent by myosin in the attachment state (t_{on}) using the formula t_{on} = 1/rate of ADP release + 1/rate of acto-myosin dissociation by ATP. We can therefore compare t_{on} of different isoforms and relate their t_{on} with the unloaded shortening velocity (Vo) of the skinned muscle fibers types from which a given myosin isoform was extracted. When we plot t_{on} versus the reciprocal of Vo we find a clear linear relationship in which slow fibers have the lowest Vo and myosin 1 the longer time of attachment, and the type 2B fibers have the highest Vo and myosin 2B the shortest time of attachment (Fig. 9b).

Single molecule mechanics (or optical trap, OT) is an approach in which displacement (δ), and duration (t_{on}) of the elementary interaction events can be studied in myosin molecules dispersed on the surface of a silica bead interacting with an actin filament attached to two beads whose position is controlled by laser tweezers (Fig. 10). The approach has been developed by Finer et al. [30] and had been previously applied to the comparison of δ and t_{on} of cardiac V1 and V3 myosins [31] and of smooth and skeletal muscle myosin [32]. As extraction of myosin from bulk skeletal muscles invariably provides a mixture of isoforms, pure skeletal isoforms had never been used for single molecule analysis. Our approach of myosin extraction from single fibers,

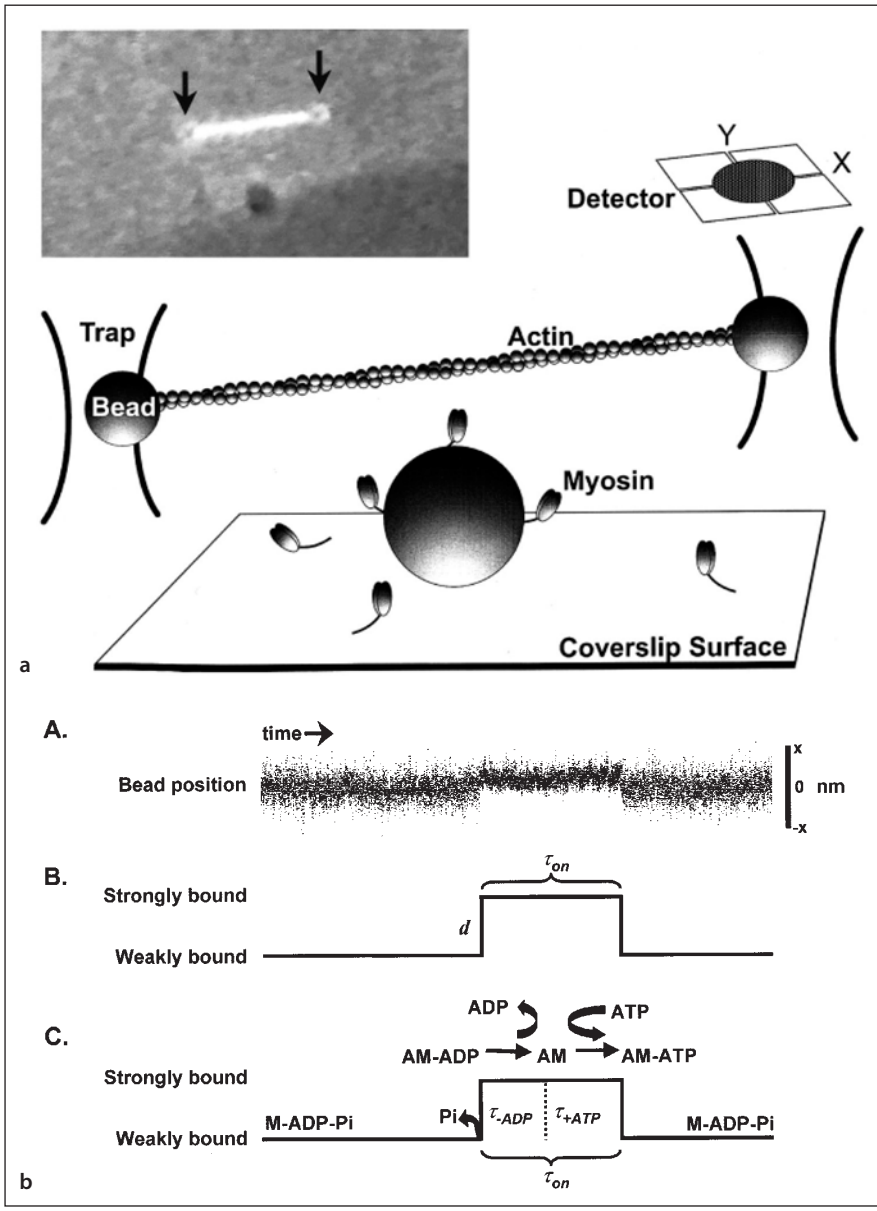

Fig. 10 Three-bead optical trap assay used to study the elementary event at the basis of force generation and shortening, namely the working stroke. Upper portion: three-bead assay. An actin filament is connected to polystyrene beads through biotin-avidin links forming a structure named dumbell. The beads are held by optical tweezers or traps. The myosin molecule is carried by a third bead stuck to the coverslide; when the actin filament comes within reach, the myosin molecule interacts with actin and subsequent working strokes can be recorded. ***A*** Position trace of the dumbbell motion. Acto-myosin interaction were detected from noise reduction of the bead position signal. ***B*** The displacement (d or δ), and duration (τ_{on}) of the elementary interaction events. ***C*** The likely coupling between the mechanical and biochemical events in the power stroke. Reprinted with permission from [33]

providing workable amounts of pure myosin isoforms, prompted the possibility to compare δ and t_{on} of different skeletal myosin isoforms using an OT set-up. However, we decided to refine our approach of myosin isoform preparation to be able to study a single motor domain of myosin. In fact, as myosin has two heads, i.e., two motor domains, δ and t_{on} of myosin can be the outcome of the interaction of one and/or two myosin heads with actin in a rather uncontrollable fashion. Only by loading in an OT the subfragment-1 of the myosin molecule (S1), i.e., a single motor domain, can differences in δ and t_{on} among isoforms be safely attributed to the features of the motor portion of the molecule. Moreover, as fast skeletal myosins are faster than smooth and cardiac myosins, we improved the spatial and temporal resolution of the OT set-up to reliably measure δ and t_{on} of such isoforms. We collaborated with Francesco Pavone and Marco Capitanio at LENS (European Laboratory of Non Linear Spectroscopy) of the University of Florence in setting up the required OT set-up for our kind of analysis, and together we could then successfully study δ and t_{on} of two isoforms of S1 having very large differences (six- to sevenfold) in unloaded shortening velocity: S1 of myosin 1 (slow) isoform from the rat and S1 of myosin 2B from the mouse. Interestingly, whereas δ was slightly larger for the slow than for the fast isoform, t_{on} was much longer for the slow than for the fast isoform (Fig. 11).

The latter findings, in full agreement with the analysis of acto-myosin kinetics in solution [29], indicate that the lower velocity of shortening of the slow isoform was due to a much longer time of attachment which was not compensated by the slightly larger δ.

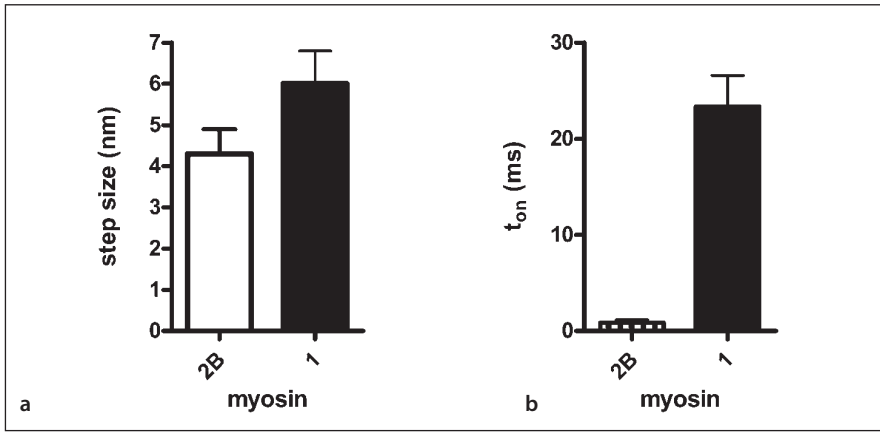

Fig. 11 Step size (**a**) and t_{on} (**b**) of isoforms 2B (fast from mouse) and 1 (slow from rat) of the fraction S1 of myosin having very large differences (six- to sevenfold) in unloaded shortening velocity. Experimental values were obtained using the optical trap approach described in Figure 10. Experiments were performed at room temperature and 50mM ionic strength. The data indicate that t_{on} and not step size can determine differences in velocity of shortening among slow and fast myosins. Data from [34]

As usual, a new question was raised from the latter results: why do slow isoforms spend more time attached to actin than fast isoforms? Luckily, a careful consideration and elaboration of the findings obtained by biochemical assays in solution and by single molecule mechanics provided a prompt answer to that question.

Slow Isoforms Spend More Time Attached to Actin as They Release ADP Slower than Fast Isoforms

The flash-photolysis approach measured the rates of the two major transitions known to determine t_{on} in sarcomeric isoforms: the rate of ADP release from acto-myosin and the rate of actomyosin dissociation by ATP. Of the two rates, the former was slower than the latter and therefore more likely to be the major phenomenon underlying the longer t_{on} of slow isoforms (Fig. 9a). On this basis and on other evidence the work by Weiss et al. [29] indicates that ADP release defines the diversity in velocity of shortening of myosin isoforms.

Strong support for this conclusion came from a careful analysis of the data collected by single molecule mechanics on the slow isoform of the rat and on the 2B isoform of the mouse. The high temporal and spatial (~300μs and 0.1nm, respectively) resolution we achieved in the analysis of the elementary mechanical events enabled the first observation of two conformational changes in the attached state of skeletal myosin, i.e., of a double step in the skeletal myosin working stroke [34]. Such phenomenon had been previously demonstrated for nonconventional or smooth muscle myosins [35-37], but not for sarcomeric myosins [35]. Therefore, until our work, it was generally believed that the chemomechanical cycle of sarcomeric myosins differed from that of unconventional and smooth myosins. The finding of two independent mechanical events in the interaction cycle of skeletal myosins was novel and confirmed the farseeing hypothesis put forward by Nytray and Geeves [22], i.e., that all members of the myosin family shared a common scheme of chemomechanical transduction.

Much evidence supported the hypothesis that the duration of the first phase of the attachment state was related to the rate of ADP release from the acto-myosin complex, and the duration of the second phase was related to the ATP-induced dissociation of the complex. One piece of evidence was the observation that the duration of the first step was independent from ATP concentration, whereas the duration of the second step was linearly related to the ATP concentration. Interestingly, whereas the rate of the first phase of the attachment phase (k1) was very different between the two isoforms studied, the rate of the second phase (k2) was very similar (Fig. 12).

Both the kinetic analysis in solution and the single molecule analysis of

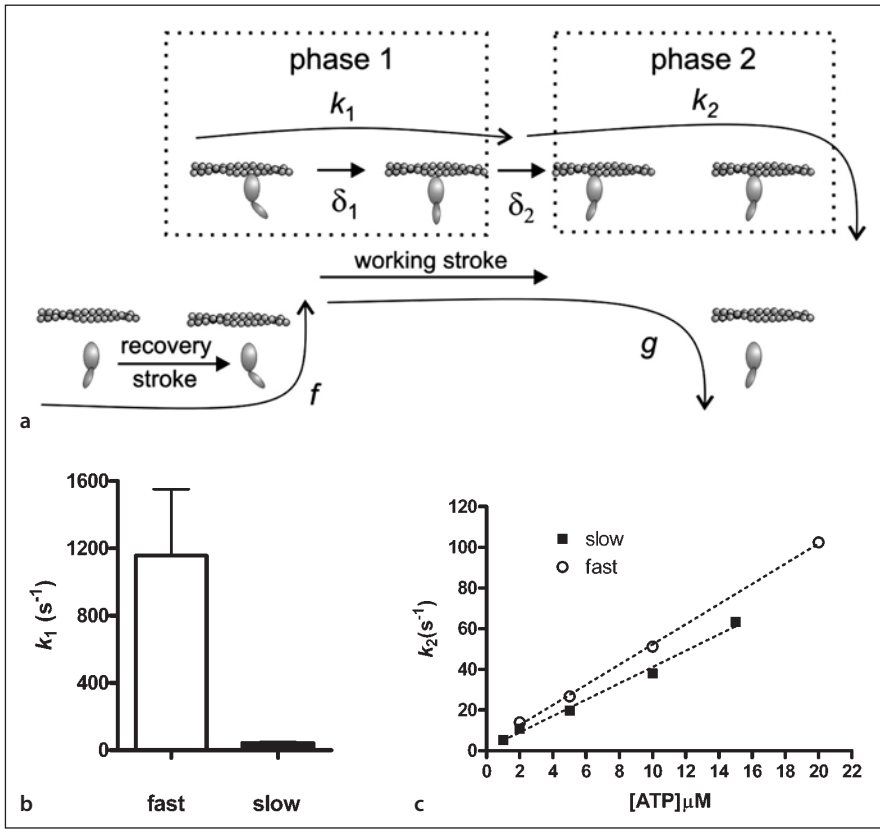

Fig. 12 Proposed mechanism of chemo-mechanical transduction for skeletal muscle myosin. (**a**) The acto-myosin detachment rate g and the corresponding rates of the two phases (K_1 and K_2) are represented. Separation of the two phases of the bound state is defined by development of a second step δ_2. The reversal of the power stroke with the attachment rate (*f*) is also represented. (**b**) Average rates of the first phase of the bound state (K_1) for fast (mouse 2B) and slow (rat 1) isoforms. (**c**) Correlation between the rates of the second phase of the bound state (K_2) and ATP concentration for both the isoforms. The data suggest that myosin isoforms differ in the rate of the first phase of the bound state likely determined by the rate of ADP release. Data from [34]

myosin isoforms indicate that slow isoforms spend more time attached to actin in a cross-bridge cycle because they release ADP at a lower rate than fast isoforms.

Conclusions and Future Studies

In searching for the mechanisms underlying the very evident diversity and adaptability of skeletal muscle performance in vivo, our work progressed from the contemporary analysis of contractile properties and MHC iso-

form content of skinned muscle fibers, to the analysis of acto-myosin kinetics of different pure isoforms in solution, and to the study of the elementary event of force generation and shortening produced by a single motor domain of slow and fast myosin isoforms. We have been developing new experimental approaches and adapting existing ones to follow a cascade of mechanisms that ultimately identified the major determinants of the diversity of muscle performance in a very fine event at molecular level, i.e., at the level of the motor domain of the different MHC isoforms.

Although myosin isoforms and the mechanisms we have highlighted play a major role, we cannot forget other factors. Muscle metabolism [2], the way muscle fibers are organized between the tendons (muscle architecture) [38], and the characteristics of the tendons [39] are also among the relevant determinants of skeletal muscle performance in vivo.

Our story is far from ending as new questions keep arising. We have recently suggested that slow and fast isoforms might not differ just in the rate of ADP release, but also in the relative contribution of the phases determining the attached state [40]: in slow isoforms the time of attachment might actually depend on the rate of ADP release, whereas in fast isoforms the rate of acto-myosin dissociation by ATP might unexpectedly play a significant role. This suggestion has been very recently supported also by Iorga et al. [41]. Moreover, our laboratory and other laboratories have been working on the molecular mechanisms on the basis of the functional diversity among myosin isoforms, comparing the amino acid sequence of different isoforms [13, 42]. The latter studies are bound to answer another important question: why do skeletal myosin isoforms show different kinetics of interaction with actin, i.e., which are the amino acid differences involved in myosin isoform diversity?

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Chapter 2

Physical Inactivity is the Main Cause of the Metabolic Syndrome

Pierpaolo De Feo, Chiara Di Loreto, Anna Ranchelli, Cristina Fatone, Paola Lucidi and Fausto Santeusanio

Introduction

The metabolic syndrome is characterized by the coexistence of visceral adiposity, impaired fasting glucose or overt diabetes mellitus, reduced HDL cholesterol, and increased blood pressure and triglycerides. In Western and developing countries the prevalence of the metabolic syndrome is rising because the explosion of the twin epidemics: obesity and diabetes [1-5]. Visceral adiposity plays a key role in the subsequent manifestation of diabetes and of the full metabolic syndrome. The present article sustains the hypothesis that obesity, diabetes, and the metabolic syndrome are increasing mainly because people no longer need to be physically active in their daily lives [1-5].

There is enough evidence in literature demonstrating that physical inactivity is the main cause of the metabolic syndrome. Gerald Reaven in a recent review on the metabolic or X syndrome concludes: "Obesity is not a component of syndrome X, because in contrast to the other variables, it is not a consequence of insulin resistance but only increases the likelihood of an individual becoming insulin resistant and developing the associated adverse consequences. In the same vein, physical inactivity acts similarly to obesity in increasing the likelihood that insulin resistance will develop, and results of prospective studies have shown that physical inactivity seems to be as potent as obesity, if not more so, in increasing risk of developing type 2 diabetes mellitus or CVD" [6, 7]. Accordingly, there is enough evidence in literature demonstrating that physical activity is an effective therapeutic tool for prevention and management of type 2 diabetes mellitus. Intervention trials have demonstrated that in subjects with impaired glucose tolerance diet plus exercise programs reduce by ~60% the risk of developing diabetes [8, 9]. In subjects with overt type 2 diabetes, diet and exercise produce greater weight loss and allow greater reductions in hypoglycemic medications than diet alone [10-12]. Many studies have shown that maintaining

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a regular regimen of physical activity improves quality of life, reduces the risk of mortality from all causes [1-4], prevents type 2 diabetes mellitus in subjects with impaired glucose tolerance [8, 9], and enhances glucose control in subjects affected by type 2 diabetes mellitus [10-12].

Mechanisms Mediating the Beneficial Effects of Exercise

Exercise reduces blood glucose through an increase of insulin-dependent and insulin-independent glucose transport to working muscles [13]. Exercise increases the translocation of glucose transporter 4 (GLUT 4) to the surface of muscle cells [14]. There is evidence for the presence of two distinct pools of GLUT4 in skeletal muscle, one responding to exercise and one responding to insulin [15, 16]. Muscle contraction increases the AMP/ATP and creatinine/phosphocreatinine ratios, which rapidly activate adenosine monophosphate protein kinase (AMPK), a key mediator of fatty acid oxidation [17] and glucose transport [18] in mammalian cells. During muscle contraction, AMPK appears to produce the translocation of GLUT 4 of either the insulin-dependent [16] or the insulin-independent [15] pools. In type 2 diabetic subjects, physical training increases insulin-stimulated nonoxidative glucose disposal [19, 20], presumably activating glycogen synthesis. The beneficial effects of regular physical activity on insulin sensitivity appear to be the final result of specific effects of exercise on GLUT 4 content, oxidative capacity, and capillary density of skeletal muscle. Preliminary data suggest that insulin-independent glucose transport, induced by exercise, is promoted by augmented endothelial and muscle production of nitric oxide [21, 22]. Since impaired nitric oxide production often complicates type 2 diabetes mellitus, physical exercise might be utilized to improve insulin sensitivity and endothelial dysfunction as well. The effects of exercise on endothelial function might also be responsible for the reduction of blood pressure induced by regular physical activity.

A central role of exercise in the prevention and treatment of the metabolic syndrome is the exquisite sensitivity of visceral fat to physical activity. Abdominal fat is quickly released to sustain ATP production during moderate intensity aerobic exercise [23]. Thus, constant physical activity results in a reduction of visceral fat and an improvement of the features of the metabolic syndrome [12].

Motivation to Physical Activity

Despite the evidence about the benefits of exercise, many diabetologists do not spend time and efforts convincing type 2 diabetic subjects to practice physical activity. It is likely that the limited diffusion of exercise as a standard therapeutic tool among endocrinologists is caused by the poor adher-

ence of older adults to comply with their recommendations. Survey studies have shown that adults with diabetes are less likely than adults in general to engage in regular physical activity [24] and that only 23% of older adults with type 2 diabetes reported >60 min of weekly physical activity [25]. There is the need for simple and reproducible strategies of counseling to motivate type 2 diabetic patients to the practice of exercise. Recently, we have demonstrated that using an individual behavioral approach, primarily based on the social learning theory [26], it is possible for physicians to motivate the majority of type 2 diabetic subjects to long-term practice of exercise [27]. The intervention consisted in a first counseling of at least 30 min conducted by a endocrinologist and designed to advise physical activity, followed, after 1 month, by home calling and every 3 months by an ambulatory visit of about 15 min [27]. The intervention was effective in reducing BMI, HbA1c, coronary risk, and treatment costs with a significant correlation between the amount of voluntary physical activity and the beneficial effects [12].

The demonstration that physical activity counseling can motivate most diabetic subjects to increase their levels of voluntary energy expenditure (EE) [27-28] outlines the importance of instituting physical activity programs as an essential part of therapy for type 2 diabetes mellitus. The ADA (American Diabetes Association) emphasizes the benefits of regular physical activity in the prevention and treatment of type 2 diabetes mellitus, referring to proposals given to general population by several scientific societies [29]. These recommendations advise individuals to engage in 30 min or more moderate-intensity physical activity on most (preferably all) days of the week. To maintain long-term weight loss, data from several studies suggest that more physical activity (60-75 min/day) is needed [30].

A rationale use of physical activity to prevent and treat type 2 diabetes mellitus requires the information about the amount of voluntary EE required to obtain significant benefits and about the minimum improvement in physical fitness that is associated with reduced mortality rates in diabetic and obese individuals. Both targets, EE and physical fitness, can be quantified using as a unit of measure the metabolic equivalent (MET). One MET corresponds to the consumption of 3.5 $ml \cdot kg^{-1} \cdot min^{-1}$ of oxygen, which is the average amount utilized by the human body in the resting state. Physical activity increases oxygen consumption (VO_2) by contracting muscles in relationship to the quantity of activated muscles and exercise intensity. Thus, measuring VO_2, it is possible to calculate the multiples of MET required for different activities in humans [31]. METs can be used either to describe the status of physical fitness by measuring the work load that a person can achieve before exhaustion (VO_{2max}) or the amount of EE consumed through physical activity over a period of time. The latter measure is commonly expressed as METs-h/week and calculated as the product of the duration (hours x week) of the different activities weighted by an estimate of MET intensity of each activity. Increased levels of EE can be

achieved either through structured leisure-time physical activity or by focusing on easy-to-perform daily activities such as walking the dog, washing the car, or avoiding the elevator as often as possible, etc. The total amount of EE will be the result of physical activity duration (PAD) and the intensity at which it is performed. It has been proposed to classify physical activities in moderate (3-6 METs), vigorous (6-9 METs) and very vigorous (>9 METs) [32]. However, such a categorization cannot be generalized because the intensity of physical activity is strictly related to the VO2max of subjects. For instance, an intensity of 10 METs is a moderate effort for elite athletes in aerobic sports who are able to maintain intensities over 20 METs for more than 1h. In order to better individualize the levels of intensity of physical activity a practical approach might be to compare the rates of perceived exertion, using the Borg's scale [33], with the objective measurement of METs achieved (see below).

How Much Physical Activity is Beneficial for Subjects with the Metabolic Syndrome?

To answer this question we have recently examined the 2-year impact of different increments in EE through leisure-time physical activity on several physiological and biochemical outcomes, on direct medical costs and on direct and indirect social costs in a group of type 2 diabetic subjects who were randomized to a physical activity counseling intervention [12]. The intervention resulted in remarkable cost savings; health benefits and financial advantages were significantly related to increased amounts of EE. Our results confirmed that the advice of several scientific societies [1-4, 29] recommending 30 min or more moderate-intensity physical activity (>10 METs/h/week) on most days, if not every day, is also valid for type 2 diabetic subjects and demonstrate a significant dose/response relationship. Post-hoc analysis showed that EE must be >10 METs/h/week for significant beneficial effects. In fact, EE ranging between 11 and 20 METs/h/week significantly reduced HbA1c, total cholesterol, triglycerides, and blood pressure with a 2.6±0.6% reduction of 10-year CHD risk. These benefits occurred in the absence of any significant weight loss, suggesting that regular aerobic physical activity improves glucose control, lipid profile, and blood pressure independently of weight reduction. This conclusion concurs with results of a meta-analyses examining the effects of physical activity on glucose control in type 2 diabetes mellitus [34] and on blood pressure in general population [35].

Regarding the effects of physical activity on body weight, our results, based on anthropometric measurements (body weight, waist circumference), confirm that visceral fat is a very sensitive target of physical activity (waist circumference vs. EE: r=-0.77) and that to induce long-term losses of body weight increments in EE >20 METs/h/week are required [1-4, 29, 36].

Constant EE >20 METs/h/week are needed to decrease BW, BMI, waist circumference, heart rate, and LDL cholesterol and augment HDL cholesterol. This amount of EE induces greater reductions in HbA1c, total cholesterol, triglycerides, and blood pressure leading to a ~4%-5% decrease in the 10-year CHD risk. Results in our type 2 diabetic subjects confirm reports of prospective studies in the general population which show an inverse linear dose-response between amount of physical activity and all-cause mortality, total CV disease, and CHD incidence and mortality [1-4, 29].

As has been shown in the general population [2], the health benefits of physical activity tend to become less evident in patients when the increase in EE is beyond a certain level, as was indicated by benefit analysis in groups with more than 20 METs/h/week [12]. Since the threshold for full benefits was observed in group G 21-30 (average EE: 27 METs/h/week), we recommend a target of 27 METs/h/week as a reasonable target of EE for previously sedentary type 2 diabetic subjects [12]. This goal corresponds to a 3-mile (~5 km) daily walk (1h/day at a pace of 3 miles/h or 45 min/day at a pace of 4 miles/h) which, according to our results, might be expected over 2 years to reduce BW by 2.4 kg, BMI by 0.9 kg/m^2, waist circumference by 4.8 cm, fasting plasma glucose by 0.9 mM, HbA1c by 1.5%, systolic and diastolic BP by 10 and 7 mmHg, resting HR by 5 bpm, triglycerides by 0.4 mM, 10-year CHD risk by 2.4% and to augment HDL cholesterol by 0.12 mM. However, it must be emphasized that greater amounts of EE resulted in added improvements in anthropometric and biochemical markers of the metabolic syndrome; there was a significant ($p<0.05$) linear relationship between the amount of EE and a series of biological parameters and cost savings as reported in Table 1. The extrapolations reported in Table 1 might be used to explain to diabetic subjects the beneficial effects expected by the increase in their weekly levels of EE.

Table 1 Expected average effects of increased EE (METs/ h/week) through leisure-time physical activity over 2 years of time in subjects with type 2 diabetes mellitus (data extrapolated from significant correlations obtained by [12]

	15	20	25	30	40	50
BW Kg	−1.2	−1.6	−2.0	−2.4	−3.2	−4.0
Waist cm	−2.4	−3.2	−4.0	−4.8	−6.2	−8.0
HB A1c %	−0.3	−0.4	−0.5	−0.6	−0.8	−1.0
PA max mmHg	−2.1	−2.8	−3.5	−4.2	−5.6	−7.0
PA min mmHg	−1.2	−1.6	−2.0	−2.4	−3.2	−4.0
Heart rate bpm	−2.4	−3.2	−4.0	−4.8	−6.2	−8.0
COLL HDL mg %	+2.1	+2.8	+3.5	+4.2	−5.6	+7.0
TG mg %	−19.0	−26.0	−32.0	−38.0	−52.0	−64.0
CHD %	−1.2	−1.6	−2.0	−2.4	−3.2	−4.0
Insulin Ut/day	−5.0	−7.0	−9.0	−11.0	−14.0	−18.0
Drugs euro/year	−300.00	−400.00	−500.00	−600.00	−800.00	−1.000.00

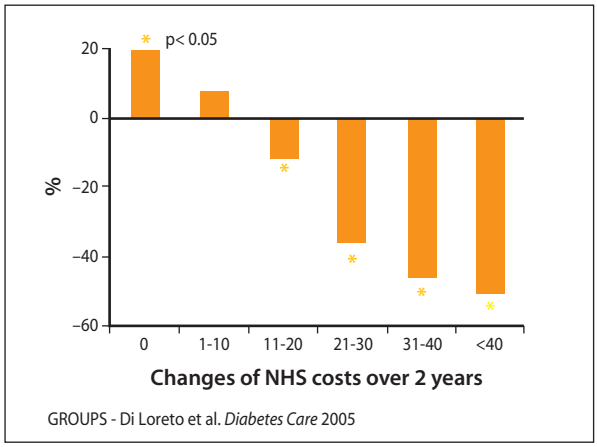

Fig. 1 Effects of increased EE (METs/week) through leisure-time physical activity over 2 years in subjects with type 2 diabetes mellitus on percent reduction in costs paid by National Health Service. Data from [12]

Cost analysis indicates remarkable financial savings (Fig. 1). Over 2 years, prescription costs, which were usually medication for diabetes, hypertension, and dyslipidemia were reduced by $259 and other health care costs by $298 per capita/year in the entire group, even allowing for the costs of counseling, which took a physician 75 min over the first year and 60 min over the second. Post-hoc subgroup cost analysis demonstrated that as with the health benefits, the greatest financial benefits were achieved by groups with EE >20 METs/h/week (average EE: 27 METs/h/week). Money saving tends to become less evident when the increase in EE is beyond a certain level. In subjects with highest EE (Group >40), savings due to lower medical costs and indirect social costs are partially counterbalanced by the increased costs of physical activity i.e., exercise apparel, footwear, and gym costs. Thus, also from the financial point of view, recommending EE amounts of 27 METs/h/week appears to be an appropriate target for previously sedentary type 2 diabetic subjects. In terms of PAD, the target of 27 METs/h/week corresponds to about 1h/day of moderate intensity physical activity (3-6 METs) or to 30-40 min/day of vigorous physical activity (6-9 METs).

How Much Physical Fitness is Required for Subjects with Type 2 Diabetes Mellitus?

Prospective studies performed have demonstrated that even modest increments of physical fitness in obese or type 2 diabetic individuals can reduce by about twofold the risk of overall mortality [37, 38]. The results of these studies agree that a reasonable target might be an improvement of 3-4 METs, which would increase from 6-7 to about 10 METs the maximal performance of middle-age type 2 male diabetic subjects [37, 38]. Such an improvement

corresponds to an amelioration of VO_{2max} of 10-13 ml $kg^{-1} \cdot min^{-1}$ that is achievable by most subjects after an aerobic training program of 6-12 months [33].

Methods to Quantify EE and Physical Fitness in Type 2 Diabetes Mellitus

Levels of voluntary physical activity can be assessed using questionnaires. A series of validated questionnaires has been reviewed by Kirska and Caspersen [39]. The Diabetes Prevention Program [9] and our study [12] used the Modifiable Activity Questionnaire [39]. Energy expenditure was calculated as the product of the duration (hours x week) of the different activities weighted by an estimate of metabolic equivalent (MET) of each activity. The major limitations of calculating EE through questionnaires are: (1) it is not possible to rule out that some patients might over report their amount of physical activity; (2) not all patients are willing to compile a diary; (3) to convert the questionnaires filled by patients into METs is time consuming. The recent availability of wearable body monitoring devices might overcome these drawbacks and offers a direct measurement of "free-living" activity more feasible and accurate than previous methods. One such device, the SenseWear ArmBand (BodyMedia, Inc.), has been validated in normal subjects in the resting state [40] and during exercise [41], and in a small group of type 2 diabetes mellitus subjects [42] as a measure of daily physical activity. The Sense Wear Armband is a multisensor piece of equipment, worn on the triceps of the right arm for up to 2 weeks continuously, that uses physiological body signals from five sensors (skin temperature, near body temperature/heat flux, galvanic skin resistance, two accelerometers) in combination with free-living activity recognition patterns to calculate energy consumption based on specific algorithms.

The results of SenseWear ArmBand were compared to the doubly labeled water (DLW) technique over a period of 10 days in six diabetic patients treated with diet only and/or oral hypoglycemic agents [42]. The results of this preliminary study are promising because it demonstrated that the correlation between the armband and DLW reached $r = 0.9696$ ($P=0.0014$) and the authors hypothesized a narrow limit of agreement (±100-300kcal/day) between the two methods. From a practical point the Sense Wear ArmBand might be used in subjects affected by the metabolic syndrome to gain information about: (1) the basal metabolic rate of patients (average EE during resting hours); (2) EE consumed through spontaneous, moderate, or high intensity physical activity (setting different ranges of METs); (3) the status of physical fitness by recording the peak of METs achieved during high intensity physical activity; (4) the hours and the quality of sleep (constant or intermittent); (5) the accuracy of a physical activity diary report. Furthermore, the report of ArmBand can be used to discuss with the patient whether the targets of

physical activity were achieved and to plan together the next steps to reach, in order to increase long-term compliance to regular activity. Accurate recording of EE is useful for a rationale individualization of long-term weight-loss programs. Knowledge of caloric consumption allows small but reliable reductions in the amount of daily caloric intake as a deficit of only 200-300 Kcal. On a long-term basis, such caloric shortage is better accepted by patients and combined with physical activity would result in a progressive and selective loss of fat mass, sparing lean body mass, and basal metabolic rate.

Conclusions

Data of literature showing that modest increments of physical fitness in diabetic subjects reduce by twofold the risk of overall mortality [37, 38] support the establishment of physical activity programs in the cure of type 2 diabetes mellitus and/or the metabolic syndrome. Since it is possible to motivate the majority of persons with metabolic syndrome to engage in the long-term practice of physical activity, it is time to move exercise from theory to daily ambulatory practice. In a recent web document (http://www.who.int/dietphysicalactivity/publications/facts/pa/en/index.htm) the WHO (World Health Organization) states: "Physical inactivity is estimated to cause 2 million deaths worldwide annually. Globally, it is estimated to cause about 10%-16% of cases each of breast cancer, colon cancers and diabetes, and about 22% of ischemic heart disease." We have to do our best to direct our patients to use human genes for the scope they have been selected over millions of years: physical activity.

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SECTION II

Mitochondrial Biogenesis and Physical Exercise

Chapter 3

Exercise-Induced Mitochondrial Biogenesis in Skeletal Muscle

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Introduction

Regularly performed endurance exercise has a number of health benefits, including improvements in cardiovascular function, muscle metabolism, and increased work capacity. The increase in endurance is a result of greater oxygen delivery and extraction by the exercising muscle. Oxygen extraction is a result of an improved capillary-to-fiber ratio, as well as a higher mitochondrial content within muscle. The increase in mitochondrial content is a well-established and dramatic adaptation within the exercised muscle, but the molecular mechanisms underlying this change in muscle phenotype are just beginning to be clarified. An understanding of the cellular processes involved could help in the development of therapeutic applications other than exercise, and may help us better comprehend the pathology of mitochondrial diseases. This increase in mitochondrial content which occurs as a result of regular exercise is referred to as mitochondrial biogenesis. The process is complex because mitochondria are composed of proteins encoded by both nuclear and mitochondrial DNA (mtDNA). The major steps involved include: (1) signaling events leading to transcription, brought about by each exercise bout; (2) transcriptional regulation of nuclear-encoded genes encoding mitochondrial proteins, mainly mediated by the coactivator PGC-1α; (3) control of mitochondrial DNA gene expression by the transcription factor Tfam; (4) mitochondrial fission and fusion mechanisms; (5) import of nuclear-derived gene products into the mitochondrion via the protein import machinery; and (6) assembly of nuclear- and mitochondrially-encoded subunits into functional holoenzyme complexes. A summary of these steps is provided in Figure 1. An additional complicating factor in mitochondrial biogenesis is the fact that mitochondrial

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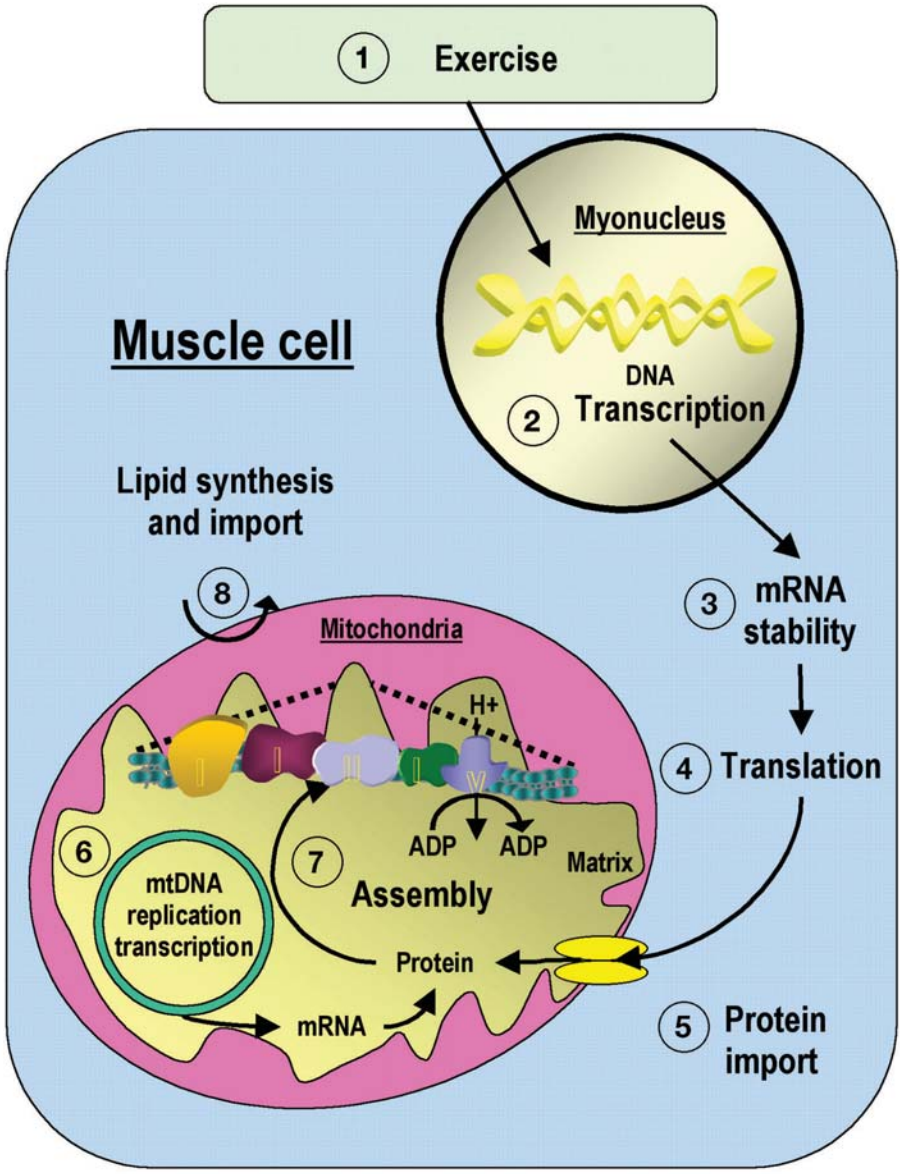

Fig. 1 The steps involved in exercise-induced mitochondrial biogenesis. (*1*) Exercise initiates a unique set of intracellular signals involving calcium, ROS, and ATP turnover to promote changes in DNA transcription in the nucleus (*2*), and mRNA stability (*3*) in the cytosol. The resulting mRNAs are translated into proteins (*4*), which are then targeted and translocated into the mitochondria via the protein import machinery (*5*). Simultaneously, mtDNA transcription (*6*) is initiated by putative signals acting on transcription factors such as mitochondrial transcription factor A (Tfam), creating mRNAs encoding 13 subunits of the electron transport chain. Nuclear- and mitochondrially-encoded proteins are then assembled (*7*) together to form complexes that are incorporated into the ETC. Finally, mitochondrial biogenesis also requires the synthesis and import of lipids (*8*) into the outer and inner mitochondrial membranes in order to increase mitochondrial volume within the cell

structure differs markedly among cell types, and even within different regions of a specific cell type. For example, in skeletal muscle, mitochondrial properties differ between those organelles located under the sarcolemma [subsarcolemmal (SS) mitochondria] and those between the myofibrils [intermyofibrillar (IMF) mitochondria]. It is now known that exercise can modify the rates of several of the steps leading to mitochondrial biogenesis, thus establishing exercise as an extremely useful model for understanding the underlying mechanisms involved in organelle synthesis. Recently, several breakthroughs in our understanding of the initiation of mitochondrial biogenesis have occurred, with the discovery of an important overall regulator of the process, PGC-1α. In this paper we will review our current understanding of mitochondrial regulatory proteins, the signals leading to mitochondrial biogenesis during exercise, as well as mitochondrial biogenesis during aging and muscle disuse. A number of other related reviews have also recently been published on this topic [1-3].

The Role Of PGC-1α in Exercise-Induced Mitochondrial Biogenesis

PPARγ coactivator-1α (PGC-1α) has been termed the "master regulator" of mitochondrial biogenesis because of its ability to induce mitochondrial biogenesis in a variety of experimental models. In mouse C2C12 skeletal muscle cells, ectopic PGC-1α expression increases mitochondrial content and oxygen consumption [4]. In addition, overexpression of PGC-1α in skeletal muscle of transgenic mice is sufficient to coordinate a host of muscle adaptations reminiscent of endurance exercise training, including increased mitochondrial content, increased proportion of Type I muscle fibers, and a corresponding increase in muscle fatigue resistance [5].

PGC-1α Coactivates the Transcription of Genes Involved in Mitochondrial Biogenesis

PGC-1α binds to and coactivates DNA-binding transcription factors, thus augmenting their activity. The primary targets of PGC-1α in the initial stages of mitochondrial biogenesis are the nuclear respiratory factors, NRF-1 and NRF-2. PGC-1α increases the expression of both these transcription factors, and coactivates NRF-1-mediated transcription [4]. NRF-1 and/or NRF-2 binding sites are found in the promoter regions of several nuclear genes encoding mitochondrial proteins, including cytochrome c, components of all five electron transport chain complexes, mitochondrial import proteins, heme biosynthesis proteins, and the mitochondrial transcription factors Tfam, TFB1M, and TFB2M [6, 7]. PGC-1α coactivates NRF-1 in transcribing

all three of these mitochondrial transcription factors, which then act to replicate and transcribe the mitochondrial genome. Thus, working through NRF-1, PGC-1α coordinates the bi-genomic regulation of mitochondrial biogenesis. In addition to NRF-1, PGC-1α also coactivates estrogen-related receptor α (ERRα) [8]. Knockdown of ERRα diminishes PGC-1α-induced mitochondrial biogenesis, while ERRα overexpression increases mitochondrial protein expression [8].

Post-Translational Modification of PGC-1α Alters its Activity

PGC-1α is recruited to promoters by DNA-binding transcription factors such as NRF-1. Upon transcription factor docking, PGC-1α undergoes a conformational change that facilitates the association of two other coactivator proteins, steroid receptor coactivator-1 (SRC-1) and CREB-binding protein (CBP)/p300 [9]. Both SRC-1 and CBP/p300 acetylate histones to create a relaxed chromatin structure that is more accessible to the transcription machinery [10]. Thus, PGC-1α bridges sequence-specific transcription factors with chromatin-modifying enzymes to regulate local gene transcription.

PGC-1α is also inhibited when bound by a repressor protein termed p160MBP. This repression is relieved by p38 MAPK (p38)-mediated phosphorylation of PGC-1α, which blocks the physical interaction of PGC-1α and p160MBP [11]. In addition to posttranslationally activating PGC-1α, p38 also increases the expression of PGC-1α. p38 is activated in skeletal muscle of exercising humans [12] as well as electrically stimulated C2C12 cells [13], conditions that also result in the upregulation of PGC-1α [13, 14]. Moreover, transgenic mice expressing constitutively active MKK6, which activates p38, have higher PGC-1α and COXIV protein levels than wild-type (WT) mice [15].

In addition to phosphorylation, PGC-1α is also subject to methylation and acetylation. Methylation of PGC-1α by arginine methyltransferase 1 (PRMT1) is necessary for maximal PGC-1α-mediated expression of cytochrome c and ERRα [16]. Reversible acetylation of PGC-1α also regulates its coactivation activity [17, 18], but the effect that this modification has on PGC-1α-mediated transcription of genes involved in mitochondrial biogenesis is not fully defined. Deacetylation of PGC-1α by SIRT1 enhances the ability of PGC-1α to coactivate the transcription of gluconeogenic genes, but has no effect on the coactivation of cytochrome c and β-ATP synthase transcription [18]. On the other hand, acetylation of PGC-1α by GCN5 represses the PGC-1α-mediated expression of these two proteins [17]. Thus, multiple posttranslational modifications can alter the ability of PGC-1α to coactivate the transcription of genes involved in mitochondrial biogenesis.

Endurance Exercise Induces the Expression of PGC-1α

PGC-1α expression is dynamically regulated by altered patterns of physical activity. In response to a single bout of exercise, PGC-1α mRNA and protein are significantly elevated in mice, rats, and humans [15, 19-23]. This increase in gene expression is evident as early as 2h postexercise [20, 21, 24]. Interestingly, PGC-1α protein has been shown to progressively increase over the course of a 53-day training program in rats [25], suggesting that multiple bouts of exercise are required to maximize PGC-1α levels.

Although exercise elicits numerous systemic changes in the body, the exercise-induced PGC-1α upregulation appears to be independent of these factors. Evidence for this comes from the fact that contractile activity alone (i.e., no humoral influence) is sufficient to increase PGC-1α expression. This has been shown in whole rats subject to chronic low frequency stimulation [13], in electrically stimulated isolated epitrochlearis muscle [22], and in C2C12 cells electrically stimulated in culture [13]. Together, these studies point to contractile activity as the main stimulus for exercise-induced PGC-1α upregulation.

Multiple Exercise-Induced Signals Converge to Increase PGC-1α Transcription

How changes in muscle activity are transduced to produce alterations in gene transcription and subsequent phenotypic adaptations is an important question in exercise physiology. The exercise-induced signals that regulate PGC-1α expression have been the subject of much investigation. The increase in PGC-1α mRNA following acute exercise is at least partly due to an increase in transcription [21], and the major exercise-induced signals appear to act on PGC-1α expression at this level. CaMK and p38 MAPK increase PGC-1α promoter activity through the activation of cAMP response element (CRE) binding protein (CREB) and activating transcription factor 2 (ATF2), respectively [15, 26]. Both of these transcription factors bind the same DNA element, namely the CRE. In addition, the PGC-1α promoter contains a binding site for myocyte enhancer factor 2 (MEF2), a transcription factor that is activated by both CaMK and p38 [27]. Electrical stimulation of skeletal muscle in mice activates the PGC-1α promoter, and this effect is abolished when either the MEF2 or CRE binding site is mutated [28]. Interestingly, PGC-1α activates its own promoter by coactivating MEF2, an effect that is augmented by the Ca2+-dependent phosphatase calcineurin [26]. These studies point to the cooperative action of MEF2, CREB, and ATF2 transcription factors in altering PGC-1α transcription in response to multiple exercise-induced signals.

Can Exercise Induce Mitochondrial Biogenesis in the Absence of PGC-1α?

It is clear that PGC-1α is sufficient to induce mitochondrial biogenesis. However, whether it is necessary for exercise-induced mitochondrial biogenesis is not fully resolved. Leone et al. [29] reported that mitochondrial volume is lower in skeletal muscle of PGC-1α knockout (PGC-1α-/-) mice than in WT mice, with a concomitantly reduced expression of Tfam, cytochrome c, and COXIV. In contrast, Arany et al. [30] found no difference in mitochondrial volume in skeletal or cardiac muscle of PGC-1α-/- mice versus WT mice. Nevertheless, both groups showed that PGC-1α-/- mice suffer a reduced capacity to increase work output to match an increase in metabolic demand in slow-twitch muscle [29] and in heart [30]. Specifically, PGC-1α-/- mice display a diminished capacity for endurance exercise and fatigue resistance [29] and exhibit signs of cardiac dysfunction at an early age [30]. Thus, it is clear that PGC-1α plays a vital role in the ability of muscle to adapt to heightened energy demands, but the effects of PGC-1α on other tissues such as brain [31] represent potential confounding variables in these studies. Using an inducible PGC-1α knockout construct to specifically target skeletal muscle of mice at the time of experimentation, and placing the mice on a voluntary running program may further define the exact role of PGC-1α in exercise-induced mitochondrial biogenesis.

In response to exercise, the pre-existing pool of PGC-1α is activated, leading to a coordinated upregulation of proteins involved in mitochondrial biogenesis. Since one of these proteins is PGC-1α itself, this action forms an autoregulatory positive feedback loop leading to an expansion of the PGC-1α pool. Due to the central role played by PGC-1α in exercise-induced mitochondrial biogenesis, and its dynamic regulation by exercise-associated molecular events, PGC-1α represents a nodal point between human movement and alterations in gene expression with tremendous potential for therapeutic manipulation.

The Role of Calcium, AMP Kinase, and Reactive Oxygen Species (ROS) Signaling to Mitochondrial Biogenesis

Ca^{2+} Signaling and Exercise

In skeletal muscle, Ca^{2+} acts as an essential regulatory and signaling molecule. It is through the actions of Ca^{2+} that the characteristic contractile properties of muscle are determined. However, it is the alternate patterns of α-motoneuron activation that ultimately generates the various Ca^{2+} waveforms, which are then responsible for the distinctive programs of gene

expression within fast or slow myofibers. α-Motoneurons that innervate slow oxidative myofibers fire almost continuously, creating a cytosolic Ca^{2+} range that oscillates between 100 and 300 nM [32]. Alternately, α-motoneurons that innervate fast-glycolytic fibers usually fire intermittently, with Ca^{2+} concentrations reaching as high as ~1 μM. Experiments involving the cross-innervation of a muscle fiber (e.g., taking a slow oxidative myofiber and innervating it with a fast-glycolytic α-motoneuron) or electrically stimulating a muscle would alter the rate and pattern of the Ca^{2+} waveform within the tissues. This alteration has been strongly implicated in muscle phenotype adaptations to contractile activity [33, 34].

Gene expression of respiratory proteins in skeletal muscle has been linked to intracellular Ca^{2+} signaling mediated by Ca^{2+}-dependent regulatory enzymes, calcineurin, Ca^{2+}/calmodulin-dependent protein kinase (CaMK), and protein kinase C (PKC). Ca^{2+}-regulated calcineurin stimulates transcription through the regulation of the transcription factors NFAT and MEF2. Furthermore, CaMKIV activity enhances calcineurin-dependent MEF2 activation through the disruption of MEF2-class II histone deacetylases (HDACs) interactions, which frees up bound MEF2 [27]. Calcineurin dephosphorylates NFAT in response to increased intracellular calcium and regulates gene expression. While the calcineurin and NFAT signaling pathway functions in skeletal myotube formation, myofiber-type switching, and myocyte hypertrophy [35], it may not play a direct role in PGC-1α-induced mitochondrial biogenesis [36]. When the calcineurin inhibitor cyclosporin A (CSA) was injected into rats throughout an exercise training protocol there was no reduction of the exercise-induced increase in PGC-1α expression [36]. However, CSA was found to potentially inhibit a posttranscriptional step in the expression of COX-I and COX-IV, therefore providing a possible explanation for the observed decrease in skeletal muscle substrate oxidation. In contrast, transgenic mice possessing a constitutively active CaMKIV exhibited increased mitochondrial biogenesis in skeletal muscle along with enhanced regulation of mitochondrial gene expression [37]. This experiment demonstrated that elevated PGC-1α expression followed by mitochondrial biogenesis is dependent on CaMK, which is regulated by increases in cellular Ca^{2+} [37]. However, the response of CaMKIV knockout mice to endurance training, including an upregulation of PGC-1α, is indistinguishable from that of WT mice [15]. Nevertheless, other CaMK isoforms, more abundantly expressed in muscle, may still be important for the exercise-induced upregulation of PGC-1α [38]. In addition, when intracellular Ca^{2+} levels in muscle cells are increased using the ionophore A23187, [5, 7] there is a concurrent increase in cytochrome c gene expression through the PKC-dependent pathway [39]. Together these observations demonstrate the regulation of mitochondrial content by effector proteins that first sense changes in intracellular Ca^{2+} levels, then manipulate the expression of tran-

scription regulatory factors, such as MEF2, NFAT, and PGC-1α to induce increases in mitochondrial biogenesis.

Reactive Oxygen Species and Exercise

Aerobic exercise is intrinsically linked to increased oxygen consumption. During exercise the body as a whole uses ~10-fold more oxygen than at rest, while in muscle tissue oxygen consumption may increase by 50 to 100-fold [40]. Various studies have demonstrated a connection between this increase in oxygen consumption during exercise and the formation of reactive oxygen species (ROS). In addition it has been shown that macromolecular structures within the cell can be damaged by elevated ROS. In fact, oxidative stress contributes to the accumulation of somatic mutations and oxidative damage to mtDNA. This has been apparent in mitochondrial diseases [41], tumorgenesis [42], aging [43], degenerative diseases [44, 45], and diabetes [46]. However, skeletal muscle not only has the ability to produce ROS, but also has an elaborate system to regulate these reactive molecules and prevent their damaging effects. The cellular antioxidant defense system includes the mitochondrial and cytosolic forms of superoxide dismutase (MnSOD and CuZnSOD, respectively), glutathione peroxidase (GPx), and catalase [47]. The cell also contains several known scavengers of ROS such as vitamin E, ascorbate, and glutathione. MnSOD and CuZnSOD convert superoxide radical ($O_2^{\cdot-}$) to H_2O_2, which is then transformed to water by GPx, or to water and oxygen using catalase. Skeletal muscle has a larger proportion of SOD enzymatic activity in high oxidative type I fibers, compared to low-oxidative type IIb fibers. The high oxidative fibers also respond to endurance training with a more robust induction of SOD activity following endurance training in comparison to fibers with low oxidative capacity [48].

Under normal conditions Ambrosio et al. [49] demonstrated that the majority of ROS formation originates from the mitochondrial respiratory chain. The measured percent of oxygen converted to ROS is approximately 1%-4% of that which is consumed by the ETC [50]. However, a growing body of evidence has shown that the increase in ROS production due to exercise may also be generated by alternative sources. McArdle et al. [51] showed that ROS are also released into the extracellular fluid of the muscle following bouts of contractile activity. However, Jackson et al. [52] proposed that since superoxide is a charged and highly reactive molecule, it would not easily pass though the mitochondrial membrane and therefore would not affect the levels of extracellular superoxide. This was also observed in experiments that examined ROS production in genetically modified mice with reduced levels of mitochondrial MnSOD antioxidant activity [51]. With contractile activity, these mice displayed an elevated level of mitochondri-

al ROS while exhibiting a lack of change in extracellular ROS. Therefore, it was proposed that the flavoprotein oxidoreductase system, located at the plasma membrane, is a predominant generator of extracellular superoxide during contractile activity [53].

Signaling pathways involving ROS have also been shown to induce mitochondrial biogenesis. MtDNA copy number has been shown to change with rising levels of ROS in aging tissues in the brain [54], lung [55], and skeletal muscle [56]. This increase in mtDNA within aging cells may represent a feedback response that compensates for mitochondrial mutations, or impaired respiratory chain functioning [57]. The increase in mtDNA was accompanied by an induction in mitochondrial mass. This response appeared to be mediated by PGC-1α and NRF-1, as the expression of both increased following exogenous ROS treatment [58]. Altogether, these observations clearly demonstrate that either endogenous or exogenous oxidative stressors can induce increases in mitochondrial abundance and mtDNA copy number. This may be due to an alteration in the redox state that could trigger a signal between mitochondria and the nucleus that ultimately upregulates the various genes involved in mitochondrial biogenesis.

There are several signaling pathways that may be involved in the induction of mitochondrial biogenesis in response to ROS, such as the well-known transcription factors AP-1 and NF-κB [2]. These transcription factors may be responsible for the increased expression of NRF-1 and PGC-1α; however, few links have been found to support this hypothesis. In addition, the activation of the phosphatidylinositol 3′-kinase-Akt (PI3K-Akt) pathway was found to be associated with ROS signaling events resulting in increased mitochondrial biogenesis in hepatic mitochondria following LPS-induced liver damage [58]. These results indicated that P13K-Akt might also play an essential role in the activation of mitochondrial biogenesis through oxidative signaling pathways in liver.

AMPK Activation with Exercise

5′-AMP activated protein kinase (AMPK) has been described as an energy-sensing enzyme that actively responds to cellular conditions that are associated with energy depletion. Thus, AMPK is activated by a high AMP:ATP ratio, such as that which occurs during repeated muscle contractions. AMPK has been shown to be activated by exercise in both animals [59] and humans [60]. AMPK is a heterotrimer that consists of a catalytic α subunit and two regulatory subunits, β and γ [61]. Skeletal muscle expresses both an α1 and α2 isoform of AMPK; however, there are also two known β isoforms and three γ isoforms. The signaling cascade that involves AMPK phosphorylation and activation is the result of a reduction in the ATP:ADP ratio and a con-

current elevation in AMP due to myokinase activity. AMPK is allosterically activated by AMP up to 10-fold [62] and is antagonized by ATP which competes for the same binding site [63]. AMPK can also be phosphorylated and activated by one or more upstream protein kinases that create a more than 100-fold activation. The relative extent of α2 AMPK activation by exercise is altered with training. AMPK is preferentially activated in human vastus lateralis muscle following exercise on a cycle ergometer at 60%-70% of VO_2 max [64]. Activation of α2 AMPK also occurs with 5-aminoimidazole-4-carboxamide riboside (AICAR) treatment. AICAR is taken up by cells and phosphorylated by cellular adenosine kinase to ZMP, an analog of AMP. Pharmacological activation of AMPK by AICAR increases PGC-1α mRNA [22] and protein [13]. The upregulation of PGC-1α transcription and translation is accompanied by the increased DNA binding activity of NRF-1 in rats with elevated levels of activated AMPK [65]. As noted above, NRF-1 is a transcriptional regulator of proteins involved in mitochondrial biogenesis, and it is also strongly upregulated by PGC-1α [4]. Moreover, mice genetically engineered to lack AMPK activity do not display an increase in PGC-1α or mitochondrial content in response to an increased AMP:ATP ratio in skeletal muscle during energy deprivation [66]. In addition, chronic activation of AMPK using AICAR in resting rats has resulted in increases in mitochondrial enzymes such as δ-aminolevulinic synthase, cytochrome c, citrate synthase, and malate dehydrogenase in skeletal muscle [67]. Thus, AMPK activation is another important regulator of mitochondrial biogenesis under conditions of energy deprivation in muscle cells.

Mitochondrial DNA (mtDNA) Transcription Factors

Mitochondria possess their own circular genome of about 16.5 kb termed mitochondrial DNA (mtDNA). mtDNA encodes proteins that function as subunits for respiratory complexes I, III, and IV [6]. However, this represents less than 1% of the total number of gene products that are found within the organelle. Indeed, the proteins that regulate the replication and transcription of mtDNA are nuclear-encoded, and need to be imported into the organelle. One of the most important of these regulatory proteins is mitochondrial transcription factor A (Tfam). Tfam regulates both mtDNA copy number and transcriptional activity. The importance of Tfam is evident from the phenotype exhibited by Tfam knockout mice. Tfam knockout is embryonic lethal, and mtDNA copy number and respiratory chain complex activities are reduced in the heart of heterozygous Tfam knockout mice.

Exercise increases the expression and function of Tfam in muscle. Gordon et al. [68] demonstrated that chronic contractile activity of the rat mus-

cle led to an increase in Tfam mRNA level after 4 days, leading to an accumulation intramitochondrial Tfam protein, an increase in Tfam-mtDNA binding and mtDNA transcript levels encoding COX subunit III, and a higher COX enzyme activity by day 7. A similar increase in Tfam expression has been found following endurance training in humans [69]. Thus, the increase in Tfam expression during the progression of exercise training contributes substantially to mitochondrial biogenesis in skeletal muscle.

Mitochondrial Biogenesis During Chronic Muscle Disuse

Chronic muscle disuse has been applied as an experimental paradigm to investigate adaptations in skeletal muscle since the 1950s [70]. Primarily these models were first administered to delineate how inactivity mediates muscle atrophy [71, 70], but by the early 1970s research began to focus on alterations in protein expression, and the functional capabilities of disused skeletal muscle [72-77]. Currently, there are a number of conditions that represent reduced contractile activity. These include space flight [78, 79], limb immobilization [80-83], denervation [84-86], and bed rest [87]. Under these conditions, a plethora of adaptations in skeletal muscle occur, including a reduction in mitochondrial content and function, with an increase in cellular susceptibility to apoptosis. Furthermore, these alterations are usually most evident within subsarcolemmal (SS) mitochondria, and less so with intermyofibrillar (IMF) mitochondria. Thus, chronic muscular inactivity is a perturbation that can modify skeletal muscle mitochondria, with detrimental metabolic and performance implications.

Alterations in Mitochondrial Function with Chronic Muscular Inactivity

There is strong evidence to suggest that chronic muscle disuse decreases mitochondrial content and whole muscle oxidative capacity. Chronic muscle inactivity has been shown to disrupt the expression of both the nuclear and the mitochondrial genomes [88], and thereby inhibit mitochondrial biogenesis. In particular, prolonged muscle disuse has been shown to decrease cytochrome c mRNA in both slow and fast twitch muscles [80, 89]. This reduction in cytochrome c mRNA exceeds the rate of overall muscle protein loss [80], suggesting that inactivity specifically targets mitochondrial proteins. Furthermore, the enzymatic activities of cytochrome c oxidase [88], succinate dehydrogenase (SDH), citrate synthase (CS) [88], and malate dehydrogenase [75] are all decreased with chronic reductions in muscle use. As a consequence, mitochondria from disused skeletal muscle display a decreased ability to generate ATP [85]. Highly oxidative muscles, such as the soleus, become

more dependent on glycolytic pathways for ATP production during periods of muscle immobilization [77]. This shift in substrate utilization can be partially attributed to a decline in the expression of fatty acid transport proteins, resulting in a decreased import [90]and oxidation [77]of long-chain fatty acids into skeletal muscle. These changes are accompanied by an upregulation in the lactate dehydrogenase-A isoform, which promotes the conversion of pyruvate to lactate [91]. Therefore, chronic muscular inactivity has an impact on mitochondrial protein expression, which influences ATP provision and substrate utilization and muscle energy metabolism.

Adaptations which occur during muscle disuse are not equally distributed between the SS and IMF mitochondrial populations. It has been known for many years that these two subfractions possess different biochemical properties [92], but very little is known about the molecular mechanisms which govern how each subfraction responds to muscle disuse. Muscle disuse brings about a rapid decline in SS mitochondrial content, and compromises their ability to generate ATP within 48h of disuse [93]. Conversely, IMF mitochondria exhibit a slower, more gradual decrease in response to reductions in muscular activity [83]. As a result of these adaptation differences, IMF mitochondria comprise a greater proportion of the total mitochondrial content during muscle disuse [81]. In addition, SS and IMF mitochondria also differ in their susceptibility to apoptosis, with IMF mitochondria having a greater response to apoptotic stimuli [94]. Therefore, IMF mitochondria could potentially play a more influential role in mitochondrially-mediated apoptosis during muscle disuse [94].

Mitochondrial Apoptotic Susceptibility

A decrement in the oxidative capacity of mitochondria is not the only physiological change which occurs during muscle inactivity. Mitochondria house a number of proapoptotic proteins, such as cytochrome c and apoptosis inducing factor (AIF) which, when released into the cytosol, stimulate signaling pathways that culminate with myonuclear death [95]. The release of these proteins is regulated, in part, by the mitochondrial permeability transition pore PTP (mtPTP) which is composed of the voltage-dependent anion channel (VDAC), the adenine nucleotide translocator (ANT), and cyclophilin D (Cyp D). The mtPTP is thought to be formed when Ca^{2+} accumulates within the matrix of the mitochondria, causing Cyp D to associate with ANT and to stimulate pore opening. It has been suggested that, in denervated muscle, the increased opening of the mtPTP can be attributed to a greater sensitivity of Cyp D to Ca^{2+} activation [84]. This effect on mtPTP opening is likely further exacerbated by an elevation in ROS. ROS are also well-known triggers of mtPTP opening. An increase in oxidative stress has been

demonstrated in soleus and in mixed muscles during 4 weeks of muscle disuse produced by hindlimb unloading [96]. As a consequence of this, the elevated levels of ROS are free to degrade cardiolipin [97], to oxidize unprotected mtDNA [95], and to stimulate the formation of the mtPTP [98].

The mitochondrial apoptosis-induced channel (MAC) is a lesser known, but equally important channel created when proapoptotic Bax or Bak proteins translocate to the mitochondria, oligomerize, and permeabilize the outer mitochondrial membrane [99]. Once permeabilized, mitochondria are able to release small proteins such as cytochrome c through the MAC, but bigger apoptotic proteins, such as AIF, are not able to be released through this pore [99]. Muscle disuse results in an increase in whole muscle Bax levels [86, 100], which could augment MAC permeability to cytochrome c, and account for the higher levels of cytosolic cytochrome c in denervated skeletal muscle [86]. This will contribute to a greater degree of apoptosis in chronically disused skeletal muscle. Thus, it is evident that, while a chronic muscle disuse decreases mitochondrial content and inhibits mitochondrial ATP-generating capabilities, the contribution of mitochondria to apoptosis appears to be increased, contributing to the muscle atrophy observed under these conditions.

Mitochondrial Content and Function During Aging

In the early 1990s, work from Muller-Hocker and colleagues provided evidence of mitochondrial defects in aged skeletal muscle [101, 102]. Using histochemical analyses in human muscle, they demonstrated the presence of "ragged red-like" fibers (RRFs) with the proliferation of subsarcolemmal mitochondria presenting a lack of cytochrome c oxidase activity. Subsequently, an age-related impairment in muscle oxidative capacity involving defects in mitochondrial activity has been reported in several studies. Thus, in skeletal muscle from older humans and animals, the activities of several complexes of the electron transport chain and citrate synthase [103-106], oxygen consumption, and ATP production [107, 108] have been shown to decrease with age.

However, concerns have emerged about the origin of this age-related impairment. First, some of the previous studies have ignored factors that can influence mitochondrial and muscle functions such as physical activity. Indeed, when subjects were matched for physical activity, age-related decreases observed in old subjects were suppressed [109, 110], underlining the importance of having an exercise-controlled population in aging studies. Second, based on the knowledge of mitochondrial diseases, extensive work has sought to identify the genetic causes of this enzyme impairment in aging muscle. An array of mtDNA mutations (large-scale deletions and point mutations), previously identified in mitochondrial diseases or pres-

ent in the mitochondrial genes and D-loop, have been shown to accumulate in mtDNA of aging muscle [111, 112]. Even though those mutations accumulated exponentially with advancing age, the fraction of mutated relative to WT mtDNA was not viewed as high enough to reach the threshold required to induce the overall decline in oxidative metabolism seen in aged skeletal muscle. However, this accumulation of mutated mtDNA had physiological relevance when analysis was shifted from whole muscle to single cells. Several studies have documented a significant impairment of mitochondrial function associated with increased mtDNA mutations at the level of individual muscle cells [113, 114]. This focal accumulation of mtDNA mutations and the ensuing mosaic of defective mitochondria and RRFs (1%-2 % of muscle fibers in aged individuals) [115, 116], appeared to be sufficient to induce muscle fiber breakage and to contribute to the age-related decline in muscle mass [117, 118].

Further investigation is nevertheless needed to relate the series of events connecting defective mitochondria, muscle fiber breakage, and muscle atrophy. Answers may lie in the pivotal role that mitochondria play in reactive oxygen species (ROS) production and apoptosis regulation. An early proposal from Harman [119] suggesting that increased ROS could be involved in age-related alterations has been and is still thoroughly investigated, since mitochondria are both the source and the target of those by-products. ROS production has generally been shown to be elevated in aged skeletal muscle[120, 121], and it is known to damage mtDNA, organelle phospholipids, and proteins [122-124]. In contrast, results concerning the expression of antioxidants enzymes (e.g., MnSOD, glutathione peroxidase, catalase) activities are not altogether clear. Some studies show decreases, while others illustrate increases or no changes with aging [125-127]. Nevertheless, because of the general increase in oxidative damage observed in mitochondria, an imbalance between ROS production and scavenging abilities may exist, thus favoring the accumulation of defective mitochondria within muscle. Moreover, since ROS are potent activators of the mitochondrial apoptotic pathway, an imbalance in ROS production in defective mitochondria may increase the potential to trigger apoptosis in aged skeletal muscle. Although further investigations remain to be performed to assess the relationship between ROS and apoptosis, a line of evidence supports a higher incidence of apoptosis in aged skeletal muscle [128, 129] that can contribute to tissue atrophy.

Potential of Exercise to Attenuate Age-Related Mitochondrial Dysfunction

Although it has long been established that exercise training increases, and muscle disuse decreases, the activity of mitochondrial oxidative enzymes in

skeletal muscle, a lack of consideration of this notion in aging studies has led to discrepancies in our overall understanding of the effect of aging on muscle mitochondrial function. Indeed, some of the age-associated alterations found in mitochondrial activity can be the result of a reduction in the level of voluntary physical activity as individuals age [109, 130]. In this regard, it is notable that the adaptation to exercise is not limited to young individuals, since older athletes can increase the activity of mitochondrial oxidative enzymes to a similar extent as result of training [131, 132]. This likely happens through increases in expression of the coactivator PGC-1α and the specific transcription factors NRF-1 and Tfam, main regulators of mitochondrial biogenesis and mitochondrial protein expression [133]. One can assume that if mitochondrial function deteriorates with age, mitochondrial biogenesis induced by exercise should allow for an attenuation of this age-related decline, and therefore may have a protective role. However, despite the fact that exercise-induced increases in enzyme activities and mitochondrial content have been reported in aging individuals, less is known about the effects of exercise on the expansion of mtDNA mutations, ROS balance, and apoptosis in aged skeletal muscle. For example, in patients suffering from mitochondrial diseases due to mtDNA mutations, the introduction of an exercise program to improve muscle oxidative capacity and mitochondrial function has been approached with caution. In those patients, exercise induced mitochondrial biogenesis, but also increased both WT and mutant mtDNA, worsening the heteroplasmy ratio in muscle fibers [134]. Thus, one might expect that this phenomenon may also occur in older individuals.

In response to a bout of exercise, total oxygen consumption is increased by 10- to 15-fold in skeletal muscle and can ultimately result in an elevation in ROS production [135]. It has also been shown that the rate of production of ROS from muscle mitochondria from exercised rats was increased when compared to rested animals [120, 136]. Several lines of evidence support the fact that exercise may be beneficial in attenuating an aging-induced ROS imbalance. Old animals that were submitted to an 8-week treadmill exercise program, or 1 year of swimming, were found to have reduced oxidative damage compared to untrained old rats, notably due to alterations in antioxidant defenses [137, 138]. At the mitochondrial level, recent work from Leeuwenburgh's group has reported a 10% decrease in mitochondrial hydrogen peroxide production [139] in animals resulting from lifelong voluntary wheel running. This may occur through the exercise-induced increase in mitochondrial content, a better redistribution of electrons through the electron transport chain, and/or a better coupling between oxygen consumption and ATP synthesis in the exercised muscle of old animals. The precise mechanism for this effect remains to be determined.

An increased incidence of apoptosis has been documented in skeletal muscle submitted to acute, prolonged exercise. Spontaneous wheel running in

mice evoked an elevation in DNA fragmentation and the expression of proapoptotic proteins and proteolytic enzymes [140]. The exact role of this induction is still unclear, but it may contribute to the remodeling and regeneration of muscle tissue during the recovery period. In contrast to acute exercise, recent work has indicated that apoptosis may be attenuated by a program of regular exercise. Rats trained for 8 weeks presented a reduction in TUNEL-positive nuclei, as well as a decreased Bax to Bcl-2 ratio in soleus muscle [141]. In addition, the use of exercise along with IGF-1 treatment has been shown to attenuate the increase in TUNEL-positive nuclei brought about by hindlimb suspension [142]. Although there are no data available yet concerning effect of exercise on aged skeletal muscle, our expectation is that exercise has the potential to attenuate apoptosis. This is because chronic exercise increases mitochondrial biogenesis and oxidative capacity, and it promotes the expression of antioxidant (e.g., MnSOD) and antiapoptotic proteins (e.g., Bcl-2 and Hsp70) preventing apoptosis activation. However, further investigation is required to clearly establish the potential of exercise to thwart age-induced apoptosis in skeletal muscle.

Conclusions

A comprehension of mitochondrial biogenesis is now recognized as relevant to an understanding of a large number of cellular pathological conditions. Exercise can play a significant role in accelerating the rate of mitochondrial biogenesis, and likely serves to attenuate the mitochondrial dysfunction which arises during aging and conditions of muscle disuse, thereby improving work performance, resistance to fatigue, and the quality of life.

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Chapter 4

Genetic Vs. Acquired Fitness: Cardiomyocyte Adaptations

Ulrik Wisløff, Per Magnus Haram and Ole Johan Kemi

Fitness

Exercise and Health

The human genome was selected through natural selection to maximize fitness in the early ancestral environment, a time in which physical activity was the key for survival. Our genome has not changed much the last 100,000 years, and exercise still remains essential for optimal gene expression and avoidance of disease [1-3]. Physical inactivity is now established as an independent risk factor for cardiovascular morbidity and mortality, an effect that is similar to that of high blood pressure, high levels of blood lipids, and smoking combined [4]. The human body is therefore not ideally suited for a Western lifestyle, where inactivity is the norm with a daily energy expenditure corresponding to only 38% of what our Paleolithic ancestors had [1-3]. An inactive lifestyle will therefore alter gene expression and perturb homeostasis in several organ systems towards the unphysiological end of the range and lead to complex disease scenarios such as the metabolic syndrome. In the present mini-review, we focus upon adaptations in heart function both in healthy individuals and in individuals with the metabolic syndrome and present data derived mainly from studies using appropriate animal models.

The Metabolic Syndrome

In 1979, Kannel and McGee [4] discovered increased incidence of cardiovascular disease in patients with diabetes. Almost a decade later, Reaven [5] described the metabolic syndrome as consisting of three or more of the fol-

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lowing criteria; central obesity, atherogenic dyslipidemia, raised blood pressure, insulin resistance or glucose intolerance, prothrombotic state, and proinflammatory state. According to the International Diabetes Foundation (http://www.idf.org) the new consensus worldwide definition of the metabolic syndrome, for persons to be defined as having the metabolic syndrome they must be centrally obese and have any two of the following four factors: raised triglyceride level, reduced HDL cholesterol, raised blood pressure, raised fasting plasma glucose, or previously diagnosed type 2 diabetes. The metabolic syndrome is now present in at least 25% of the US population, according to updated statistics from the American Heart Association (http://www.americanheart.org). The metabolic syndrome is a multifactorial disease caused by interactions between multiple genetic and environmental factors, and several studies link impaired aerobic metabolism to the pathogenesis of the metabolic syndrome in humans [6, 7]. A limitation in studies indicating a cause-effect relationship between the metabolic syndrome and aerobic metabolism in humans is that one cannot exclude the possibility that the observed impairment in metabolism may be caused by other health behaviors not measured. An animal model therefore seems to be the preferable model to test whether there is a cause-effect relationship between impaired aerobic capacity and occurrence of the metabolic syndrome. As such, an introduction to aerobic capacity and metabolism is warranted, including a summarized presentation of the cardiovascular system as to how it may determine aerobic capacity.

Aerobic Capacity

Aerobic capacity consists of maximal oxygen uptake (VO_{2max}), anaerobic threshold (Th_{an}), and work economy [8].

Most previous works regard VO_{2max} as the single best indicator of an individual's cardiorespiratory endurance capacity [8]. Although traditionally related to endurance performance such as cross-country skiing and running, VO_{2max} has recently been established as a strong predictor of cardiovascular morbidity and mortality [9]. Improved VO_{2max} can be acquired through endurance training and is associated with salutary adaptations in multiple organ systems. An assessment of VO_{2max} offers a precise measure of the capacity to transport and utilize oxygen; that is the functional capacities of the lungs, cardiovascular system, and muscle mitochondria combined. At maximal aerobic exercise, the majority of evidence demonstrates a VO_{2max} that is supply limited [10-14]. This appears to be evident in highly trained athletes [15] and in average fit humans [16]. Consequently, cardiac output and more precisely stroke volume, has a major influence on VO_{2max} [13-18], whereas maximal heart rate as an inherited and largely

unchangeable entity, has no such influence. This conclusion is based on the observation that the capacity of skeletal muscle to consume oxygen markedly surpasses the capacity of the heart to supply oxygen. It is estimated that only one third of the muscle mass in man can fully utilize the oxygen-delivering capacity of the heart [13, 14, 18]. If a larger muscle mass is intensely engaged in the exercise, sympathetic vasoconstriction occurs in the arterioles of the exercising limbs to avoid a reduction in blood pressure [11, 12]. Blood flow in healthy arteries is therefore mainly restricted by cardiac output, along with the ability of arteries to dilate. The capacity of the muscle capillary network is never reached at maximum exercise [11, 12, 18, 19], but a denser capillary network exists in endurance athletes. This might prolong the transit time of erythrocytes to allow for increased extraction rates of oxygen and substrate exchange [20]. At the skeletal muscle level, the oxidative capacity of mitochondria could restrict VO_{2max} not only through restrictions in the systemic supply of oxygen, but also by limitations in extraction of oxygen, and diffusive oxygen transport from the muscle capillary to the mitochondrial cytochrome. Approximately 98% of the oxygen we metabolize is handled by our mitochondria, and exercise training increases mitochondrial density, size, and enzyme activity [21]. Two important metabolic effects of enhanced mitochondrial enzyme activity include (1) increased capacity to oxidize fat at a higher rate (thus sparing muscle glycogen and blood glucose) and (2) a decreased lactate production during submaximal exercise [22-24]. These muscle adaptations are important in explaining the improvement in endurance performance that occurs with regular exercise training [25], since metabolic adaptations in skeletal muscle are critical for improving submaximal endurance performance. There also exists evidence that untrained humans are demand-limited and improvement in VO_{2max} early in the training period is produced by peripheral factors [18, 26, 27].

Th_{an} determines the fraction of VO_{2max} that may be sustained for an extended period of time [8, 28], and represents the highest intensity during dynamic exercise with large muscle groups, in which production and clearance of lactic acid are approximately the same during a steady rate work condition [8, 28, 29]. The factors determining Th_{an} are not well known, but muscle fiber type distribution, the potential for fat metabolism, and expression and distribution of skeletal muscle lactic dehydrogenase isoenzymes and monocarboxylate lactate transporters may be important determinants [8, 28, 29].

Work economy is referred to as the ratio between work intensity and oxygen consumption [30-32]. At a given work intensity, oxygen uptake may vary considerably between subjects with similar VO_{2max}. This is evident both in highly trained [30] and in untrained subjects [17]. In elite endurance athletes with a relatively narrow range in VO_{2max}, work economy has been found to differ as much as 20% [33] and to correlate with performance [30,

34]. The causes of intraindividual variations in gross oxygen cost of activity at a standard work intensity are not well understood, but it seems likely that anatomical traits, mechanical skill, neuromuscular skill, and storage of elastic energy are important [29, 34].

Athlete's Heart

Endurance training associates with functional and morphological changes in the heart, such as increased left ventricular chamber size, wall thickness, and mass [35, 36]. Furthermore, the athlete's heart is associated with increased maximal cardiac output with enhancement in both the diastolic filling and ventricular ejection rate [37-39].

Cardiomyocyte Dimensions and Contractile Function

Since myocardial tissue from trained humans is not easily available, data at the cellular level have to be derived from experimental models. Several animal models of endurance exercise have been shown to mimic important aspects of human physiology and could help determine the cellular and molecular mechanisms of training-induced improvements of cardiac function [40-44]. In a rat model of endurance training, increased dimensions and improved left ventricle contraction and relaxation can be observed in isolated cardiomyocytes. This demonstrates that improved intrinsic (i.e., without influence of the neuro-hormonal system) cardiomyocyte function can contribute to both the systolic and diastolic improvements that occur in the athlete's heart.

Training-induced elongation of left ventricular cardiomyocytes occurs in the absence of changes in sarcomere length [45] and the changes in cardiac contractile function induced by endurance training are due in part to cardiomyocyte length-independent changes in contractile function. Several lines of evidence support this notion. Schaible and Scheuer [46, 47] demonstrated that treadmill training increased end-diastolic volume, stroke work, ejection fraction, and midwall fractional shortening in the absence of changes in end-diastolic wall stress in perfused working rat hearts. Furthermore, isometric force development by rat left ventricular papillary muscle maintained at optimal length is increased by endurance training [48-50]. Recently, Diffee and Chung [51] showed that training increased the velocity of loaded shortening and increased peak power output in the

single permeabilized cardiomyocyte preparation. At slow stimulation frequencies (0.067-0.2 Hz) and low temperatures (23-29°C), there is little evidence of training-induced improvement in the shortening characteristics of cardiomyocytes [45, 52]. However, training-induced adaptations, such as increased degree of fractional shortening and reduced relengthening time, become more evident as both the stimulation frequency and temperatures approach in vivo conditions [42, 53, 54]. For the rat, this is 300 to 600 beats per minute at 37°C. There seems to be a progressive increase in cardiomyocyte contractility in response to regular exercise training until a plateau of training effects have been reached. This time-scale coincides with those of exercise training-induced changes on VO_{2max} and cardiomyocyte hypertrophy [42, 43].

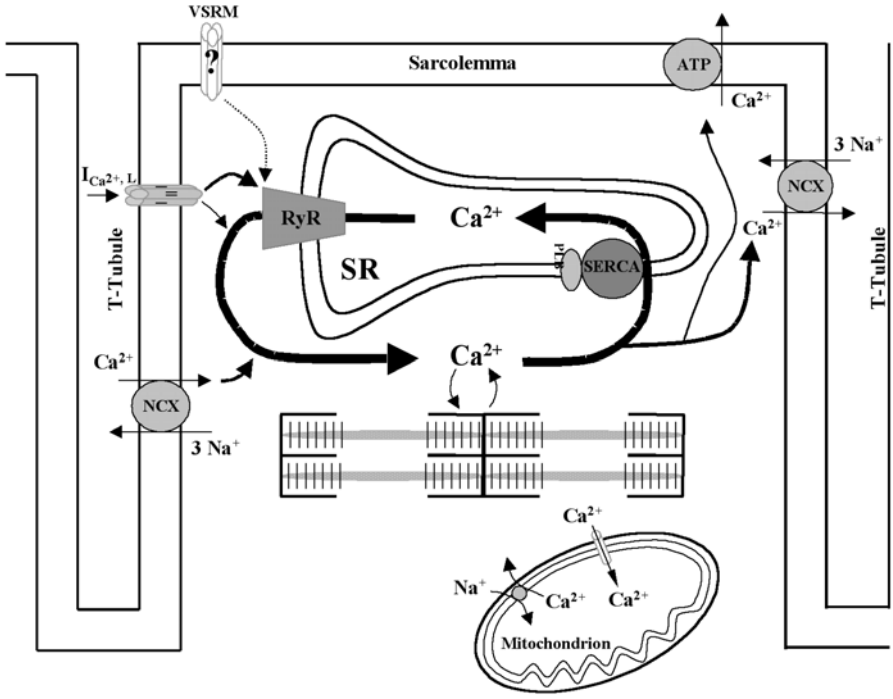

Fig. 1 The main mechanisms that contribute to the excitation-contraction coupling and removal of Ca^{2+} from the cytosol after contraction. VSRM, voltage sensitive release mechanism; ATP, adenosine triphosphate; NCX, Na^+-Ca^{2+} exchanger; SR, sarcoplasmatic reticulum; SERCA, SR Ca^{2+} ATPase; $I_{Ca^{2+},L}$, inward Ca^{2+} current via L-type Ca^{2+} channels; PLB, phospholamban; RyR, ryanodine receptor. For details, see text. Data from [55]

Intracellular Calcium Transients

In cardiac muscle, the force of contraction depends on the peak intracellular calcium (Ca^{2+}) concentration during systole, the sarcomere length, and the responsiveness of the myofilaments to Ca^{2+} [55]. Impairment of Ca^{2+} handling is a major cause of both contractile dysfunction and arrhythmias in pathophysiological conditions [56]. A brief increase in cytoplasmic Ca^{2+} concentration allows Ca^{2+} to bind to the myofilament protein troponin C, which activates the myofilaments. This is often called the Ca^{2+} transient and this transduces the chemical signal and energy (ATP) into cardiomyocyte shortening in a Ca^{2+}-dependent manner. During the action potential, Ca^{2+} enters the cell mainly via voltage-activated Ca^{2+} channels (dihydropyridine receptors or L-type Ca^{2+} channels) as an inward Ca^{2+} current (I_{Ca2+}). L-type Ca^{2+} channels are located primarily at sarcolemmal-sarcoplasmatic reticulum (SR) junctions where the SR Ca^{2+} release channels (the ryanodine receptors) reside. In addition, the sodium (Na^+) - Ca^{2+} exchanger (NCX) contributes to Ca^{2+} influx and efflux with a stoichiometry of three Na^+ to one Ca^{2+} that produce an ionic current either inward (forward mode: during high intracellular Ca^{2+} concentrations) or outward (reverse mode: during positive membrane potentials and high intracellular Na^+). The Ca^{2+} entering the cardiomyocyte from the outside contributes directly only to a minor degree to myofilament activation, and its main effect is to stimulate Ca^{2+} release from the intracellular pool of Ca^{2+}: the SR. This is normally termed Ca^{2+}-induced Ca^{2+} release (CICR) (Fig. 1). For relaxation and filling of the heart to occur, the intracellular Ca^{2+} concentration must decline. This requires Ca^{2+} transport out of the cytosol by four pathways involving SR Ca^{2+}-ATPase (SERCA2), sarcolemmal Na^+-Ca^{2+} exchange, sarcolemmal Ca^{2+}-ATPase, and mitochondrial Ca^{2+} uniport [55]. The SERCA2 and NCX are quantitatively the most important Ca^{2+} extruders. In rat ventricular cardiomyocytes, the SERCA2 removes about 92% of the activator Ca^{2+} from the cytosol, whereas the NCX removes 7%, with only about 1% each for the sarcolemmal Ca^{2+}-ATPase and mitochondrial Ca^{2+} uniporter. Although there is a certain degree of species-dependence to the quantitative distribution, the qualitative mechanisms are similar between species, including humans. In heart failure, the expression of SERCA2 is normally reduced and NCX increased, and both changes tend to reduce the Ca^{2+} content in SR and thus limit SR Ca^{2+} release. This may be a central cause of systolic deficit in heart failure [55, 57].

Cardiomyocyte shortening in healthy endurance-trained rats is associated with lower peak systolic and diastolic intracellular Ca^{2+} and reduced time for the Ca^{2+} decay from systole [43-45]. Gene analysis approaches demonstrate a marked upregulation of SERCA2 and NCX in trained hearts [43, 44, 58-61]. Chronically elevated NCX levels are known to reduce systolic Ca^{2+} [62] and

may contribute to the reduced peak systolic Ca^{2+} observed in cardiomyocytes from endurance-trained rats. Furthermore, increased Ca^{2+} uptake capacity of the SR due to increased SERCA2 expression could account for the increased rate of decay of the Ca^{2+} transient observed after regular exercise training [43].

Myofilament Calcium Sensitivity

An additional mechanism for the increased contractile force in the cardiomyocyte is that exercise training may result in an increase in the sensitivity of the myofilaments to activation by Ca^{2+}. An increase in Ca^{2+} sensitivity would result in a greater isometric tension generation at the same intracellular Ca^{2+} level. In healthy rats, treadmill running induces an increased cardiomyocyte sensitivity to Ca^{2+}, both in intact [44, 45] and permeabilized [44, 51, 63] cardiomyocytes, with more pronounced changes in endocardial than epicardial cardiomyocytes [64]. There are also indications that permeabilized cardiomyocytes from trained hearts are less affected by low pH at constant Ca^{2+} than sedentary counterparts [43, 44]. As previously reported [65], low pH decreases and alkaline pH increases myofilament shortening in cardiomyocytes from sedentary and trained cardiomyocytes. In an analogous way to intracellular Ca^{2+}, this indicates that a component of the enhanced cardiomyocyte contractility could be attributed to the more alkaline intracellular pH in the trained cardiomyocytes at high stimulus frequencies.

In the following we sum up data from studies investigating whether rats selected on the basis of low versus high intrinsic exercise performance also differ in VO_{2max}, mitochondrial oxidative pathways, and cardiovascular risk factors linked to the metabolic syndrome. Furthermore, we propose a close link between cardiomyocyte function and VO_{2max}, both in normal individuals and individuals with either inherited (intrinsic) high or low aerobic capacity.

Inherited or Acquired Aerobic Capacity: Links Between VO_{2max} and the Cardiomyocyte

Our data demonstrate that the level of VO_{2max}, whether inherited or acquired, is closely related to cellular structure and function in the heart. Furthermore, it documents that endurance training improves cardiovascular health even in rats with genetically derived metabolic syndrome that closely resembles the condition of metabolic syndrome in humans.

Inherited Low Maximal Oxygen Uptake, Cardiovascular Risk Profile, and Metabolic Syndrome

A specific aim of our research has been to determine whether rats selected on the basis of low versus high intrinsic exercise performance also differed in VO_{2max}, mitochondrial oxidative pathways, and cardiovascular risk factors linked to the metabolic syndrome. After eleven generations of selective breeding based upon aerobic treadmill running, contrasting rat lines of Low Capacity Runners (LCR) and High Capacity Runners (HCR) were obtained [66, 67]. HCR were superior to the LCR for distance run to exhaustion (347%) and VO_{2max} (60%). LCR demonstrated a cluster of risk factors for cardiovascular disease, i.e., higher levels of factors such as body mass, visceral adiposity, blood pressure, insulin, glucose, free fatty acids, and triglycerides. This risk profile resembles the metabolic syndrome as described in humans [66]; thus, the LCR rat model serves as an experimental model for this condition that is not based upon single-gene, chemical, or physical manipulation, but on artificial selection over generations that mimics evolutionary processes where cosegregation is preserved and unknown parts of the genome may be affected. Moreover, HCR showed higher levels of economy of running, adaptation to exercise, nitric oxide-induced vascular dilation, and had five higher measures of heart function. The low aerobic capacity in LCR was associated with decreased amounts of transcription factors required for mitochondrial biogenesis and in the amounts of oxidative enzymes in skeletal muscle. Impairment of mitochondrial function may link a low level of fitness to cardiovascular and metabolic disease. Although several lines of evidence have demonstrated strong associations between physical fitness and major cardiovascular risk factors [9], our experiments clearly indicate that low aerobic capacity constitutes a physiological basis which predisposes for clinical manifestations of disease such as the metabolic syndrome [66].

Intrinsic Maximal Oxygen Uptake

A central hypothesis of our work is that diverging aerobic capacity represents a continuum between health and disease. Untrained female LCR rats of generation 11 had VO_{2max} levels of approximately 45 mL $kg^{-0.75}$ min^{-1} [65] similar to that observed in rats with postinfarction heart failure [43]. Furthermore, their HCR counterparts, representing the other end of the continuum, had a supra-normal VO_{2max} of ~70 mL $kg^{-0.75}$ min^{-1}, whilst VO_{2max} of normal Sprague Dawley rats was ~60 mL $kg^{-0.75}$ min^{-1} [42-44]. Although LCR males and females weighed 39% and 24% more than HCR males and females, respectively, multiple regression analysis revealed that body weight did not

account for more than 7% and 14%-20% of the variations in distance run in females and males, respectively [66]. Previous work in HCR and LCR rats at generation 7 showed a 12% difference in VO_{2max} between the two lines. Although a significantly smaller stroke volume was found in the LCR at hypoxic, but not normoxic conditions, the major determinant of endurance capacity was found to be a higher capacity of oxygen transfer at the tissue level [68] in line with increased capillary density, citrate synthase, and beta-hydroxyacyl-CoA dehydrogenase in skeletal muscle of HCR. These data suggest that most of the genetic adaptations for improved oxygen utilization in HCR are due to "peripheral factors" in the skeletal muscle and not in differences in heart or lung function [69]. Thus, this is consistent with the increased expression levels proteins that are important for mitochondrial function in soleus muscle of HCR [66] and the fact that VO_{2max} in untrained individuals appears to be mainly limited by "peripheral factors," whereas in trained individuals, there is a supply limitation of oxygen from the heart [13]. However, while studying HCR and LCR rats from generation 11 [66], we also found substantial differences in cardiomyocyte morphology, contractility, and Ca^{2+}-handling, as well as differences in endothelial function between HCR and LCR. These factors are all major contributors to cardiovascular health and VO_{2max}. Thus, reduced cardiac and endothelial function might likely explain, at least partially, the reduced VO_{2max} in LCR rats, and impairment of these factors may therefore be important to combat in order to decelerate the development of the metabolic syndrome.

Exercise-Induced Improvements in Maximal Oxygen Uptake

In contrast to many studies [46, 47, 70, 71] we find that regular exercise training induces a substantial increase in VO_{2max} in the rat-treadmill-model; VO_{2max} increases on average 10% per week until it levels off after 6-8 weeks of exercise training [42, 43, 72]. This is likely a result of the high aerobic intensity of the training regimen. Differences in training response reported in the literature are probably due to different training regimens used and/or insufficient control of relative exercise intensity. The load required to produce a training effect has to increase as the performance improves during the course of training [8]. The training load should, therefore, be set relative to the level of fitness of the individual. Christensen [73] demonstrated, in humans, the need for a gradual increase in training load with improved performance, in the case of the effect on heart rate, as early as 1931. He observed that regular endurance training at a given exercise rate gradually lowered the heart rate and that after a period of training at a higher load, a standard submaximal work load could then be performed with even lower heart rate. The following general principle of training is apparent in

a number of parameters, among them VO_{2max}; after adaptation to a given work load is reached, the absolute exercise intensity required to achieve further improvement has to increase [8]. A similar training regimen as used in our rat models has been applied to patients with established cardiovascular disease [74, 75] and in patients with metabolic syndrome [76]. Rognmo, et al. [74] determined the effects of moderate- and high-intensity aerobic interval training in patients with coronary artery disease (CAD) on peak oxygen uptake. Importantly, training volume was equated so that only exercise-intensity differed between the exercise groups (i.e., the two groups had similar energy expenditure at each exercise session). They found that high-intensity interval training for CAD patients was twice as effective in improving VO_{2max} as compared to the CAD patients that trained with moderate intensity. Similar results were found in patients with postinfarction heart failure exercising with intervals at 90%-95% of their peak heart rate [75] as well as in patients with the metabolic syndrome [76]. Thus, it seems that this type of interval training is also highly effective for improving VO_{2max} in humans with established cardiovascular disease. Interestingly, the level of VO_{2max} rapidly decreased when rats stopped the exercise program, losing half of its exercise-induced increase in VO_{2max} in 2 weeks. This indicates that the substantial improvement in VO_{2max} over several weeks of regular training is quickly lost when the rats revert to an inactive life-style. The number of exercise sessions necessary to maintain VO_{2max} levels is uncertain, but cutting down from 6 to 2 sessions per week is not sufficient to maintain VO_{2max} [77]. Future studies should determine the amount and intensity required to maintain the gain in VO_{2max} achieved after a program of high-intensity interval training [72].

Evidence of the Athlete's Heart

The athlete's heart is a hypertrophied heart with an increase in left ventricle volume and enhanced pumping capacity. A high level of VO_{2max}, regardless of whether it is intrinsic or acquired, associates with the athlete's heart. Left ventricular weights scaled appropriately to body mass were 19% higher in HCR vs. LCR [66]. In both HCR and LCR, regular endurance exercise increased left ventricular weights and cardiomyocyte length significantly, but the increase is significantly higher in HCR than in LCR. This suggests that the HCR not only has evolved into having a higher intrinsic aerobic fitness, but also possess a higher responsiveness to exercise training than LCR, which may be traced back to its genome. We have also demonstrated a development of the heart into the athlete's heart in normal Sprague Dawley rats, as endurance training also in this model increases left ventricular mass and cardiomyocyte length and width [72]. It is apparent from our studies and

studies elsewhere [45] that longitudinal cardiomyocyte growth is sufficient to account for the effect of training on myocardial mass and provides a cellular mechanism that explains the eccentric ventricular hypertrophy that is often elicited by programs of aerobic exercise in humans and animal models of exercise. If we plot the development of left ventricular hypertrophy and VO_{2max} in normal Sprague-Dawley rats undergoing a high-intensity training program that lasts from 2 to 13 weeks, the relationship between VO_{2max} and left ventricular hypertrophy has an exponential form (Fig. 2).

This fits with Peter Wagner's [18] hypothesis that untrained subjects are demand-limited and that improvement in VO_{2max} early in a training period is due to peripheral factors, whereas fit subjects seems to be supply limited, i.e., most improvement in VO_{2max} is therefore due to increased maximal cardiac output. In healthy human subjects [78], 8 weeks of endurance training improved VO_{2max} by 18%, which was associated with increased stroke volume and enhanced contractility. Detraining athletes for 12 weeks, however, led to a 20% decrease in VO_{2max} along with decreases in stroke volume and left ventricular end diastolic dimensions [79]. To clarify this at the cellular level, we have demonstrated a close correlation between physiological hypertrophy and contractile function in isolated cardiomyocytes [66, 72] (Fig. 3). Cessation of exercise led to a decrease in heart weights, reaching sedentary values after 2-4 weeks of inactivity. Despite this, cell length remained significantly above that observed in controls after 4 weeks of inactivity, and this was the measured parameter that most closely correlated with the changes in VO_{2max} [72]. Although no human data exist on exercised cardiomyocytes,

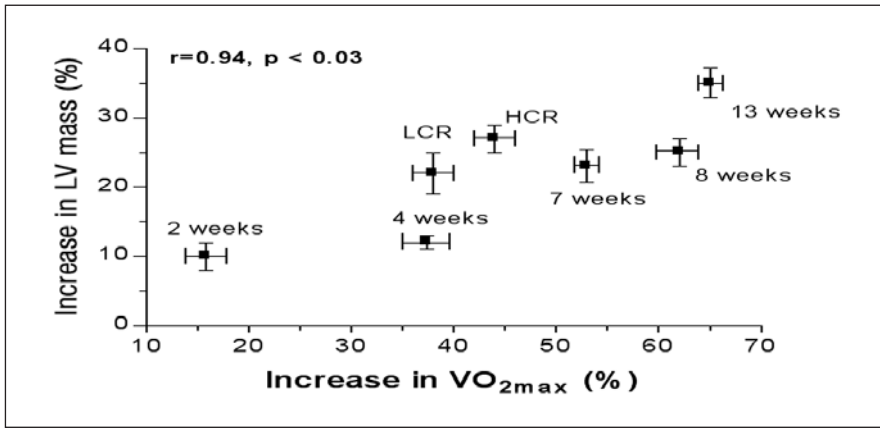

Fig. 2 Relationship between exercise-induced increases in maximal oxygen uptake (VO_{2max}; %) and left ventricular (LV) hypertrophy (%). Data are presented as mean ±SD, accounting from a total of ≥100 rats from studies in our laboratory [66, 72]. Points 2-13 weeks refer to normal Sprague-Dawley rats undergoing 2-13 weeks of endurance exercise training; note the exponential relationship between VO_{2max} and LV hypertrophy. LCR, low capacity runners; HCR, high capacity runners

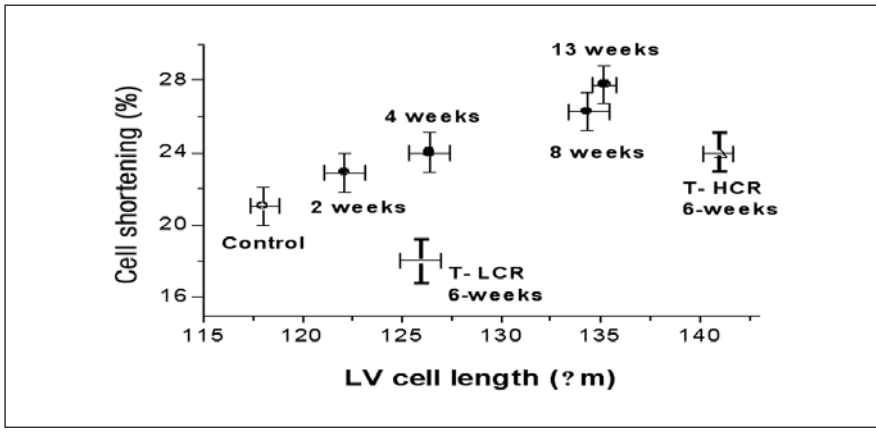

Fig. 3 Time-dependent increase in left ventricular (LV) cell length and maximal extent of shortening in cardiomyocytes isolated from endurance trained and sedentary rats. Each data point represents mean ±SD of ≥60 cells, 9 ±3 in each rat (n=6 per group); Data are from studies in our laboratory [44, 66]. Points 2-13 weeks denote normal Sprague-Dawley rats trained for 2-13 weeks, whereas control refers to sedentary untrained rats. In each cell data were calculated as the mean of 10 consecutive contractions after stabilization at 7 Hz. T, trained; HCR, high capacity runner; LCR, low capacity runner

significant reduction in cavity size and normalization of wall thickness has also been observed in detrained athletes [80], suggesting that the human athlete's heart may also regress during inactivity.

During the last 10-15 years, detailed studies of transcriptional, translational, and posttranslational regulation have characterized a host of molecular mechanisms and signaling pathways associated with cardiomyocyte growth. Numerous cascades appear to be activated or inactivated with exercise training programs, such as those that involve protein kinase B/Akt and mitogen-activated protein kinases; however, a detailed description of molecular regulation of hypertrophy is beyond the scope of this review. The reader is referred to texts elsewhere [81].

Cardiomyocyte Contractility

The level of VO_{2max} is closely related to cardiomyocyte contractile function. Cardiomyocytes from rats with a high VO_{2max}, both intrinsic and acquired, show a greater degree of fractional shortening and have shorter relengthening times than those with a low VO_{2max} [66]. These data are in line with Moore et al. [45] and previous studies in our laboratory [42, 43] showing an increased amplitude of shortening in cardiomyocytes from trained animals, but differs with those of Laughlin et al. [52], reporting no effect of training. Differences in training protocols, stimulation frequencies, and

temperature used when stimulating the cardiomyocytes might explain these contrasting results.

Previously it has been shown that training-induced elongation of left ventricular cardiomyocytes occurs in the absence of changes in sarcomere length [45]. Also, it appears that the changes in contractile function produced by endurance training are due in part to cardiomyocyte length-independent changes in contractile function. Several lines of evidence support this assumption. Schaible and Scheuer [47] demonstrated that endurance training elicited an increase in end-diastolic volume, stroke work, ejection fraction, and midwall fractional shortening in the absence of changes in end-diastolic wall stress in perfused working rat hearts. Additionally, isometric force development by rat left ventricular papillary muscle is increased by endurance training [60]. Despite this, cell length remained significantly above what was observed in controls after 4 weeks of inactivity, whereas the exercise-induced improvement in cardiomyocyte shortening regressed completely within 2 to 4 weeks of inactivity [72].

Cardiomyocyte Calcium Handling

In line with increased rate of cardiomyocyte shortening and relengthening, we find a concurrent increase in the rates of systolic Ca^{2+} release and diastolic Ca^{2+} removal in trained normal Sprague-Dawley rats [72]. However, despite increased fractional shortening in normal, healthy Sprague-Dawley rats, the Ca^{2+} amplitude remains unchanged. These data suggest that endurance training induces an increase in the Ca^{2+} sensitivity of the contractile myofilaments. Previously, it has been shown that the increased Ca^{2+} sensitivity can be attributed to a higher intracellular pH observed at physiological stimulation frequencies [43, 44]. Furthermore, permeabilized cells from trained rats shorten to a greater extent than sedentary cardiomyocytes in the presence of a constant buffered pH. These results indicate that the contractile proteins of the cardiomyocytes from trained rats have an increased intrinsic Ca^{2+} sensitivity compared to cardiomyocytes from sedentary rats. The cellular basis for these changes is unknown, but multiple biochemical alterations of the contractile proteins have been suggested, including changes in the expression of troponin I and T isoforms, and increased alpha-myosin heavy chain expression [82-84]. Furthermore, Diffee et al. [51, 64] have reported an increase in Ca^{2+} sensitivity in conjunction with increased expression of atrial myosin light chain-1. Atrial myosin light chain-1 has previously been shown to increase in human cardiac hypertrophy and has been associated with increased Ca^{2+} sensitivity.

A somewhat different pattern of Ca^{2+} handling was observed in left ventricular cardiomyocytes isolated from HCR and LCR rats. Intrinsically high

VO_{2max} was associated with similar Ca^{2+} kinetics (time to peak and decay of Ca^{2+}) as in trained normal rats. HCR had lower diastolic Ca^{2+} concentrations ($[Ca^{2+}]$) and increased systolic $[Ca^{2+}]$; thus, HCR had increased $[Ca^{2+}]$ amplitude and more Ca^{2+} available for contractile work compared to that of cardiomyocytes from LCR rats. Furthermore, the increased cardiomyocyte shortening in both trained HCR and LCR was associated with a lowering of peak systolic and diastolic $[Ca^{2+}]$ [64]. Lower peak systolic Ca^{2+} transients have been reported earlier by Moore et al. [45] and Wisløff et al. [43, 44], but not by others [52]. The reduction in peak systolic Ca^{2+} concentration in trained cardiomyocytes could be due to: (1) reduced Ca^{2+} released into cytosol via sarcolemma and SR; (2) dilution of released Ca^{2+} in the sarcoplasm due to increased average cardiomyocyte volume; and (3) increased intracellular Ca^{2+} buffering capacity. The first two possibilities are unlikely since reduced Ca^{2+} influx or diluted cytosolic $[Ca^{2+}]$ would reduce the Ca^{2+} binding to myofilaments and reduce contractility. The final possibility is feasible since only a small fraction of Ca^{2+} that is released into and removed from the sarcoplasm during an excitation-contraction coupling cycle exists as free Ca^{2+} [85]. This adaptation to training is consistent with lower diastolic and systolic $[Ca^{2+}]$ in trained cardiomyocytes. Tibbits et al. [86] demonstrated that Ca^{2+} binding sites increased by about 65% in papillary muscle from trained rats, whereas Penpargkul et al. [87] reported enhanced Ca^{2+} binding by cardiac SR from trained rats. Lower diastolic $[Ca^{2+}]$ in trained cardiomyocytes could also result from enhanced sarcolemmal ATP-dependent Ca^{2+} extrusion [88] and/or mitochondrial metabolism [89], thus effectively lowering the set point for Ca^{2+} regulation [90] in trained cardiomyocytes. Changes in myofilament Ca^{2+} affinity can dramatically affect amplitude and time course of the Ca^{2+} transient. The cardiotonic agent sulmazole increases myofilament Ca^{2+} binding affinity and peak myocardial force development, reduces peak systolic $[Ca^{2+}]$ [91], and increases Ca^{2+} transient decay. Similarly, intracellular alkalosis increases cardiomyocyte shortening by increasing myofilament Ca^{2+} sensitivity. The accompanying Ca^{2+} transient is smaller in amplitude and shorter in duration [92]. We have previously showed that trained cardiomyocytes have a significantly less acidic intracellular pH at high stimulation rates (>2 Hz) [44]. It is therefore possible that the lower systolic $[Ca^{2+}]$ after training is related to higher intracellular pH. However, this explanation is insufficient since intracellular pH is comparable between cardiomyocytes from sedentary and exercise-trained rats below 2 Hz, yet trained cardiomyocytes shorten to a greater extent [44]. Without data on intracellular Ca^{2+} buffering capacity or Ca^{2+} flux, free $[Ca^{2+}]$ cannot be directly related to the amount of Ca^{2+} released into the cytosol. Nonetheless, Ca^{2+} cycling appears to be an entity with great plastic potential, as training-induced faster Ca^{2+}-transient time-courses returned to baseline levels within 2 to 4 weeks of detraining and thus explain the regression of cardiomyocyte shortening in the same time period [72].

Increased Ca^{2+} uptake capacity of the SR caused by increased SERCA2 expression could account for the increased rate of decay of the Ca^{2+} transient [43]. In line with this, we have unpublished observations of training-induced changes in cardiomyocyte contractility and relaxation that by far are abolished by selective protein kinase inhibition. This is consistent with the notion that activation of specific protein kinases enhances cardiomyocyte contractility and relaxation by phosphorylating proteins involved in Ca^{2+} handling [55]. Exactly how this happens has not been fully investigated, but compelling evidence suggests increased channel function by phosphorylation, rather than merely changed myocardial protein levels of L-type Ca^{2+} channels or ryanodine receptors, respectively [93]. Another suggested mechanism may be related to protein kinase B/Akt, as cardiac-specific overexpression of nuclear-targeted Akt increased Serine-16 phosphorylation of phospholamban, corresponding to a larger phosphorylation of protein kinase A, which also has the Serine-16 residue as a target [94].

Conclusions

1. Aerobic capacity correlates with an individual's metabolic risk profile and cardiac adaptations, both in the sedentary state and in response to exercise.
2. Selection for low versus high intrinsic aerobic capacity generated a different load of metabolic and cardiovascular risk factors constituting the metabolic syndrome. Our data indicate that low aerobic capacity constitutes a physiological basis that predisposes the subject to clinical manifestations of disease such as the metabolic syndrome.
3. Cardiac adaptation to regular exercise is highly dynamic and depends on cellular changes. Exercise-induced improvements in VO_{2max} and cardiomyocyte function reach peak levels after 6-8 weeks of exercise training, whereas most of the exercise-induced gains acquired over 8-12 weeks of training are lost within 4 weeks of detraining.

Integrated Function and Health Effects

For patients and athletes to fully benefit from exercise training it is important to know the basis mechanism of training effects in health and disease. Improved knowledge must be sought by means of cellular and molecular cardiac biology and applied to exercise and pathological physiology. As exercise physiology and molecular biology are rapidly expanding sciences, new developments during the coming years will explain many of the effects of exercise training we already see in both the cardiac myocyte, as well as other

cells. Extending from an improved understanding of the underlying phenomena, one of the future challenges will be to implement the increasing level of knowledge into everyday practice of sports and medicine.

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Chapter 5

Molecular Modifications Induced by Physical Exercise: A Significant Role in Disease Prevention

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Introduction

The pathogenesis of metabolic syndrome is at present only partly understood; however, a sedentary lifestyle, an unhealthy diet, being overweight or obese, and still largely unknown genetic factors clearly interact to cause it [1, 2]. People suffering from metabolic syndrome share three or more of the following characteristics: augmented waist circumference, elevated plasma triglycerides, low levels of high-density lipoprotein, increased waist circumference, glucose intolerance, and hypertension. Although several studies point to insulin resistance as the principal cause in the development of metabolic syndrome and cardiovascular disease, a growing body of evidence highlights the importance of aerobic capacity as a predictor of metabolic syndrome and cardiovascular diseases [3-5]. Aerobic capacity, how well an organism can metabolize oxygen and generate energy, depends on the efficiency of oxygen delivery to tissues and the subsequent effectiveness of respiration carried out by mitochondria in those tissues, especially in skeletal muscle.

A recent study, carried out on rats selected on the basis of aerobic exercise capacity, suggests that genetically determined intrinsic low aerobic capacity increases the risk of developing elevated glucose, lipids, body fat, and blood pressure, a cluster of abnormalities often present in the elderly, which constitute the metabolic syndrome. These results are consistent with associational studies in humans subjects, implicating impaired mitochondrial function in diseases [6]. Genes regulating mitochondrial biogenesis and respiration efficiency in skeletal muscle are crucial determinants of maximal oxygen consumption, but exactly which genes are functionally

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impaired in sedentary individuals is at present unclear. A growing body of evidence points to the role of low aerobic exercise capacity in the development of metabolic syndrome and cardiovascular diseases. In humans, skeletal muscle accounts for 40% of total body weight and 50% of total energy expenditure and it is a primary site of glucose disposal and fatty acid oxidation. Thus it is not surprising that mitochondria dysfunction in skeletal muscle tissue plays a relevant role in the pathogenesis of obesity, insulin resistance, and type 2 diabetes mellitus [7], as well as in other diseases [8].

The incidence of obesity and insulin resistance is rapidly increasing, along with progression to type 2 diabetes and cardiovascular diseases [9]. Increased deposition of lipids in muscle and liver are markers of insulin resistance [10, 11], but whether this is causal in the development of insulin resistance is less clear. Some data suggest that insulin resistance can be attributed to alterations of molecules such as adiponectin, resistin, TNFα, interleukin-6, visfatin, or retinol-binding protein-4. While the primary cause of type 2 diabetes is unknown, it is clear that insulin resistance plays a major role in its development. Cross-sectional studies have shown the presence of insulin resistance one to two decades before the onset of the disease [12, 13]. Finally, perturbations that reduce insulin resistance prevent the development of diabetes [14]. Recently, it has been hypothesized that impaired mitochondrial function leads to accumulation of lipid metabolites and altered insulin signaling [4, 15, 16] (Fig. 1).

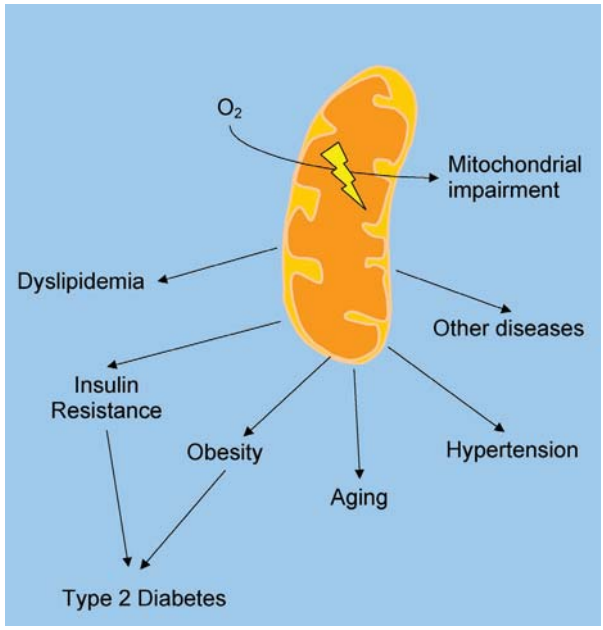

Fig. 1 Mitochondrial impairment is associated with dyslipidemia, obesity, insulin resistance, hypertension and/or type 2 diabetes, key factors involved in metabolic syndrome. This is in strong agreement with the hypothesis that mitochondrial dysfunction may be a primary cause in the development of metabolic disorders

Skeletal Muscle Metabolic Alterations Associated with Insulin-Resistant States

In type 2 diabetes patients treated with steady-state plasma concentrations of insulin, muscle glycogen synthesis was ~50% lower than in normal individuals and accounted for almost the entire insulin-stimulated glucose uptake in both normal and diabetic subjects [17]. These studies demonstrate that under hyperglycemic, hyperinsulinemic conditions, muscle glycogen synthesis is the major pathway for glucose utilization in both normal and diabetic subjects and that impairment in muscle glycogen synthesis may have a key role in causing insulin resistance in patients with type 2 diabetes. Intracellular glucose-6-phosphate is an intermediary metabolite between glucose transport/phosphorylation and glycogen synthesis, hence the intracellular concentration of glucose-6-phosphate will be determined by the relative activities of these two steps. In patients with type 2 diabetes, the decreased activity of glycogen synthase should lead to increased glucose-6-phosphate concentrations compared with those of normal individuals [18]. In type 2 diabetic patients, increases in glucose-6-phosphate in response to insulin stimulation were significantly blunted, suggesting that either decreased glucose transport or decreased glucose phosphorylation activity could be involved in the development of muscle insulin resistance. In order to determine whether failing the boost glucose-6-phosphate levels is a primary or an acquired defect, lean normoglycemic insulin-resistant offspring of parents with type 2 diabetes were also studied [18]. These individuals have a ~40% likelihood of developing diabetes later in life. In these subjects a ~50% reduction was shown in the rate of insulin-stimulated whole body glucose metabolism, which has been attributed to a decrease in the rate of muscle glycogen synthesis [18]. Collectively, these data suggest that defects in insulin-stimulated muscle glucose transport/phosphorylation activity are very early events in the pathogenesis of type 2 diabetes. To examine the role of the glucose phosphorylation step in determining muscle glucose-6-phosphate concentrations, the hexokinase activity in type 2 diabetic individuals was evaluated. Intracellular glucose is an intermediary metabolite between glucose transport and glucose phosphorylation, and its concentration reflects the relative activities of these two steps in muscle glucose metabolism. In patients with type 2 diabetes, the intracellular glucose concentrations have been found to be lower than the concentrations expected if hexokinase II was the primary rate-controlling enzyme for glycogen synthesis [19]. These data strongly suggest that, in type 2 diabetic patients, defective insulin-stimulated glucose transport activity is the primary factor responsible for the development of insulin resistance.

In a cross-sectional study of young, normal weight offspring of type 2

diabetic patients, an inverse relationship between fasting plasma fatty acid concentrations and insulin sensitivity was found, which is in agreement with the hypothesis that impaired fatty acid metabolism contributes to insulin resistance in patients with type 2 diabetes [20, 21]. Competition between fatty acids and glucose for substrate oxidation was observed in rat heart more than 40 years ago. Randle et al. [22] speculated, for the first time, that increased fat oxidation led to insulin resistance associated with obesity and hypothesized that intracellular fatty acid accumulation would lead to an increase in the intramitochondrial acetyl coenzyme A/coenzyme A and NADH/NAD^+ ratios, leading to inhibition of pyruvate dehydrogenase and increasing concentrations of intracellular citrate. The citrate is a negative modulator of phosphofructokinase, the most important rate-controlling enzyme in glycolysis. Thus, citrate accumulation would lead to increasing intracellular glucose-6-phosphate concentrations through the inhibition of hexokinase II activity. The inhibition of hexokinase II activity would result in an increase in intracellular glucose concentrations and decreased muscle glucose uptake. Recently, Schulman and colleagues [23] have recently shown that maintaining high levels of plasma fatty acid concentrations for 5 h resulted in a ~50% reduction in insulin-stimulated rates of muscle glycogen synthesis and whole body glucose oxidation compared with the control subjects. Contrary to the prediction of Randle's model, where fat-induced insulin resistance would result in an increase in intramuscular glucose-6-phosphate concentrations, these new data suggest that increases in plasma fatty acid concentrations first induce insulin resistance by inhibiting glucose transport and/or phosphorylation activity and that this event causes the reduction in muscle glycogen synthesis and glucose oxidation. Obese individuals [24], patients with type 2 diabetes [25], and lean, normoglycemic insulin-resistant offspring of type 2 diabetic individuals [18] show a reduction in insulin-activated glucose transport/phosphorylation activity, similar to normal subjects maintained at high plasma fatty acid levels. Hence, this evidence suggests that accumulation of intramuscular fatty acid metabolites may play a key role in the pathogenesis of insulin resistance in obese patients and patients with type 2 diabetes. Furthermore, elevated plasma fatty acid concentrations significantly reduced intracellular glucose concentrations [26], implying that the rate-controlling step for fatty acid-induced insulin resistance in humans is glucose transport. These data are in disagreement with the mechanism proposed by Randle, which postulates an increase in both intracellular glucose-6-phosphate and glucose concentrations. The reduced glucose transport activity found in these subjects could be the result of fatty acid effects on the glucose transporter-4 (GLUT-4). In skeletal muscle GLUT-4 mRNA were found normal in multiple insulin-resistant disease state, including obesity and type 2 diabetes [27, 28] such as in lean and non-diabetic subjects. This has given rise to the inference that

defects in GLUT-4 translocation cause insulin resistance in muscle, and supportive data are available [29, 30]. An important study of Garvey et al. [31] demonstrated that in diabetic subjects a greater proportion of GLUT-4 was abnormally localized in denser membrane vesicles and it is not normally transferred in the cellular membrane limiting the uptake of the glucose from the cell. The decreased GLUT-4 translocation to the plasma membrane may be the result of a direct mechanism involving alterations in the trafficking, budding, fusion, or activity of GLUT-4 or it may be due to fatty acid-induced alterations in upstream insulin signaling events. The latter possibility has been analyzed in muscle biopsy samples, in which an accumulation of intracellular lipid metabolites such as diacylglycerol was found. This condition was shown to activate a serine kinase cascade that leads to defects in insulin signaling and action [26, 32].

An attractive hypothesis that explains the cause of several forms of insulin resistance in humans holds that a serine/threonine kinase cascade may be activated by increasing intracellular fatty acid metabolites like diacylglycerol [32-37]. The serine kinase activation may lead to phosphorylation of critical serine sites (Ser 307, Ser 612) on IRS-1 [38-40]. Serine phosphorylated forms of IRS-1 fail to associate with and activate PI3K cascade, resulting in decreased activation of glucose transport and other down-stream events. If this hypothesis is correct, any alteration that leads to an increase in intracellular fatty acid metabolites in muscle, through increased delivery from excess caloric intake, sedentary life style, or alterations in adipocyte fatty acid metabolism and/or through decreased mitochondrial oxidation, might be expected to induce insulin resistance in muscle [40].

The capacity of chronic exercise training to reverse this defect in glucose transport/phosphorylation activity was then examined [41]. After exercise training, insulin sensitivity and insulin-simulated muscle glycogen synthesis normalized in the insulin-resistant offspring, and this could be attributed to the correction of their defects in muscle glucose transport/phosphorylation activity. These data strongly suggest that aerobic exercise might be useful in reversing insulin-resistance in these pre-diabetic individuals and that it may prevent the development of type 2 diabetes.

Mitochondrial Dysfunction Might Underlie Type 2 Diabetes

Increased deposition of lipids in muscle and liver is a marker of insulin resistance [10], but whether this is causal in the development of insulin resistance is less clear. More recently, it has been hypothesized that impaired mitochondrial function leads to the accumulation of lipid metabolites and alters insulin signaling [4, 11, 16, 42]. Recent experiments have shown that insulin resistance in the elderly could be attributed to intramyocellular lipid

content, which in turn is linked to a reduction in mitochondrial oxidative-phosphorylation activity [3]. The reduction in mitochondrial function and lipid accumulation in muscle can probably be ascribed to an age-related reduction in mitochondrial content caused by accumulated mutations in mtDNA, which are known to occur with aging [43]. Furthermore, recent studies have shown that a reduction in mitochondrial activity is associated with an increase in intramyocellular lipid content in young, lean, insulin-resistant offspring of parents with type 2 diabetes, a group of individuals that has a strong tendency to develop diabetes later in life [4]. Taken together, these data suggest that alterations in nuclear encoded genes that regulate mitochondrial function and biogenesis may establish the genetic basis for inheritance of type 2 diabetes.

Mitochondria are the site of oxidative energy production in eukaryotic cells. Mitochondrial biogenesis involves the coordinated action of both nuclear and mitochondrial encoded genomes. Peroxisome proliferator-activated receptor α coactivator α (PGC-1α), an inducible transcriptional coactivator, has been implicated as a major regulator of the mitochondrial biogenic program. PGC-1α interacts with nuclear respiratory factor 1 (NRF-1), stimulating transcription of many mitochondrial genes as well as mitochondrial transcription factor A (TFAM), a direct regulator of mitochondrial DNA replication and transcription. A coordinated reduction of PGC-1α-responsive genes involved in oxidative phosphorylation was found in vastus lateralis muscle biopsies from non-diabetic relatives of subjects with type 2 diabetes and in subjects with overt type 2 diabetes compared with glucose-tolerant controls [16, 44]. Additional investigations have also shown reduced mitochondrial function in non-diabetic relatives of subjects with type 2 diabetes and in subjects with overt type 2 diabetes. This mitochondrial impairment has been assessed by multiple methods including ATP phosphorylation, mitochondrial size, citrate synthase (CS) activity, rotenone-sensitive nicotinamide adenine dinucleotide:oxygen (NADH:O_2) oxidoreductase, and mitochondrial copy number [4, 7, 45, 46]. An important question is whether mitochondrial dysfunction is an inherent property of insulin-resistant subjects or whether it is acquired and can be reversed by exercise training. Aerobic exercise training is sufficient to increase mitochondrial enzyme activity and the expression of nuclear-encoded genes involved in regulating mitochondrial transcription, including PGC-1α, NRF-1, and TFAM, in young and old lean individuals [47, 48].

Most of these investigations have not evaluated training levels and have not adequately matched groups for gender and other physical characteristics that may have a substantial impact on mitochondrial metabolism. A recent study on individuals characterized by similar VO_{2max} and body fat percentage has shown that insulin-resistant obese subjects had significantly reduced expression of PGC-1α and COX1, indicating reduced mitochondr-

ial biogenesis. Furthermore, CS activity, a marker of mitochondrial content and function, was also reduced [49]. Taken together, these data lead to the hypothesis that mitochondrial dysfunction could be causal in the development of insulin resistance.

Decline in Skeletal Muscle Mitochondrial Function Associated with Aging

Impaired oxidative phosphorylation by skeletal muscle mitochondria has been postulated to contribute to age-associated insulin resistance and fat accumulation within skeletal muscle [3]. This impaired mitochondrial functional capacity associated with aging has been attributed to a reduced mitochondrial content, as reflected by lower mtDNA content [50]. Many age-related declines in physiological function can be partially attributed to mitochondria dysfunction [51]. There is a significant loss in the number of muscle fibers as well as biochemical and morphological abnormalities in aging skeletal muscle [52, 53]. Age-related muscle wasting, muscle weakness, and reduced aerobic capacity result in many metabolic disorders and diminished physical performance in humans [54-56]. The specific mechanisms leading to the age-related changes are currently unknown. Mitochondria are primary sites of reactive oxygen species formation that causes progressive damage to mtDNA and proteins [53, 57]. Increased prevalence of mtDNA mutations [58, 59], decreased mtDNA abundance [60, 61], and progressive decline in mitochondrial respiratory chain function [62, 63] have been proposed as underlying causes of mitochondrial dysfunction in aging. This finding is based on the hypothesis that cumulative oxidative damage could be the cause of aging [64]. Furthermore, oxidative damage has been associated with increased mtDNA mutations and deletions in older muscles [43, 58]. The importance of mtDNA damage has recently been demonstrated in mice in which accumulation of mtDNA mutations resulted in accelerated aging [65]. Oxidative damage to proteins, lipids, and other cellular components may also affect the function of aging cells [66]. The rate of synthesis of contractile and mitochondrial proteins in human skeletal muscle was shown to decline with advancing age and this may alter muscle metabolic capacity in older people [54-56]. The activity of oxidative enzymes and content mRNA transcripts encoding mitochondrial proteins are also reduced in older muscle [47, 55, 60, 61]. Content and function of specific proteins in muscle depends on protein synthesis and breakdown. Mitochondrial protein synthesis declines with age in human muscle [55]. This decline may be due to reduced mRNA template availability because both COX3 and COX4 transcript levels decline significantly as we age [47, 61]. It has recently been reported that mRNA abundance of three nuclear-derived transcription factors that regulate mitochondrial biogenesis, PGC-1α, NRF-1,

and TFAM, do not change with age in human muscle. These findings demonstrate that despite age-related functional decline, skeletal muscle capacity for mitochondrial biogenesis remains high in older muscle when stimulated by regular aerobic exercise. Hence, further work on the effect of aging on the action of these and other nuclear signals that regulate mitochondrial biogenesis is needed. These studies collectively raise the question of whether age-related mitochondrial defects are the result of normal aging or conversely, whether they are at least partially acquired through lifestyle and factors other than aging per se.

A robust improvement in skeletal muscle mitochondrial content and function was found in elderly men and women in response to a program of moderate intensity physical exercise [47, 48]. Kelley et al. [7] observed an impaired bioenergetic capacity of skeletal muscle mitochondria in type 2 diabetes and obesity, including smaller mitochondria and reduced electron transport chain activity. The electron transport chain activity in the healthy older participants at baseline was three-fold less than that observed for younger lean individuals but similar to that seen in middle-aged obese participants without type 2 diabetes [67]. In particular, the lower electron transport chain activity in these older men and women was more pronounced in sub-sarcolemmal mitochondria than in inter-myofibrillar mitochondria. In these individuals, exercise training improved mitochondrial content and mitochondrial function; however, this improvement was more pronounced in sub-sarcolemmal than in inter-myofibrillar mitochondria [48]. Sub-sarcolemmal mitochondria likely provide energy for cellular processes of substrate transport and cell signaling in skeletal muscle [68], and exhibit higher rates of fatty acid oxidation [69]. Thus, sub-sarcolemmal mitochondria may be specifically linked to physical inactivity, low oxidative capacity, and insulin resistance. Further work on the functional significance of how different mitochondrial subpopulations in skeletal muscle respond to exercise stimulation might provide new insight for designing specific interventions, including exercise, for the prevention and treatment of skeletal muscle functional changes associated with aging.

New Prospects in the Study of Skeletal Muscle Adaptation in Response to Physical Exercise

Among physiological stimuli, exercise is extremely effective in modulating muscle gene expression. Adult skeletal muscle tissue displays high plasticity in response to repeated bouts of contractile activity and endurance exercise training is strongly correlated with increased steady-state levels of many mRNAs encoding mitochondrial proteins and with increased mitochondrial density [70-72]. Activation of gene expression and increase in mito-

chondrial volume therefore appear to be the main instructive mechanisms responsible for the subsequent structural and biochemical adaptations of the mitochondrial compartment in exercised skeletal muscle, known as mitochondrial biogenesis [73, 74].

Recent results point to the transcriptional coactivator factor PGC-1α and its downstream nuclear receptors as important mediators in the control of mitochondrial biogenesis [75]. PGC-1α induced by exercise interacts with nuclear respiratory factors (NRF-1 and NRF-2) and mitochondrial transcription factor A (TFAM) to promote mitochondrial DNA replication [16, 76-78]. The PGC-1α is also known to upregulate the cytochrome c oxidase subunit Vb (COX5B) [44], a nuclear encoded protein of the mitochondrial respiratory chain, and controls the transcript levels of the mitochondrially encoded cytochrome c oxidase subunit II (MT-CO2) through the NRF-1/TFAM pathway [79].

The needle biopsy technique described by Bergström [80] is the most commonly used to assess the vastus lateralis at the cellular level, and, in fact, it has become an essential tool in biomedical research. This technique is useful for biochemical, histochemical, and histomorphometric muscle analysis [81]. Although only infrequent and limited complications have been reported, this technique may be painful for some subjects, and it requires a 5-10 mm skin incision [82]. The relative invasiveness of the procedure makes it difficult to obtain repeated biopsies from the same subjects to study in, for example, time-course response of the skeletal muscle to interventions such as exercise training, diet, or anabolic drug supplementation [83]. Less invasive alternatives to the Bergström biopsy have been proposed in different medical areas such as oncology [84], neuromuscular diseases [85, 86], cardiac failure [87, 88], and pulmonary diseases [82].

Very recently, ultrasound-guided fine needle muscle aspiration (FNA) followed by real-time PCR nucleic acid quantification has been proposed as a new methodology for studying gene expression in human muscle [89]. For the first time, it has been possible to quantify mRNAs and mtDNA from a small biological sample trapped in a fine needle following skeletal muscle aspiration and to show that gene expression of this tissue is related to maximal oxygen consumption. Muscle FNA sampling is sufficient to evaluate the mitochondrial DNA content and to quantify the expression of many genes involved in mitochondrial biogenesis and oxidative phosphorylation in a group of healthy middle-age subjects with a wide range of aerobic capacity. Until now, analysis of gene expression in human skeletal muscle has required Bergström biopsy for tissue sampling. However, the widespread use of this technique has been limited because it is invasive, requires local anesthesia, and can induce muscle hematomas.

Three major findings support the validity of the FNA methodology [89]. Firstly, the increased PGC-1α expression levels found in trained subjects

were accompanied by a corresponding increase in the expression of cytochrome c oxidase transcripts encoded either by the mitochondrial (MT-CO2) (Fig. 2) or the nuclear (COX5B) genome (Fig. 3). These results are in agreement with previous data showing PGC-1α to be an important regulator of energy metabolism and mitochondrial biogenesis in tissues relying mainly on oxidative metabolism for ATP production such us skeletal muscle, heart, brown fat, and liver [44, 77, 90]. In addition, recent studies have shown that the transcriptional regulator PGC-1α is nonfiber type specific, suggesting that the expression of genes encoding mitochondrial proteins does not match the differences in mtDNA content [91-93]. This is consistent with the absence of a correlation shown between PGC-1α expression and the

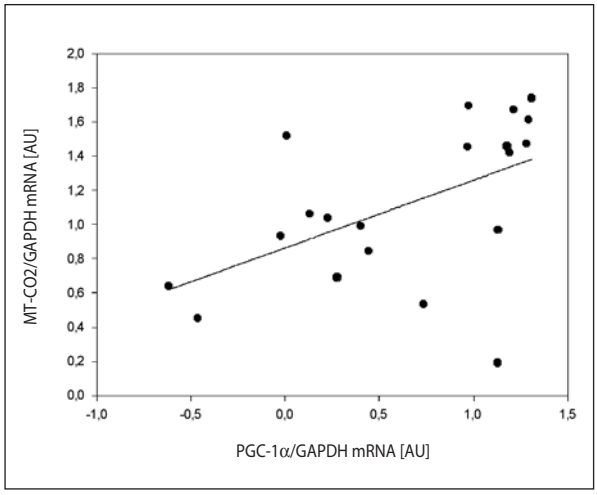

Fig. 2 Correlation studies between PGC-1α and MT-CO2 mRNA expression, obtained by FNA procedure, in healthy subjects exhibiting a large range of physical performance ($R = 0.53$; $p = 0.017$)

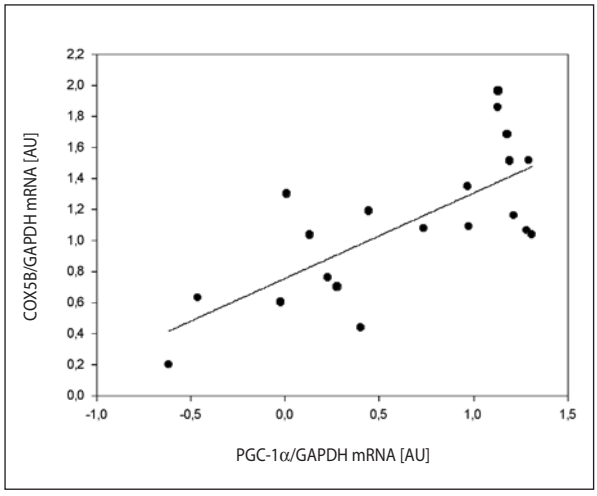

Fig. 3 A corresponding increase in PGC-1α and COX5B mRNA levels is shown in healthy subjects exhibiting a large range of physical performance ($R = 0.73$; $p < 0.001$)

percentage of MHCI expression present in the aspired sample. The third finding demonstrating the reliability of the FNA approach is the significant correlation shown between the mtDNA content and the percentage of MHCI expression. In fact, in humans, the type I fiber is characterized by high MHCI expression levels and the highest mitochondrial content, while type II fiber presents low mitochondrial content and high MHCIIa/x expression levels [92, 94]. In addition, the possibility of relating the mtDNA/nDNA content to a physiological parameter such as VO_2 peak was evaluated. This analysis showed that muscle mtDNA content (expressed as mtDNA/nDNA ratio) of healthy subjects increased with their level of physical performance and was linearly correlated with oxygen uptake [89] (Fig. 4).

When compared with the results obtained by the Bergström biopsy technique, the current gold standard, muscle FNA followed by real-time PCR nucleic acid quantification provides excellent agreement for MT-CO2, COX5B, and PGC-1α relative expression [95]. These are the first available findings regarding the application of a painless technique for sampling skeletal muscle tissue and clearly show how the muscle cells trapped in a fine needle can be used to gain insight into the molecular mechanisms underlying skeletal muscle adaptations in response to environmental stimuli.

These data show the feasibility of using a minimally invasive technique to obtain vastus lateralis samples from healthy volunteers. The FNA technique was very well tolerated; subjects reported no pain and none of them showed side effects. In fact, the FNA procedure can be performed without a skin incision and anesthesia, and is therefore more readily accepted by the sub-

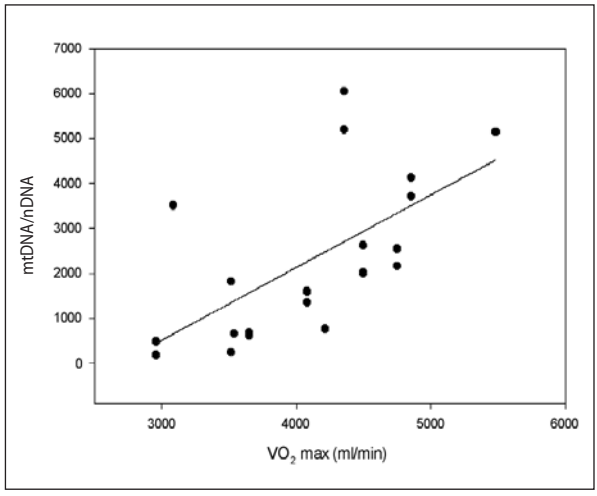

Fig. 4 Relationship between VO_2 max and mitochondrial DNA content (mtDNA/nDNA) as assessed by breath-by-breath analyzer and FNA technique

jects. This is particularly important when repeated sampling is required. Another advantage of FNA is that the risk of complication due to hematoma formation can be considered null, making this procedure very safe. To have a complete overview of the advantages and drawbacks of the FNA procedure, the remote possibility of collecting blood during withdrawal should be considered. If this occurs, the sample is not representative of muscle gene expression and the procedure must be repeated.

Conclusions

A growing body of evidence points to skeletal muscle alterations, in particular, mitochondrial impairment, as a primary risk factor in the development of insulin resistance, obesity, type 2 diabetes, cardiovascular diseases, and neurodegenerative disorders. The FNA procedure represents a new tool that can be used to gain insights into the cellular strategies underlying muscle alterations characteristic of these pathologic states. Furthermore, repeated sampling, made possible by FNA, may provide important clues to better understand skeletal muscle adaptive response in healthy human subjects to physiological conditions such as aging, nutrition, and exercise training. Research over the past four decades on physical activity has established the formidable effectiveness of exercise training for improving many of the pathologic modifications related to metabolic and cardiovascular diseases. However, additional studies are clearly needed to better understand the molecular adaptations of skeletal muscle to physical exercise. Hence, the applications of the FNA technique will contribute to our understanding of muscle plasticity and the development of intervention programs, including exercise, for the treatment and prevention of these diseases.

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SECTION III

Physical Exercise and Oxidative Stress

Chapter 6

The Contribution of Reactive Oxygen Species in Sarcopenia and Muscle Aging

Stefania Fulle and Giorgio Fanò

Introduction

In recent years, age-related diseases and disabilities have become of major interest and importance for health. This holds particularly for the Western community, where the remarkable improvement of medical health, standard of living, and hygiene have reduced the main causes of death. Despite numerous theories and intensive research, the principal molecular mechanisms underlying the process of aging are still unknown. Most, if not all, attempts to prevent or stop the onset of typical degenerative diseases associated with aging have so far been futile. Solutions to the major problems of dealing with age-related diseases can only come from a systematic and thorough molecular analysis of the aging process and a detailed understanding of its causes.

The mitochondrial theory of aging represents one of the leading theories on skeletal muscle aging [1, 2]. According to this theory, the aging process is mediated by a vicious cycle of events ultimately leading to cellular senescence. Central to this vicious cycle is an increase in oxidative stress, mediated by an increased production of reactive oxygen species (ROS), and/or a reduced antioxidant capacity (Fig. 1). The electron transport chain is thought to be the main producer of ROS in skeletal muscle, and it has been demonstrated that ROS, produced by the mitochondria, are maintained at a relatively high level inside the mitochondrial matrix [3].

After reaching a peak in mass, force, and strength (and of functionality) in early adult years, skeletal muscle gradually declines beginning at about 45 years. Consequent to the age-related decrease in muscle mass, is a complex process which is commonly defined as "sarcopenia". This age-related condition, which includes a progressive loss of mass and strength, is associated with a decline in the fibers functional capacity and it is the result of many cellular changes [4].

V. Stocchi (ed), *Role of Physical Exercise in Preventing Disease and Improving the Quality of Life*

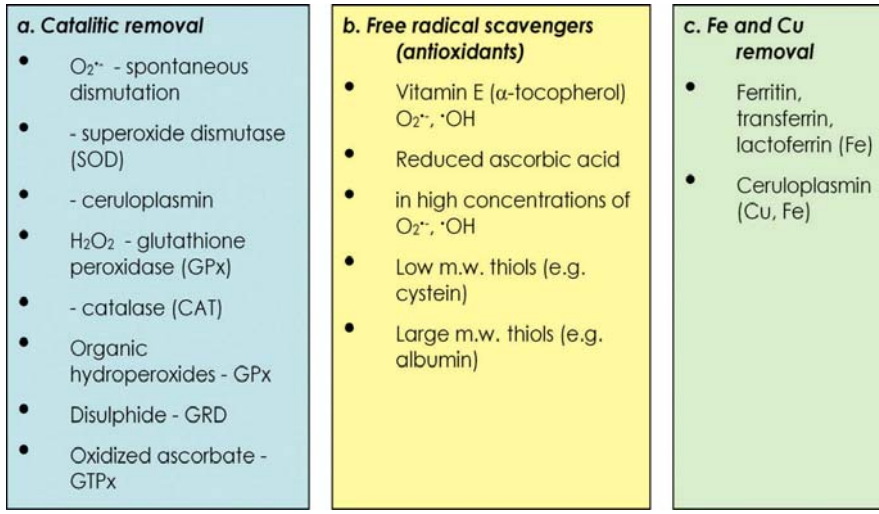

Fig. 1 Antioxidant defense mechanisms of the organism

Sarcopenia is considered an event with a multifactorial etiology: (1) mitochondrial deletion, i.e., replication errors in mitochondrial DNA that lead to an energetic deficit and fiber atrophy; (2) protein synthesis alterations with an imbalance between protein degradation and ability of the fibers to synthesize proteins; (3) loss of repair ability of the satellite cells, caused by an alteration in the proteic growth factors (mainly IGF-1, mIGF-1, HGF) and hormones (GH, Testosterone, estrogens).

The loss of muscular mass (up to 40%), more evident in the legs and in males, is attributable to the loss and atrophy of the fibers that constitute the muscle. This condition negatively affects motility and strength development. This process starts to appear at 40-50 years of age and increases toward 75 years of age. Sarcopenia is a highly prevalent condition in older people, with 35% of the older US population having a moderate degree of sarcopenia and 10% having a severe degree of sarcopenia. The burden that sarcopenia places on the healthcare system further demonstrates its public health effect [5].

Longitudinal studies have shown that muscle strength, which is in large measure determined by muscle mass, is predictive of functional limitations and disability. Thus, it seems logical to assume that sarcopenia precedes disability, but it is also plausible that physical disability itself could lead to sarcopenia. Physical disability would lead to a lower physical activity level, resulting in decreased stimulus to skeletal muscle, which in turn could cause significant muscle wasting over time [6].

Sarcopenia is histologically characterized by type II myofiber atrophy, myofiber type grouping, and fiber necrosis and also for this reason all mus-

cles don't present the same degree of tissue loss, which is most notable in weight-bearing lower limb and trunk muscle groups. Even if this functional state debuts and develops faster in inactive subjects it is still also present in physically active ones [7].

Data

The senescence of skeletal muscle is additionally characterized by a significant decrease in endogenous antioxidant mechanisms with a consequent increase in oxidative damage, which is directly correlated to a functional deficit in the control of Ca^{2+} homeostasis by myofibers [8].

Studies previously performed in our and other laboratories show that in human *vastus lateralis* muscle, a direct correlation exists between age and oxidative damage to biological molecules, such as DNA, proteins and lipids with alterations in peroxidation of membrane lipids and oxidative damage to DNA [9]. This is more evident in male subjects (ca. fourfold in DNA) compared to female subjects [10] (Fig. 2). Although protein targets seem to be the more resistant to oxidative damage, it appears that all the biological substrates of ROS are involved in age-dependent oxidative damage (higher in males). Additional factors, such as different muscle activity based on

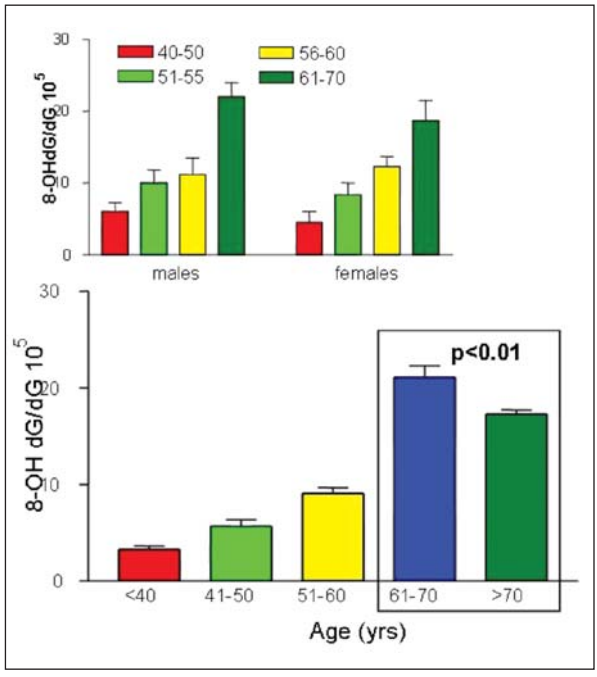

Fig. 2 Direct correlation between age and oxidative damage of DNA. This is more evident in male skeletal muscles as compared with female samples, in which an age-related increase of oxidative damage (up to fourfold for DNA) was observed. The data reported are expressed as 8-hydroxy-2-deoxyguanosine/deoxyguanosine ratio, i.e., the molar ratio between OH^8dG and dG multiplied • 10^5 ($OH^8dG/dG \cdot 10^5$)

sex (males>females), may play a role. The glutathione-dependent antioxidant enzymatic pathway does not undergo age-related modifications, except for a decreased activity of glutathione transferase (GST). On the other hand, the glutathione-independent enzymatic systems, constituting Catalase (Cat) and superoxide dismutase (SOD), display suppressed Cat activity, but no significant differences in SOD activity related to age or gender [11] (Fig. 3). It is important to note that ROS are not only involved in muscle damage, but also in modulate skeletal muscle contraction by acting on the functional status of Ca^{2+} channels. In fact, the Sarcoplasmic Reticulum (SR) Ca^{2+} channel (RyR1) may display high oxidation status due to oxidative stress, which alters its opening capacity [12, 13]. Finally, the ROS increase due to changes in antioxidant enzyme activity may also induce the observed modifications in membrane fluidity [14], and affect the functional capacity of muscle by contributing to the onset of fatigue and weakness [15, 16].

The remarkable capacity of regeneration of muscle tissue is linked to formation of new fibers deriving from undifferentiated precursors. In skeletal muscle there are quiescent mononucleated myogenic cells, called satellite cells [17], located between the sarcolemma and the basal lamina. Satellite cells contribute to muscle pre- and postnatal growth and also to muscle fiber regeneration after injury. These cells remain quiescent until external stimuli trigger them to re-entry into the cell cycle thereby becoming capable of proliferating and differentiating in complete skeletal muscle fibers [18].

During aging, there is a decrease in the antioxidative capacity of skeletal muscle that results in an abnormal accumulation of ROS. In aged human

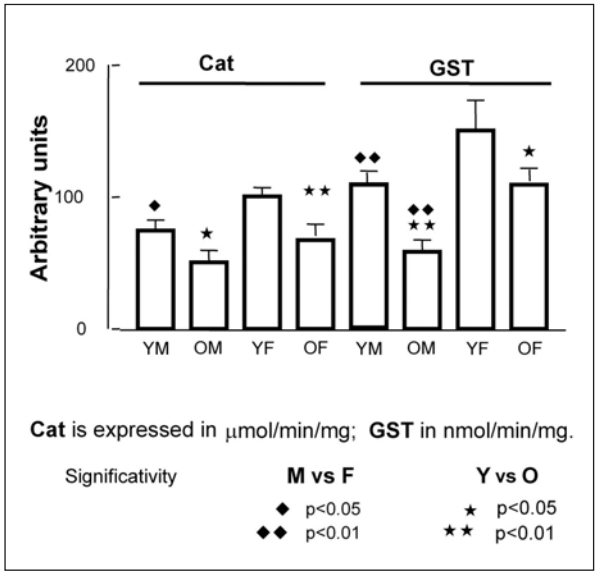

Fig. 3 The enzymatic activity measured in the whole homogenates derived from muscle biopsies. Data reported in this figure clearly indicate that Cat and GST activities (expressed in μmol/min/mg and nmol/min/mg respectively), were significantly depressed in samples derived from the old group as compared with the young group. (*Y*, young; *M*, males; *O*, old; *F*, females)

muscles, the oxidative damage seems also to affect satellite cells altering their functional status. In fact the activity of two main antioxidative enzymes, Cat and glutatione transferase, is drastically reduced in satellite cells derived from the elderly, compared to that observed in satellite cells from young subjects, and the cell membrane fluidity is modified in relation to age [19].

An alteration of antioxidative machinery could affect the oxidative status of critical functional sites of target proteins, such as calmodulin, Ca^{2+}-ATPase and Ryanodine receptor (RyR) which modulate signal transduction, as well as calcium homeostasis. In fact, the oxidative modifications of these calcium regulatory proteins contribute to the increase in intracellular calcium concentration ($[Ca^{2+}]_i$) observed during biological aging both in the muscle tissue [20] and in the satellite cells [19] (Fig. 4). In response to this high $[Ca^{2+}]_i$ these cells try to counteract with an increase of Ca^{2+}-ATPase activity (Fig. 5). All together these data indicate that the destabilizing oxida-

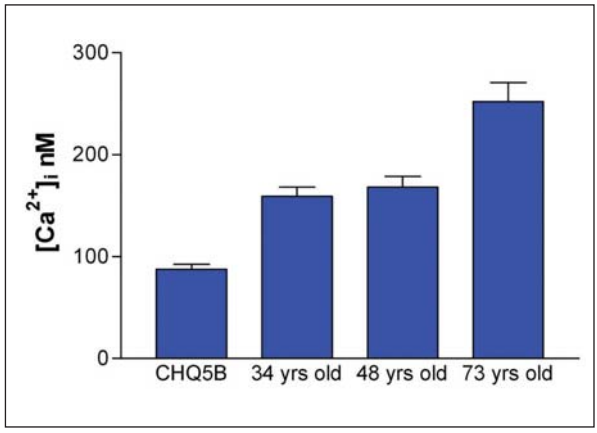

Fig. 4 Basal levels of $[Ca^{2+}]_i$. The bars represent $[Ca^{2+}]_i$ in nM measured in conditions by video imaging in myoblasts derived from muscles (vastus lateralis and gluteus medius) of different subjects

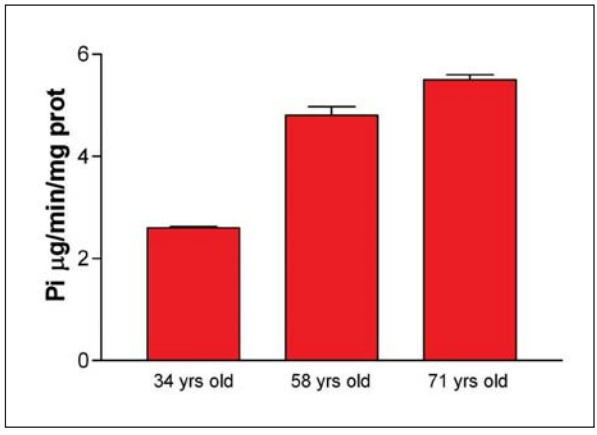

Fig. 5 Activity of the enzyme Ca^{2+}-ATPase type 1 (SERCA 1), which controls the capacity to recover Ca^{2+} released by terminal cisternae. Vesicles prepared from 7-day differentiated myotubes from subjects of different ages. The old sample exhibited a significant increase in sarcoendoplasmic reticulum Ca^{2+}-ATPase activity, compared to the younger samples

Table 1 Myogenic purity and fusion index percentage

Sample	Myogenic purity (%) (+ve desmin)	Fusion index (%)
CHQ5B (newborn)	85	85
VL28M	49	48
VL29M	71	73
VL34M	62	54
GM48M	53	43
GM69M	60	45
GM71M	47	20
VL73F	53	40
GM76M	47	43
VL81M	71	40
VL81F	76	22
VL87F	60	20

The data represent the percentage of myogenity of myoblast cultures calculated at 2-3 PDL and the fusion percentage of myotubes differentiated for 7 days. Myogenity was calculated by counting the number of cells positive for desmin and reported as a percentage of total cells (1000-1500). The efficiency of differentiation was determined by counting the number of nuclei in differentiated myotubes as percentage of the nuclei total number (700-1000) (fusion index). The fusion index was calculated in 7-day differentiated myotubes. The table shows a decrease in the value during aging; whereas the number of myogenic cells does not change, the capacity of these cells to fuse seems to decrease. In the first column are reported the cultures derived from the different subjects. (VL, vastus lateralis; GM, gluteus medium; number, years of subject; *M*, male; *F*, female)

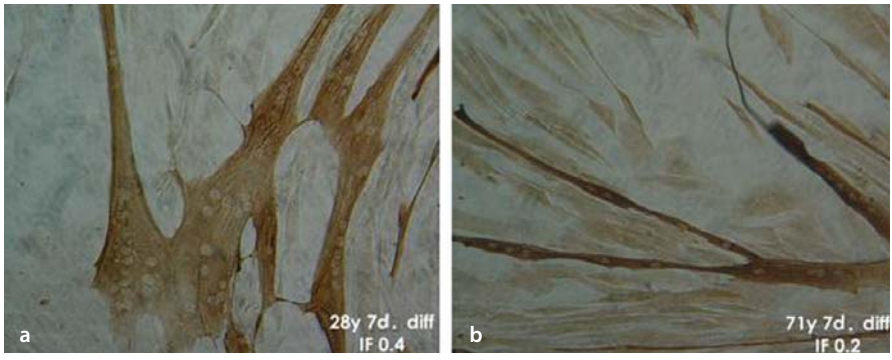

Fig. 6 Myotubes differentiated for 7 days. The cells derived from 28-year-old man (**a**) and 71-year-old man (**b**); example of MF20-positive (*brown*) and MF20-negative (*pale grey*) cells. Revelation is obtained by biotin-streptavidin complex method

tive damage observed in skeletal muscle aging [11] is also applicable to satellite cells, which are closely related both anatomically and functionally to differentiated cells. A decrease in the antioxidative capacity of main scavenger enzymes (Cat and Glutathione-S-Transferase) may be a contributing factor in the change of the functional status and may help account for the discrepancy between myogenic potentiality and myogenic capacity displayed by cell cultures derived from the elderly in our experiments (Table 1, Fig. 6). If this is true, the genesis and maintenance of sarcopenia may be derived, at least in part, from a decrease in the muscle repair capacity of satellite cells.

Considerations

As previously reported by Ji a few years ago "Many critical questions remain regarding the relationship of aging and exercise as we enter a new millennium. For example, how does aging alter exercise-induced intracellular and intercellular mechanisms that generate ROS? Can acute and chronic exercise modulate the declined gene expression of metabolic and antioxidant enzymes seen at old age? Does exercise prevent age-dependent muscle loss? What kinds of antioxidant supplementation, if any, do aged people who are physically active need? Answers to these questions require highly specific research in both animals and humans" [21].

Some of these questions can be considered as answered; however, a lot of issues still need to be resolved, such as whether the sarcopenic process can be avoided or at least slowed down.

Physical activity and exercise have several beneficial effects on the health of both young and elderly subjects.In addition to decreasing the risk of several chronic diseases [22] such as coronary artery disease, hypertension, noninsulin-dependent diabetes mellitus, anxiety, depression, etc., exercise might also decrease the risk of the muscle's functional decline and loss of mass (sarcopenia) with less risk of falls and consequent disability [23]. In fact, it seems that Ca^{2}-mobilization during muscle activity may be a signal that enhances the activity of specific transcription factors [24, 25]. However, while there is no doubt that exercise has positive effects at any age, senescent muscle seems to be more susceptible to oxidative stress during exercise due to the age-related ultrastructural and biochemical changes that facilitate ROS formation. Furthermore, muscle repair and regeneration capacities are reduced with old age and this could potentially enhance the accrual of cellular oxidative damage.

Conclusions

The *sarcopenia* has a multifactorial genesis correlated to at least four functional alterations:

1. Mitochondrial activity
2. Muscle protein synthesis
3. Satellite cells regenerative capacity
4. Ca^{2+} homeostasis

All (at least partially) are caused by hazardous ROS, and unfortunately accumulate with age. The consequential effects derived from the sarcopenia can be partly mitigated by physical exercise. Therefore, it is important that the elderly engage in physical activity, and choose a type of moderate exercise that is suitable to their abilities.

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SECTION IV

Mitochondrial Alteration in Aging and Diseases

Chapter 7

Mitochondria: The Dark Side

Daniel Edgar and Aleksandra Trifunovic

Mitochondria

Mitochondria are small organelle found in almost every cell of an organism (Fig. 1). They are the size of bacteria and form a dynamic network that is constantly changing. A typical eukaryotic cell contains about 2,000 mitochondria, which occupy roughly one fifth of its total volume [1]. Mito-

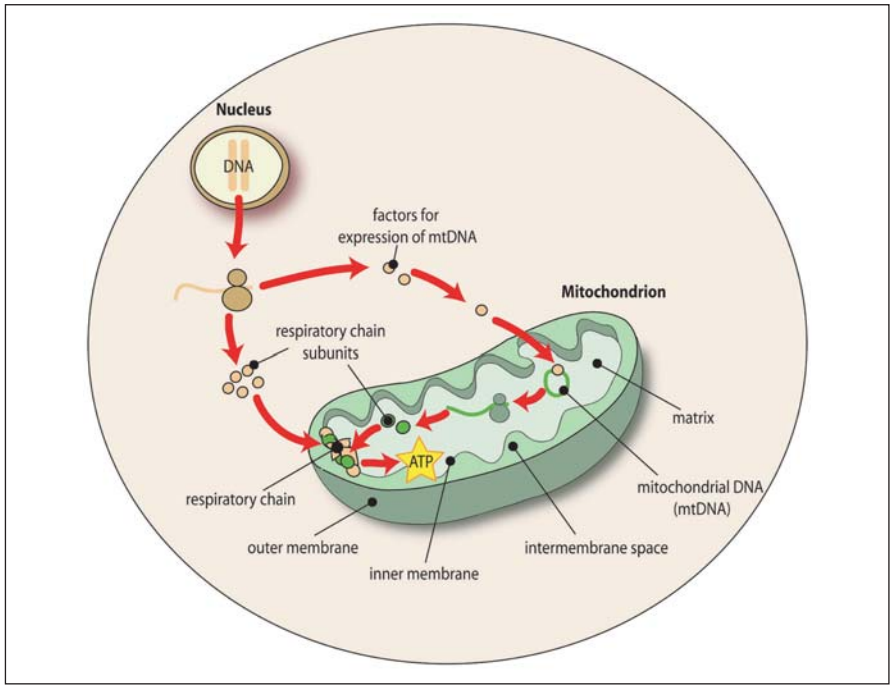

Fig. 1 Mitochondrial respiratory chain is assembled from subunits with dual origin. Thirteen of them are encoded by mtDNA, while the rest is encoded by nuclear DNA, translated in cytoplasm and imported into mitochondria. Mutations in genes encoding proteins involved in any of these steps could mitochondrial dysfunction

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chondria are considered to be the power generators of the cell, converting oxygen and nutrients into adenosine triphosphate (ATP), through a process of oxidative phosphorylation. Although mitochondria are involved in various other important cellular processes such as the beta-oxidation of fatty acids and the biosynthesis of pyrimidines, amino acids, nucleotides, phospholipids, and heme, ATP synthesis is likely to be the most important function of these organelles. Without mitochondria, higher animals would likely not exist because their cells would not be able to obtain enough energy. In fact, mitochondria enable cells to produce 15 times more ATP than they could otherwise. Mitochondrial energy production is a foundation for health and well being. It is necessary for physical strength, stamina, and consciousness [1]. Even subtle insufficiency in mitochondrial function can cause weakness, fatigue, and cognitive difficulties [2]. Furthermore, chemicals which strongly interfere with mitochondrial function are known to be potent poisons.

Mitochondria are unique because they are the only organelles in animal cells containing their own DNA, mitochondrial DNA (mtDNA). As mitochondria are also the only organelle containing ribosomes and are only formed by the division of other mitochondria, it is generally accepted that they were originally derived from endosymbiotic bacteria [3]. In particular, the premitochondrion was probably related to the rickettsias, a group of intracellular parasitic bacteria causing diseases, typhus among many others [4].

Mitochondrial Structure

Mitochondria have two functionally distinct membrane systems: an outer and inner membrane, separated by the intramembrane space (Fig. 1) [5]. This microenvironment houses proteins that play major roles in cellular physiology, in mitochondrial energetics and in the cell death, such as cytochrome c and creatine kinase. Mitochondria are entirely enclosed by the outer membrane, which contains channels made of protein complexes through which molecules and ions can move in and out. The inner membrane is folded to form cristae of which the number and shape differ between mitochondria, depending on the tissue and organism in which they are found, and serve to increase the surface area of the membrane (Fig. 1) [5]. Enclosed by the inner membrane is an internal space, known as the matrix. This matrix contains soluble enzymes that catalyze the oxidation of pyruvate and other small organic molecules with parts of the Krebs cycle occurring within mitochondria. In addition, the matrix contains several copies of the mitochondrial genome (mtDNA) (Fig. 1).

Oxidative Phosphorylation

As we have already pointed out, probably the most important function of mitochondria lies in the fact that they can generate energy in the form of ATP through a process called oxidative phosphorylation. The mitochondrial respiratory chain consists of five different protein complexes named I, II, III, IV, and V that are embedded into the lipid bilayer of the inner mitochondrial membrane. In mitochondria, ATP is produced in a two-step process. In the first step, electrons from NADH and FADH2 are transferred across the electron transport chain. This creates an electrochemical gradient that allows protons to be pumped across the inner membrane. The electron carriers (complex I-IV) transport electrons in a stepwise fashion from NADH to O2. Three of these carriers (Complex I, III, and IV) are also proton pumps, and simultaneously pump H+ ions (protons) from the matrix to the intermembrane space. The protons that are pumped create a proton gradient across the membrane, the mitochondrial transmembrane potential, usually estimated at 150-180mV negative to the cytosol [6]. In the second step, the osmotic energy of the proton gradient is dissipated through complex V generating ATP [7]. ATP is then transported out of the mitochondria by the adenine nucleotide translocase (ANT1) [8].

Mitochondrial Genetics

Mitochondria are the only organelles in animal cells, besides the nucleus, that contain their own DNA. Individual cells have around 1,000-10,000 copies of the mitochondrial genome. In 1981, the human mitochondrial DNA sequence was elucidated and found to be a 16569 bp circular, double-stranded molecule that encodes 13 protein subunits with roles in oxidative phosphorylation, and the 22 tRNAs and 2 rRNAs required for mitochondrial protein synthesis [9]. The strands of the DNA duplex are distinguished as "heavy" and "light" based on their G/T composition and hence different densities in denaturing cesium chlorides gradients. Most of the protein-encoding genes (12 of 13), as well as the two rRNA genes and 14 of the tRNA genes are encoded by the heavy strand, whereas the light strand codes for eight tRNAs and a single polypeptide. A small region of about 1 kb, called the displacement loop (D-loop), is the only noncoding region of mammalian mtDNA. The D-loop contains promoters for the light and heavy strand as well as the origin of heavy-strand replication. As mtDNA encodes only 37 genes, the rest of the mitochondrial proteins, approximately 1,500 in total, are encoded by nuclear DNA, translated in the cytoplasm and transported into mitochondria (Fig. 1).

Mitochondrial Disorders

Mitochondrial disorders are one of the most common inborn errors of metabolism, with a frequency of about 1 in 5,000 [10]. The term "mitochondrial disorders" is used to describe diseases caused by defects in mitochondrial oxidative phosphorylation (OXPHOS) and not defects in the numerous other cellular processes that are located within mitochondria. Many mutations in either mtDNA or nDNA genes coding for mitochondrial proteins are known to lead to major and catastrophic diseases in humans (Fig. 2). The first patient suffering from a mitochondrial disorder was described by Luft et al. [11] in 1962. Since then thousands of patients have been diagnosed with different kinds of mitochondrial disorders. Mitochondrial disorders are very heterogeneous from a clinical, genetic, biochemical, and molecular point of view [10]. They are usually multisystemic, with the brain and muscle being the most commonly affected tissues. Due to the complexity of mitochondrial disorders they are usually classified by their genetic defect rather then clinical manifestation. Therefore, the

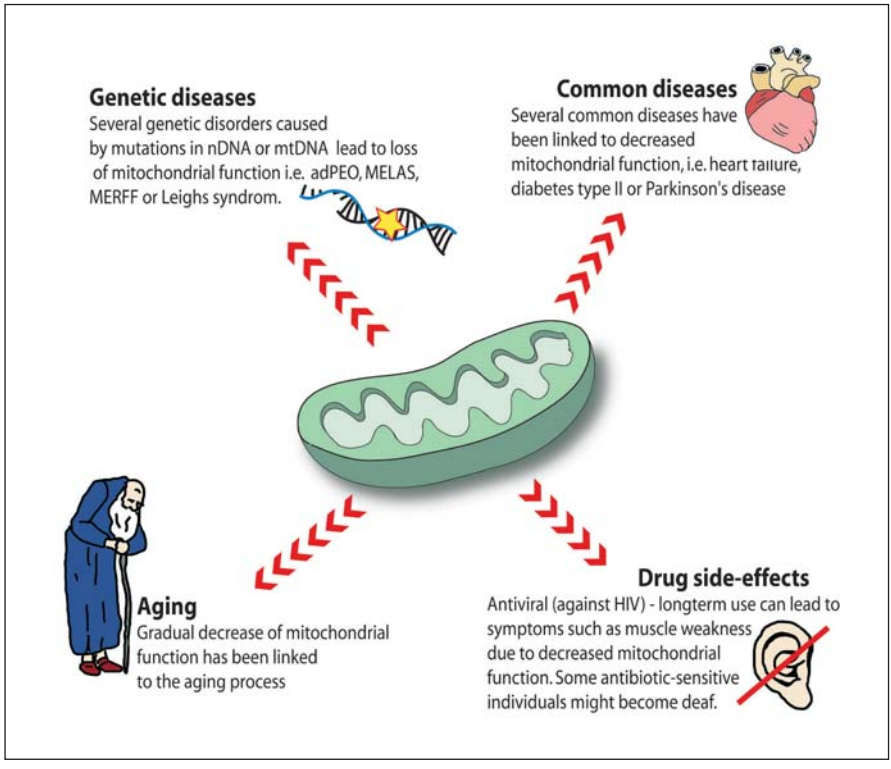

Fig. 2 Mitochondrial dysfunction is associated to different diseases as well as course of normal aging

simplest classification of mitochondrial disorders is by defining the mutations in the mtDNA or nDNA of the patient [10].

Mitochondrial DNA Diseases

The mutation rate of mitochondrial DNA is estimated to be about tenfold higher than that of nuclear DNA [12], and this difference is generally attributed to increased DNA damage from elevated concentrations of endogenous reactive oxygen species produced as byproducts of oxidative phosphorylation [13, 14]. In addition, mitochondrial DNA is not protected by histones and mitochondria appear to lack some DNA repair systems present in the nucleus.

Since the whole human mtDNA sequence has been known for more than 20 years, an increasing number of mtDNA mutations causing mitochondrial defects have been mapped [9]. Unfortunately, a couple of unique features of mtDNA genetics and inheritance still make it very difficult to predict the course of the disease, make prenatal diagnoses and/or perform genetic counseling in everyday clinical practice. First of all, mtDNA does not follow the Mendelian rules of inheritance. In most animals, as in humans, mtDNA is maternally inherited. Therefore, a mother carrying an mtDNA mutation can transmit it to her children, but only her daughters can further transmit it to the next generation. As each cell contains ~10,000 copies of mtDNA, a pathogenic mutation could be present in all or just a few copies of the molecule. Existence of two or more different populations of mtDNA in a single cell is called heteroplasmy, in contrast to homoplasmy, where all mtDNA molecules are identical. This leads us to yet another problem of the mtDNA complexity: the threshold effect. The threshold effect represents the minimal critical level of a pathogenic mutation in mtDNA that should be present in the cell or tissue to have a deleterious effect. A certain proportion of mutant mtDNA must be present before reduction of OXPHOS activity is observed, and the threshold is lower in tissues that are more dependent on oxidative metabolism. It has been shown that there are different thresholds for different types of mtDNA mutations, ranging from 90% for some tRNA mutations [15, 16] to 60% for mtDNA deletions [17]. The last but not least problem of mtDNA genetics is mitotic segregation. Random distribution of mtDNA molecules during cell division can lead to increased amounts of mutant mtDNA molecules in one of the daughter cells. This can lead to a cell carrying low levels of mutated molecules giving rise to one of relatively high levels, which in turn will affect oxidative phosphorylation in that cell.

Although genetically distinct, most mtDNA diseases share common features such as lactic acidosis, a mosaic pattern of cells deficient in

cytochrome c oxidase activity, and massive mitochondrial proliferation in muscle resulting in ragged-red fibers [18]. Mitochondrial DNA diseases commonly have a delayed onset and progressive course. Mutations in mtDNA are divided into two groups: mtDNA point mutations and mtDNA rearrangements.

MtDNA Point Mutations

MtDNA point mutations include both missense mutations in protein-coding genes and mutations that affect global protein synthesis (mutations in rRNA and tRNA genes). Mutations in mtDNA protein-encoding genes have been mainly associated with three diseases: Leber's hereditary optic neuropathy (LHON), Leigh's syndrome, and neurogenic muscle weakness, ataxia and retinitis (NARP) (for review see Zeviani 2004 [19]). Most frequent disease phenotypes associated with mutations in tRNA-coding genes are mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS) [20], and myoclonic epilepsy with ragged-red fibers (MERRF) [21]. Mutations in one of the rRNA genes (12S rRNA) is associated with sensoryneural and aminoglycoside-induced deafness [22]. Although producing a broad range of different phenotypes, mtDNA point mutations often do not have a clear-cut relationship between the clinical signs and a mutation in a specific gene. This is especially common for the mutations in tRNA-coding genes. Mutations in the same tRNA can cause a large variety of different syndromes. For example, point mutations in tRNALeu(UUR) can result in diseases ranging from pure myopathy, cardiomyopathy, MELAS, PEO, maternally inherited diabetes mellitus, and deafness to combined MELAS/MERRF syndrome [23]. On the other hand, mutations in different tRNAs could lead to development of the same syndrome: MELAS syndrome is usually caused by mutations in tRNALeu(UUR), but can also arise from mutations in tRNAPhe and tRNAVal [23, 24].

MtDNA Rearrangements

Most common mtDNA rearrangements are single big deletions of mtDNA that span over one or more tRNA genes [25]. While mtDNA point mutations are maternally inherited, mtDNA deletions are usually sporadic. The occurrence of big mtDNA deletions is usually in oogenesis or early embryogenesis. In rare cases when deletions are combined with partial duplications, large mtDNA deletions can be maternally transmitted to the next generation. There are three main mitochondrial disorders caused by a single mtDNA deletion: Kearns-Sayre syndrome (KSS), sporadic progressive external ophthalmoplegia (PEO), and Pearson's syndrome [26].

Nuclear DNA Mutations

Mitochondrial disorders caused by a mutation in nuclear encoded genes are a very heterogeneous group. Not only are most of the ~80 structural proteins of the OXPHOS system encoded by nDNA, but all the proteins needed for their import from the cytoplasm and assembly in mitochondria are also nDNA encoded. Defects in any of these proteins could lead to functionally impaired OXPHOS and therefore to mitochondrial disease. Furthermore, defects in any protein affecting stability and/or integrity of mtDNA could lead to the same deleterious effect.

Mutations in Structural Components of the OXPHOS Complexes

Until now, mutations in nuclear genes that encode different complex I subunits have been the most common mutations described for nuclear OXPHOS genes. Finding new mutations in complex I subunits has proven to be very challenging since it is a "giant" of the OXPHOS system, consisting of at least 46 different subunits [27]. Nevertheless, several new mutations associated with mitochondrial diseases have been discovered in recent years [28]. Most of these mutations have been associated with severe neurological disorders with lactic acidosis and most often Leigh's syndrome [29]. Unfortunately, for many isolated complex I deficiencies, mutations have still not been mapped, leading us to conclude that there are many unknown factors involved in the structure and assembly of complex I. Although mutations in complex II have also been associated with Leigh's syndrome, they are found to be more important in their association with inherited paragangliomas and pheochromocytomas [30]. Recently, the first mutation in a nuclear-encoded subunit of complex III was described. This mutation was found in an infant suffering from lactic acidosis and hypoglycemic episodes, and on the molecular level resulted in reduced amounts of cytochrome b [31].

Mutations in Genes Involved in Assembly of OXPHOS Complexes

Although no mutations in nuclear-encoded subunits of complex IV, Cytochrome C Oxidase (COX) have been reported, mutations in genes coding for proteins and enzymes involved in COX assembly such as SURF1, SCO1, SCO2, COX10, and COX15 have been found. Most common of these mutations is in the surfeit gene (SURF1) that causes accumulation of early intermediates and reduction of fully assembled COX. This mutation predominantly affects the brain and leads to Leigh's syndrome [32]. Mutations in SCO2 [33] and COX15 cause infantile cardiomyopathy and brain defects, while mutations in SCO1 [34] and COX10 [35] affect liver and kidney tissues, respec-

tively. Mutations in BCSIL, a protein essential for complex III assembly causes GRACILE syndrome (growth retardation, aminoaciduria, cholestasis, iron overload lactic acidosis, and early death) [36]. Finally, a single patient with lactic acidosis, dysmorphic features, and progressive encephalopathy has been described with a mutation in ATP12, an assembler of complex V [37].

Mutations in Nuclear Genes Affecting mtDNA Stability

Autosomal dominant progressive external ophthalmoplegia (adPEO) and mitochondrial neurogastrointestinal encephalomyopathy syndrome (MNGIE) are diseases caused by defective interplay of the mitochondrial and nuclear genome. Most of the adPEO patients carry mutations in one of three genes: ANT1 (muscle-heart specific isoform of mitochondrial adeninenucleotide translocator), Twinkle (mtDNA helicase), or POLG1 (catalytic subunit of mtDNA polymerase) [38]. On the molecular level adPEO is associated with multiple mtDNA mutations [39]. AdPEO is clinically characterized by ophthalmoplegia (progressive muscle weakness affecting eye muscle), very often associated with ataxia, hypogonadism, severe depression, endocrine dysfunction, hearing loss, and peripheral neuropathy [39, 40]. Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is associated with a loss of thymidine phosphorylase (TP) and is characterized by PEO, severe gastrointestinal syndrome, peripheral neuropathy, leukoencephalopathy, and mitochondrial dysfunction. Mitochondrial DNA analysis showed mtDNA deletions, mtDNA depletion, or both [41]. It is interesting to point out that TP is not a mitochondrial protein, and yet its dysfunction specifically affects mitochondrial function and mtDNA integrity.

Defects in Lipid Components of Mitochondria

Cardiolipin is a major phospholipid component of the inner mitochondrial membrane and plays a role in the activity of several OXPHOS complexes, mainly complex I and IV [42]. Cardiolipin concentrations are markedly reduced in Barth's syndrome characterized by mitochondrial myopathy, cardiomyopathy, growth retardation, and leukopenia [42]. Barth's syndrome is caused by a mutation in tafazzin, a protein homologous to phospholipid acyltransferases, which is suggested to have an important role in cardiolipin synthesis.

Mutations in Mitochondrial Proteins Indirectly Affecting OXPHOS

This is a group of diseases that are not directly associated with OXPHOS defects, and yet can cause decreased energy production [43]. These disorders are shedding new light on the field of mitochondrial disease research,

showing us that we should broaden our perspective of what defects can cause mitochondrial energy failure. Several different diseases could be put in this group, of which Freidreich's ataxia, (caused by a mutation in frataxin, a protein involved in iron homeostasis) and autosomal dominant optic atrophy, (caused by mutations in OPA1, dynamin-related guanosine triphosphatases) are probably the most common ones [43].

Mitochondria and Common Disorders

Mitochondrial dysfunction is increasingly recognized as an important factor contributing to common human diseases, including neurodegenerative disorders, diabetes, heart failure, and cancer (Fig. 2). These diseases are almost always connected with a progressive reduction of mitochondrial oxidative capacity and energy production, but also with the increased oxidative damage to the cells. Oxidative damage to the cells is mainly caused by reactive oxygen species (ROS) or reactive nitrogen species (RNS). ROS are the main byproducts of oxidative phosphorylation in mitochondria and it is primarily formed as superoxide ($O_2^{\cdot-}$). There are at least 9 known sites were ROS are produced in the mitochondria, but the sites at complex I and complex III in the respiratory chain are considered the most important [44]. Originally it was estimated that between 2% and 4% [45] of the electron flow gave rise to ROS but now it is believed that in fact it is closer to 0.2% under physiological conditions [46]. ROS come in many forms, and can cause damage to lipids, proteins and DNA in the cell. Enzymes like superoxide dismutase (SOD), catalase, or glutathione peroxidase (GPX) rapidly remove superoxide and hydrogen peroxide to avoid oxidation of cellular components [47]. Under pathological conditions the overproduction of oxidants may overwhelm the cellular antioxidant capacity, resulting in oxidation of cellular molecules and can lead to the cell death. Due to their highly reactive nature, it is very difficult to quantify reactive species directly and evidence for their existence in disease comes from the detection of oxidized proteins, lipids, and even DNA. The most investigated common pathological diseases connected with mitochondrial dysfunction are neurological disorders and diabetes.

Neurodegenerative Disorders

It has been proposed that neurons are particularly sensitive to age-dependent decline in mitochondrial function due to their high-energy demand. Since neurons are postmitotic cells, they can accumulate more somatic mtDNA mutations without the possibility to select against cells with a high

mtDNA mutation load, and apoptosis being an ultimate solution to dispose of these cells. Over the years a high amount of circumstantial evidence has accumulated showing involvement of mitochondrial dysfunction in pathology of age-related neurodegenerative disorders like Alzheimer's or Parkinson's disease.

Alzheimer's disease (AD) is the most frequent form of age-dependent dementia characterized by memory loss and on the molecular level by formation of extracellular plaques and neurofibrilary tangles. Decrease of glucose metabolism as well as reduction of mitochondrial mass and mtDNA copy numbers are reported in early stages of AD [48]. Furthermore, activities of several key OXPHOS enzymes were decreased in AD brains suggesting AD association with multiple respiratory chain defects [49, 50]. Although there is evidence for altered OXPHOS in AD, there is no convincing evidence of specific mtDNA mutations associated with AD [49, 51].

Parkinson's disease (PD) is a progressive neurodegenerative disease characterized by Lewy bodies (round intracellular inclusions in affected areas) and selective loss of dopaminergic (DA) neurons in substantia nigra. Mitochondrial involvement in the pathophysiology of PD became clear after the finding of specific complex I deficiencies only in affected areas of the brain [52]. Furthermore, treatment with different inhibitors of complex I (paraquat, rotenone, MPTP) causes parkinsonian syndrome in humans and animals [53]. Recently, some mtDNA polymorphisms were associated with a decreased risk of developing PD [54].

Diabetes Mellitus

Decreased oxidative capacity and mitochondrial dysfunction have been proposed to be a major contributor to the development of insulin resistance and type 2 diabetes [55]. Under normal conditions blood glucose levels are tightly controlled by insulin secretion from pancreatic b-cells and insulin action on liver, muscle, and fat cells. Mitochondrial oxidative metabolism provides a direct link between glucose stimulation and insulin secretion by increasing the ATP:ADP ratio in the cytosol [56] as a result of increased glucose concentration within the cell. This will trigger a cascade of events that leads to exocytosis of insulin-containing vesicles. Diabetes is usually accompanied with an increased production of free radicals or impaired antioxidant defenses [57]. Diabetes caused by mitochondrial dysfunction is estimated to account for ~1% of all cases. It can be caused by different mutations in mtDNA, but the most common is the A3243G mutation in the gene encoding tRNALeu(UUR) [58]. This mutation was present in patients with a heteroplasmy ranging from 32% to 63% and leads to loss

of both b-cells and neighboring glucagon-producing a-cells [59]. Interestingly, the same A3243G mutation causes the MELAS syndrome that can sometimes be associated with diabetes.

Mitochondria and Aging

Mitochondria were proposed to play a central role in aging by Harman over three decades ago [60]. Since then a lot of experimental evidence has been gathered that indirectly or directly supports this idea. In aged cells mitochondria are enlarged and less numerous with abnormal cristae and intramitochondrial paracrystalline inclusions [61]. Several studies have shown a decline in mitochondrial respiratory chain activity during aging [62, 63], while others have failed to make this connection [64]. Most of the studies that were done in controlled conditions on different animal models have shown a decreased activity of OXPHOS enzymes, especially complexes I and IV [65]. The results were more complex when similar experiments were done on aged healthy humans. Some groups have succeeded in showing decreased complex I and IV activity in the aged human heart or brain [66, 67]. Others have shown a trend in decreasing OXPHOS activity but have failed to cross the significance threshold [68]. Furthermore, other groups have reported no alterations of respiratory chain enzyme activity with age [64, 69].

Mitochondrial DNA Mutations and Aging

Mitochondrial DNA alterations in aging postmitotic cells have been examined extensively. Aging is associated with both mtDNA deletions and mtDNA point mutations. The highest levels of age-associated multiple mtDNA deletions are observed in postmitotic tissues with high energy demands such as heart, skeletal muscle, and brain [70, 71]. The search for mtDNA point mutations in tissue homogenates of aging individuals gave rather disappointing results with very low levels of specific mutations (0.04%-2.2%) [72]. When single cells were analyzed, mtDNA point mutations were observed to accumulate to high levels in an age-dependent and tissue-specific manner [73, 74]. Several of them accumulated up to 50% in single skin fibroblasts of individuals over 65 years of age [74]. Still, there was an open question if mtDNA mutations could be a driving force of aging or are just secondary to the aging process. Recently, we have developed a mouse model that provided the first experimental evidence for a causative link between mtDNA mutations and aging phenotypes in mammals [75]. We created homozygous knock-in mice that expressed a proofreading deficient form of the nuclear-encoded mito-

chondrial DNA polymerase (Polg). The introduced mutation was designed to create a defect in the proofreading function of Polg, leading to the progressive, random accumulation of mtDNA mutations during the course of mitochondrial biogenesis. As the proofreading in the knockin mice is efficiently prevented, these mice develop an mtDNA mutator phenotype (mtDNA mutator mice) with a three to fivefold increase in the levels of point mutations, as well as increased amounts of deleted mtDNA molecules [75]. In contrast to the mitochondrial theory of aging, we have shown that the levels of somatic mtDNA mutations accumulate at a higher rate during the time of development from oocytes to early embryonic life of mtDNA mutator mice, than during the rest of their life when mutations accumulate in rather linear fashion [76]. The mtDNA mutator mice display a completely normal phenotype at birth and in early adolescence but subsequently acquire many features of premature aging. The increase in somatic mtDNA mutations is associated with reduced lifespan and premature onset of aging-related phenotypes such as weight loss, reduced subcutaneous fat, alopecia, kyphosis, osteoporosis, anaemia, reduced fertility, heart diseases, sarcopenia, progressive hearing loss and decreased spontaneous activity [75].

Furthermore, the amount of ROS produced by embryonic fibroblasts from mtDNA mutator mice was normal, despite severe respiratory-chain dysfunction. Antioxidant defences were unaltered in adult tissues of mtDNA mutator mice. Moreover, no differences could be detected in the amount of oxidative damage to proteins and DNA and aconitase enzyme activity, a common marker for oxidative stress, was normal in mtDNA mutator mice. Our results thus challenge the direct role of ROS in the aging process, as there was no link between oxidative stress and the premature aging phenotypes in mtDNA mutator mice [76].

Our results clearly show that the premature aging phenotypes in mtDNA mutator mice are not generated by a vicious cycle of massively increased oxidative stress accompanied by exponential accumulation of mtDNA mutations. We propose instead that respiratory-chain dysfunction per se is the primary inducer of premature aging in mtDNA mutator mice.

ROS, Mitochondria, and Aging

There is no doubt that ROS production is increasing and oxidative damage is accumulating over the course of aging. There is an age-dependent accumulation of oxidative damage to the proteins, mainly in the form of protein carbonyls. In addition, several mitochondrial proteins like aconitase, ANT1, and ATPase, are known to be sensitive and can be inactivated by oxidative stress [77, 78]. Also, it has been shown that cardiolipin (a major inner membrane lipid component) is especially sensitive to oxidative stress and cardi-

olipin content decreases with age in heart, liver, and brain mitochondria [79]. Finally, mtDNA is highly susceptible to oxidative damage and several studies have shown higher age-dependent levels of 8-hydroxy 2-deoxyguanosine (8OH-dG) in mtDNA relative to nDNA [80]. Today, ROS- and ROS-induced damage to different cellular compartments are widely recognized as a possible cause of aging. Caloric restriction (CR), the most widely accepted experimental intervention for increasing lifespan in laboratory animals, also leads to decrease in the amount of ROS produced as well as oxidative damage to proteins, DNA, and lipids in the cell. It has been proposed that the decrease is most likely due to a reduction in ROS produced from complex I [81]. Another correlative link comes from the comparison of the rate of ROS production from isolated mitochondria in a variety of species with varying lifespans. In this case, ROS production and lifespan are inversely correlated [82]. Furthermore, the level of antioxidant enzymes are also inversely correlated to lifespan in different species [83, 84]. Involvement of ROS in aging has been proposed by Harman some 50 years ago [85], and this theory was later expanded upon resulting in the mitochondrial theory of aging [60]. According to this theory, ROS produced in mitochondria will damage the mtDNA, causing mutations to occur. Since the mtDNA encodes protein subunits of the respiratory chain, these will now be malfunctioning causing more ROS production during oxidative phosphorylation. This would then perpetuate itself in a "vicious circle" causing an exponential increase in mtDNA mutations and ROS production. There are several reasons why this theory may not be correct. If ROS-induced mutations are the cause for perpetuating the vicious circle, then the type of mutations seen in aging should be the ones that are caused by ROS damage. ROS causes 8OH-dG; this could lead to G-T or C-A transversions, which does not seem to be over-represented among aging associated mutations. Also, the lack of increased ROS production in mtDNA mutator mice that have elevated amounts of mtDNA mutations challenges the role of ROS in the mitochondrial theory of aging.

Conclusions

Mitochondria have an indispensable role in many different cellular processes, and energy production is probably the most important one. They are essential for maintaining cellular homeostasis, and their many functions integrate closely with the cellular metabolic network. Over the years mitochondrial dysfunction has been linked to the pathology of both mitochondrial and common disorders. Recently, we have obtained the first direct genetic evidence for involvement of mitochondria in the aging process. Mitochondria are now at the center stage in human disease and aging and we should look forward to exciting developments in this field during the coming years.

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Chapter 8

Mitochondrial Pathogenesis of Myopathies Due to Collagen VI Mutations

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Introduction

Muscle cells are individually surrounded by a basal lamina which interacts with several constituents of the extracellular matrix (ECM), which contributes to the mechanical stability of contractile cells. A major component of the muscular ECM is collagen VI, which forms a microfibrillar network in association with the basal lamina. Mutations in the genes which encode any of the three chains of collagen VI have been reported in Bethlem myopathy and Ullrich congenital muscular dystrophy.

Here we report experimental evidence on specific mitochondrial ultrastructural alterations, which characterize skeletal muscle cells of patients affected by muscle disorders caused by collagen VI gene mutations, as well as of a Col VI knock-out mouse model of the disease. These alterations appear to represent the result of mitochondrial dysfunctions dependent on alteration in the Ca^{2+} homeostasis, which can account for increased apoptosis in muscle cells. This pathogenic mechanism is in agreement with the results of a pharmacological treatment that is able not only to restore the mitochondrial structure and function in vitro, but also to revert the muscular phenotype in vivo.

Collagen VI Expression in UCMD Skeletal Muscle and in Col6a1$^{-/-}$ Mouse

Bethlem myopathy (BM) is an autosomal, dominantly inherited, mild proximal myopathy associated with contractures which contribute to disability, especially in the adult life. Cardiac involvement is absent, while respiratory muscle involvement could occur. Histopathological features are nonspecifically myopathic, and include marked variation in muscle fiber diameter, and occasional necrotic fibers. Ullrich congenital muscular dystrophy (UCMD) is an autosomal recessive disease with severe congenital muscular dystrophy, proximal joint contractures, and hyperlaxity of distal joints, char-

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acterized by an early onset. Respiratory failure is a cause of death unless treated. In muscle biopsies, the dystrophic pattern includes type I fiber predominance, increased number of internal nuclei, focal areas of necrosis, increased endomysial connective tissue, and increased presence of regenerating fibers [1]. BM (OMIM≠158810) is caused by dominant mutations in *Col6A1, Col6A2* [2], and *Col6A3* [3] genes. UCMD (OMIM≠254090) is caused by recessive mutations in *Col6A3* gene [4], although dominantly inherited mutations have been reported [5, 6].

By analyzing the distribution of mutations it has been possible to draw some conclusions on genotype–phenotype correlations with either BM or UCMD [1]. These correlations are mainly based on the knowledge of the complex assembly of collagen VI and on the variety of interactions of the collagen VI fibers with components of the ECM.

Collagen VI assembly involves the association of three genetically distinct subunits, α1 (VI), α2(VI), and α3(VI) into a monomer, followed by the formation of disulphide bonded antiparallel dimers, which aggregate to form tetramers, also stabilized by disulphide bonds. On the surface of the cell, once secreted, tetramers associate end to end to form beaded microfibrils with a typical 100/105-nm periodicity [7, 8].

By transmission electron microscopy, collagen VI microfibrils are shown to form, in the ECM, both networks and fibers of stacking elements (Fig. 1).

In normal skeletal muscles, collagen VI is shown by immunofluorescence and electron microscopy to form a highly branched filamentous network in the ECM, which is in intimate contact with the basement membrane. Among the numerous constituents of the ECM, collagen VI interacts with collagen I, decorin, biglycan, fibronectin, and perlecan (Fig. 2). By these multiple interactions collagen VI network plays a key role in tissue architecture development, maintenance, and repair [9, 10]. In fact, in UCMD cases in which some mutant mRNA expression is maintained, the secreted collagen VI is not capable of forming extensive networks, and the microfilaments may build up parallel-running thick fibrils, or circular arrays with irregular distribution of globular domains [11].

Furthermore, we found an almost complete absence of immunolocalization of collagen VI in muscle biopsies of UCMD patients in which recessive mutations in collagen VI had been initially demonstrated [4], while merosin was normally expressed. This finding demonstrated that UCMD can be screened by immunofluorescence using monoclonal antibodies directed against one of the three subunits in the patient muscle biopsies (Fig. 3). Furthermore, collagen VI was almost completely absent in the ECM secreted by fibroblasts cultured in vitro, obtained from skin biopsies of UCMD patients, while a moderate expression of collagen VI was present inside the cells [11].

Targeted inactivation of the *Col6a1* gene in mice causes a myopathic disease resembling human BM [12]. When fibroblasts from *Col6a1*$^{-/-}$ mice were cultured in vitro, the secreted ECM, in which collagen VI was absent, was

characterized by an altered three-dimensional organization of fibronectin fibrils. A similar abnormal fibronectin deposition was observed in fibroblast cultures from patients affected by BM, suggesting that collagen VI mediates the three-dimensional organization of fibronectin in the ECM [13].

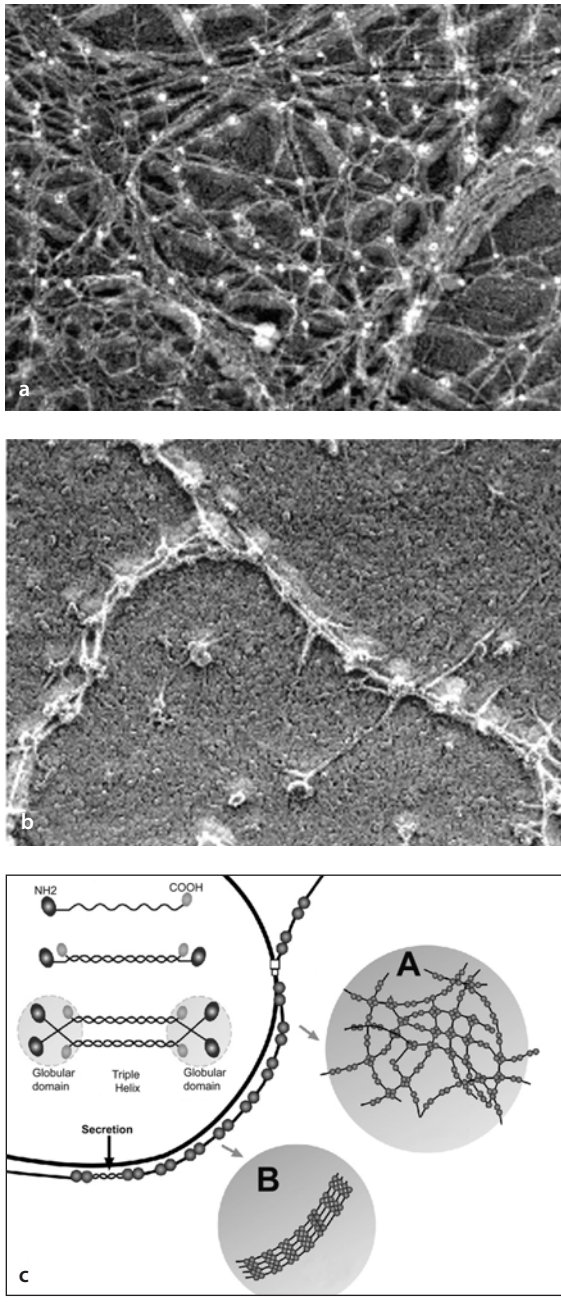

Fig. 1 Rotary shadowing of the ECM secreted by normal fibroblasts in which collagen VI fibrils, recognized by immuno-gold labeling, show the typical 105-nm period, and form either a network (**a**), or a stack of parallel fibrils (**b**), as indicated in the drawing (**c**), where the steps of collagen VI biosynthesis and assembly are also reported

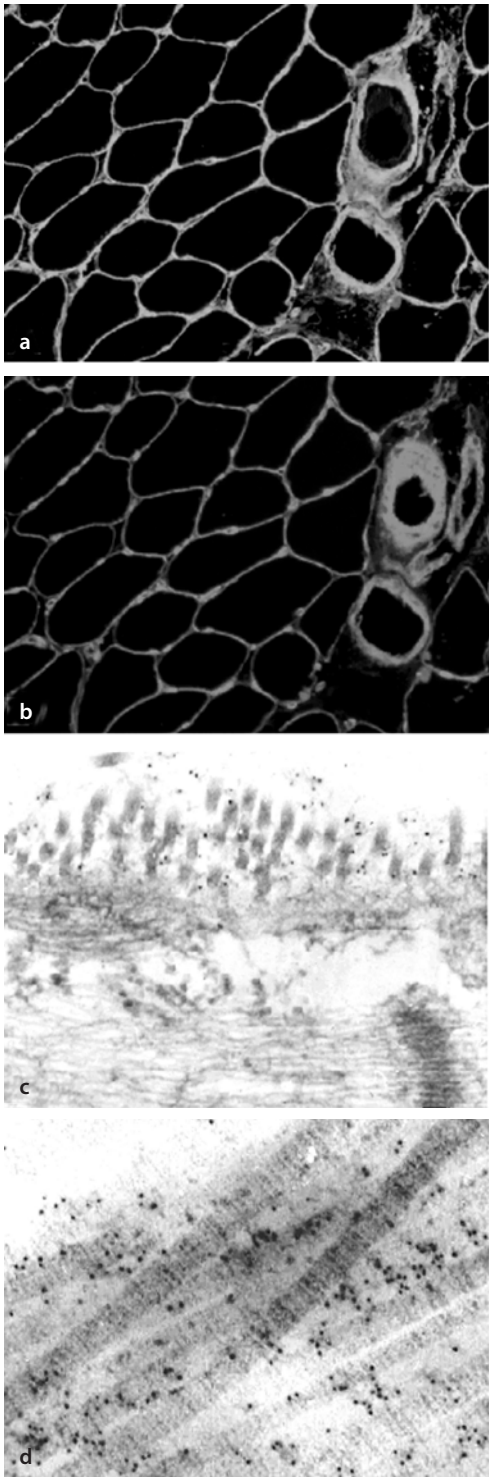

Fig. 2 Immunolabeling of collagen VI in normal skeletal muscle. Sections of skeletal muscle stained with antibodies against collagen VI (**a**) and perlecan (**b**); the two proteins are colocalized at the level of the muscle basal lamina. (**c**) Electron microscopy of a detail of the basal lamina of a muscle fiber; by immunogold collagen VI is identified at the level of the network of microfilaments connecting the basal lamina with the ECM, in which some collagen fibers are visible. (**d**) At higher magnification, gold particles decorate collagen VI microfilaments in close contact with fibrillar collagen

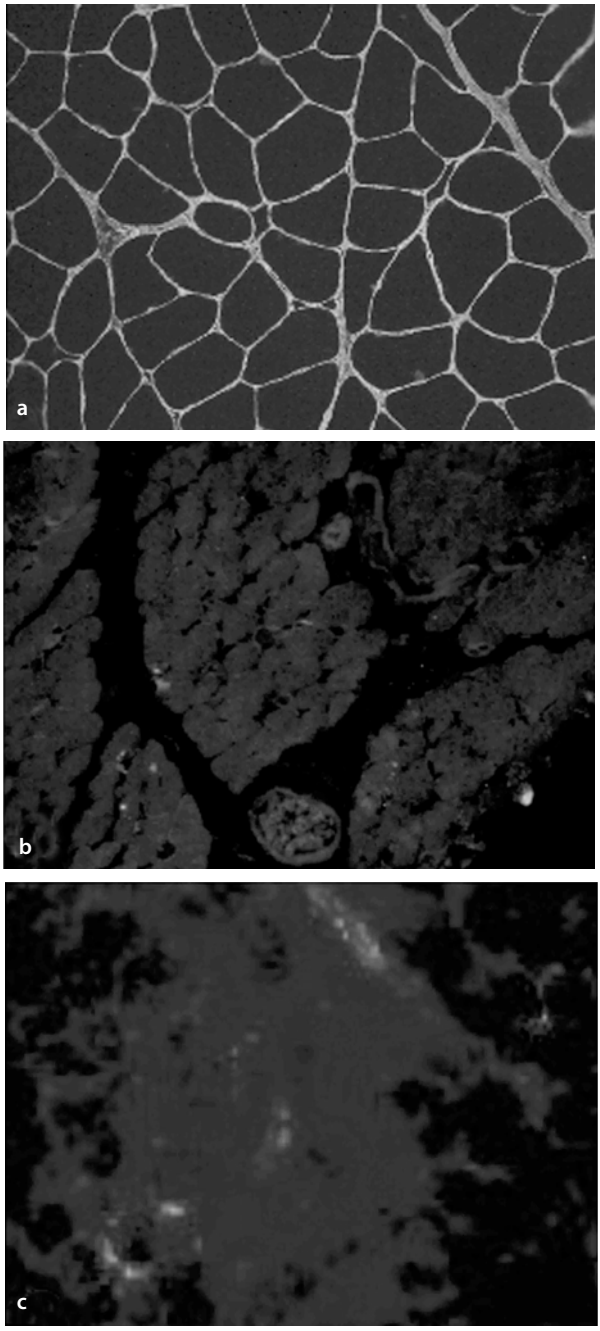

Fig. 3 Immunolabeling of skeletal muscle cryosections. (**a**) In control, the typical labeling is present all along the basal lamina surrounding each myofiber. (**b**, **c**) In these two muscle biopsies from UCMD patients characterized by a severe phenotype, collagen VI appears either absent or highly reduced

Mitochondrial Alterations and Apoptosis in Col6a1$^{-/-}$ Mice Muscle Fibers

Col6a1$^{-/-}$ mice showed a muscle phenotype that strongly resemble BM, owing to the presence of Evans blue-positive fibers [12] , while isolated muscles presented reduced contractile strength, isometric tetanic and twitch tension [14]. About one-third of muscle cells showed structural alterations, suggesting that collagen VI-deficient muscles contain differently affected fibers that might represent various steps of the dystrophic process. In agreement with these observations we found that about 30% of muscle fibers presented ultrastructural defects of mitochondria and SR. Mitochondria showed a swollen appearance, with abnormal tubular cristae and a reduced matrix density, while SR cisternae were dilated especially at the level of triadic system. In these cells showing mitochondrial alterations, several myonuclei presented the typical early hallmarks of apoptosis, such as peripheral chromatin condensation and irregular profiles. The presence of apoptotic nuclei in *Col6a1*$^{-/-}$ muscle, evaluated by TUNEL staining, attained a level sevenfold higher than in wild-type animals [14].

The increased apoptosis observed in *Col6a1*$^{-/-}$ mice fibers could be caused by mitochondrial dysfunctions as a consequence of the genetic defect. In fact, the mitochondrial involvement in triggering apoptosis has been largely documented. The release of mitochondrial proteins, including cytochrome c, is essential to form a cytosolic protein complex that activates effector caspases. Apoptotic stimuli, such as intracellular Ca^{2+} overload due to inefficiency of Ca^{2+} reuptake into the SR, could trigger the opening of the permeability transition pore (PTP), causing ion imbalance and structural alterations in mitochondria, leading to the release of proapoptotic factors [15].

However, mitochondrial transmembrane potential (Dy_m) of *Col6a1*$^{-/-}$ mice fibers was not significantly changed with respect to wild-type fibers [14]. Because ATP synthase could maintain Dy_m operating in a reverse mode [16], the effect of oligomycin, an inhibitor of ATP synthase, has been investigated. Interestingly, oligomycin-treated *Col6a1*$^{-/-}$ fibers showed quick and marked mitochondrial depolarization, not inducible in wild-type myofibers, indicating that a latent mitochondrial dysfunction in *Col6a1*$^{-/-}$ myofibers results in a higher proton permeability. Furthermore, oligomycin-treated *Col6a1*$^{-/-}$ fibers showed an increased percentage of apoptotic nuclei [14]. These results indicate that in collagen VI-deficient muscle fibers latent mitochondrial defects, partly masked by an increased ATP hydrolysis to maintain the mitochondrial transmembrane potential, result in the release of proapoptotic factors in a reduced, but significant, percentage of myofibers. The mitochondrial latent defects can be unmasked by treatments that inhibit ATP synthase, so that the rapid drop of Dy_m, results in an increased amount of cells undergoing apoptosis.

Pharmacological Inhibition of PTP Restores Mitochondrial Functions and Reverts the Muscle Phenotype

The reported alterations observed in *Col6a1*$^{-/-}$ myofibers, including Dy_m changes, increased apoptotic rate and ultrastructural mitochondrial defects, were normalized when *Col6a1*$^{-/-}$ myofibers were plated on collagen VI [14], indicating that the response to oligomycin was the result of a reversible alteration, involving the PTP opening.

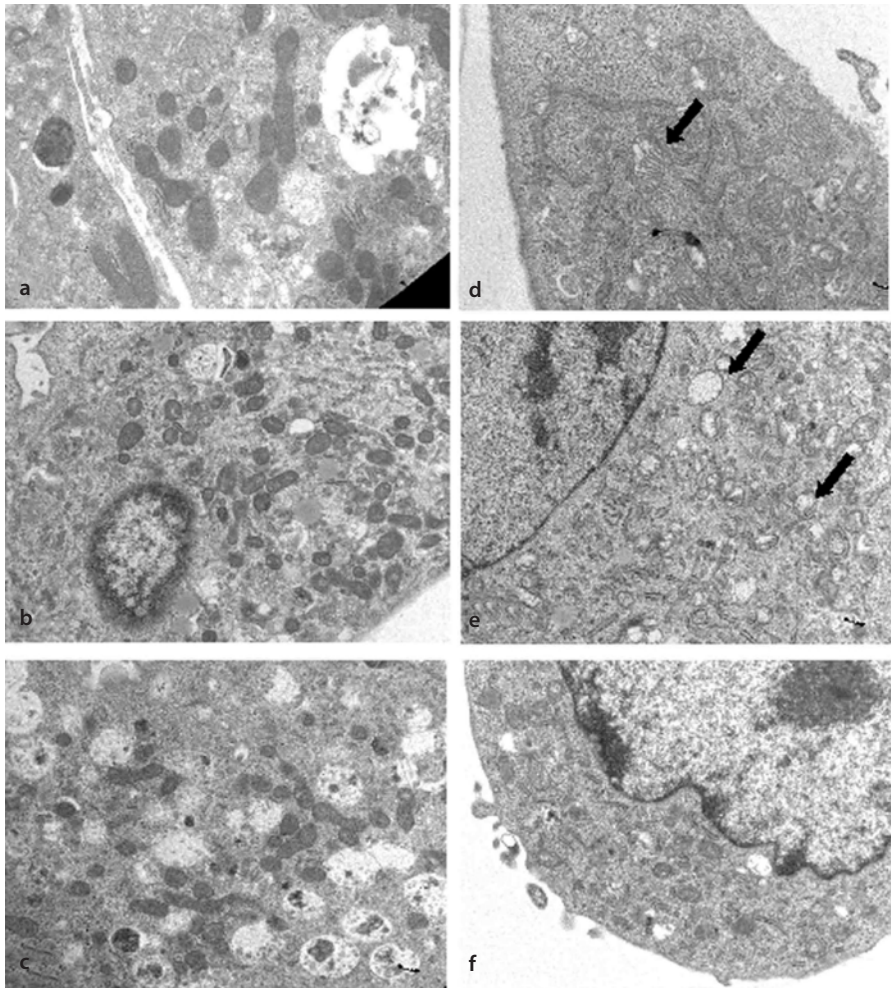

Fig. 4 Electron microscopy of samples of muscle fibers from wild-type (**a**-**c**) and from *Col6a1*$^{-/-}$ mice (**d**-**f**), at basal conditions (**a**, **d**), treated with oligomycin (**b**, **e**), or with oligomycin and cyclosporine A (**c**, **f**). With respect to wild-type animals (**a**), a certain percentage of *Col6a1*$^{-/-}$ mice muscle cells present mitochondria with a swollen appearance (**d**, *arrows*). The percentage of cells with swollen mitochondria is greatly increased in *Col6a1*$^{-/-}$ mice after the exposure to oligomycin (**e**), which is ineffective on wild-type cells (**b**). The treatment of cell cultures with oligomycin and cyclosporin A prevents the mitochondrial swelling (**f**)

Cyclosporin A (CsA) is a potent PTP inhibitor, utilized as immuno-suppressor to prevent transplant rejection. When *Col6a1*$^{-/-}$ myofibers were treated with CsA, the oligomycin sensitivity was completely abolished, so that both the Dy_m dropping, the apoptotic rate, and the mitochondrial structural alterations were reverted to levels of the wild-type cells or to those of *Col6a1*$^{-/-}$ myofibers plated on collagen VI (Fig. 4).

The crucial role of PTP in the pathogenesis of the myopathy in vivo has been highlighted by the results obtained after intraperitoneal injection of 5mg CsA per Kg body weight every 12h for 4 days in *Col6a1*$^{-/-}$ mice. In fact, the in vivo pharmacological treatment resulted in a reduction of apoptosis to basal level in both muscles and isolated myofibers, an increased capacity of Ca^{2+} retention in isolated mitochondria, and a complete disappearance of both mitochondrial and SR abnormalities [14].

Mitochondrial Defects can be Reverted by CsA in UCMD Cultured Myofibers

In order to evaluate the reliability of a pharmacological treatment of patients affected by genetic diseases of collagen VI, the responsiveness to CsA should be demonstrated also in cell cultures obtained from human patients.

In skeletal muscles of UCM patients, cytochrome c oxidase activity (COX) reaction revealed an altered pattern with respect to control; while in control ATPase (pH 10) highly positive fibers presented a low level of COX activity, UCMD patient samples presented a COX activity concentrated in some fibers, irrespective to ATPase staining. By Nomarsky interference microscopy, the mitochondria, stained by COX, showed an elongated shape in controls, while an apparent fragmentation was detected in UCMD cells. At the electron microscope, COX reaction resulted in a uniform staining of the mitochondrial membrane profiles, while in UCMD cells swollen mitochondria were almost negative (Fig. 5).

The apparent mitochondrial fission, occurring in a reduced percentage of cultured cells obtained from UCMD patients, was significantly increased in UCMD cells exposed to oligomycin. This effect was greatly reduced when UCM cells were treated with CsA and oligomycin (Fig. 6). Furthermore, we confirmed the results previously obtained in *Col6a1*$^{-/-}$ mice cells; in fact, the percentage of UCMD cells showing swollen mitochondria was greatly increased by oligomycin, while CsA prevented the appearance of mitochondrial alterations.

These results indicate that the pathogenic mechanism leading to a myopathic phenotype is similar in UCMD and in *Col6a1*$^{-/-}$ mice cells and that, at least in vitro, the latent mitochondrial defects present in UCMD cells, unmasked by the block of the ATPase activity, can be prevented by CsA, with a mechanism similar to that found in *Col6a1*$^{-/-}$ mice.

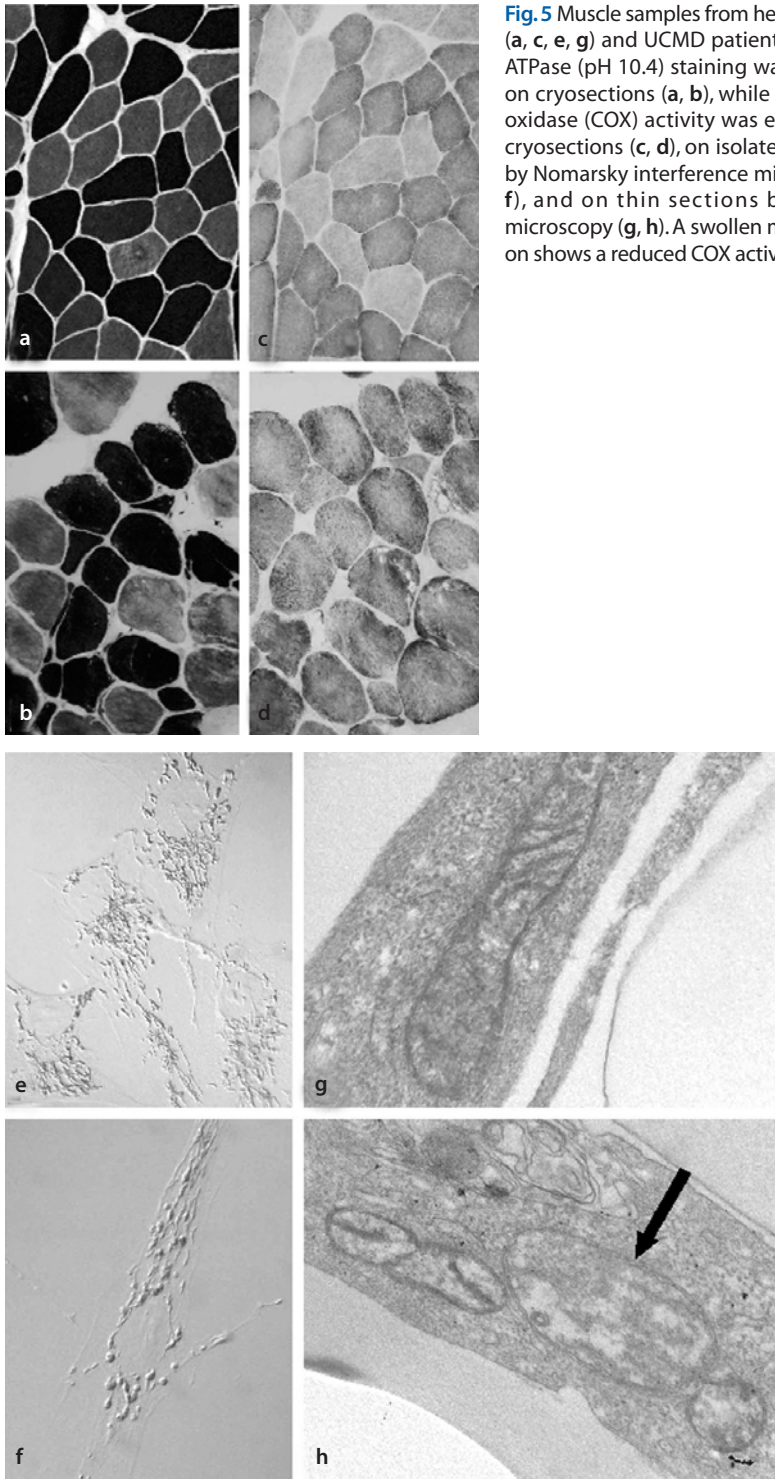

Fig. 5 Muscle samples from healthy donors (**a**, **c**, **e**, **g**) and UCMD patients (**b**, **d**, **f**, **h**). ATPase (pH 10.4) staining was evaluated on cryosections (**a**, **b**), while cytochrome oxidase (COX) activity was evaluated on cryosections (**c**, **d**), on isolated myofibers by Nomarsky interference microscopy (**e**, **f**), and on thin sections by electron microscopy (**g**, **h**). A swollen mitochondrion shows a reduced COX activity (**h**, *arrow*)

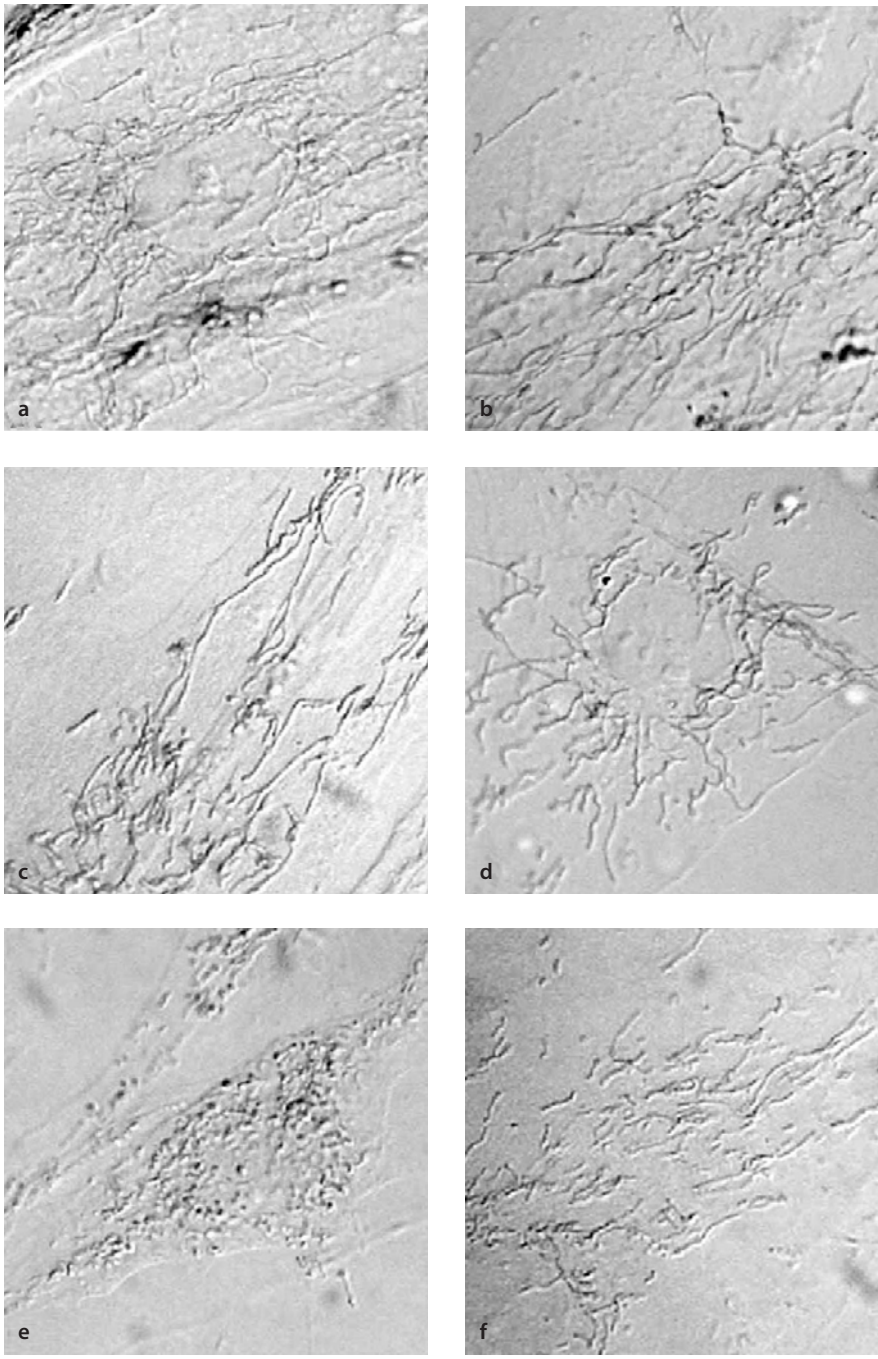

Fig. 6 Nomarsky interference microscopy of muscle cells from an healthy donor (**a**-**c**) and a UCMD patient (**d**-**f**) showing mitochondrial response to oligomycin (**b**, **e**) or oligomycin and cyclosporine A (**c**, **f**). Mitochondria are stained by COX

Conclusions

The reported experimental evidence demonstrated a link between a genetic disease affecting the expression of a ECM protein and the induction of apoptosis by mitochondrial dysfunction. Both mitochondrial defects and apoptosis can be prevented by CsA, which acts by blocking the mitochondrial PTP, to a level similar to that obtained by plating *Col6a1*$^{-/-}$ mice cells on collagen VI. The way by which an altered ECM organization could be sensed by mitochondria is still a matter of speculation. It is conceivable that normal interactions involving ECM, integrins, and intracellular signaling pathways are impaired when ECM is lacking collagen VI. This could result in an imbalance of Rac, which modulates mitochondrial ROS production [17], or a modification of the expression levels of Bcl-2-related protein [18].

A further mechanism, involving laminin, dystrophin, and the actin cytoskeleton could also be involved, given that the fusion mechanism and the regulation of mitochondrial distribution are under the control of cytoskeletal motors and small GTPase [19]. Since mitochondrial fusion/fission mechanisms are involved in the control of apoptosis [20], and the integrity of the mitochondrial net is essential for muscle cell metabolism [21], the pathogenic mechanism of collagen VI-related muscle disorders appears to depend on the collagen-mitochondria connection [22] which, although not completely clarified, represents an effective target for pharmacological intervention.

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Chapter 9

Mitochondria in Cell Life and Death

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Introduction

The mitochondrion represents a unique organelle within the complex endomembrane systems that characterize any eukaryotic cell. It is realistic to state that complex life on earth has been made possible through the "acquisition" of mitochondria which provide an adequate supply of substrates for energy-expensive tasks. Higher multicellular organisms have indeed high-energy requirements necessary to carry out complex functions, such as muscle contraction, hormones and neurotransmitters synthesis and secretion, in addition to basal cellular metabolism (biomolecules synthesis and transformation, maintenance of ionic gradients across membrane, cell division). Mitochondria can fulfill this huge energy demand thanks to their extraordinary biosynthetic capacities: every day, mitochondria of a single human being can recycle up to 50 Kg of ATP. To further underline the relevance of these subcellular structures, one can also consider how these organelles have affected the physiology of the whole organism: lungs, heart, and circulatory system have evolved essentially to provide molecular oxygen to mitochondria, which consume about 98% of the total O_2 we breathe. However, beyond the pivotal role they play in ATP production, a whole new mitochondrial biology has emerged in the last few decades: mitochondria have been shown to participate in many other aspects of cell physiology such as amino-acid synthesis, iron-sulphur clusters assembly, lipid metabolism, Ca^{2+} signaling, reactive oxygen species (ROS) production, and cell death regulation. Hence, it is consequent that any mitochondrial dysfunction will inevitably lead to disease. Indeed, many pathological conditions are associated with organelle failure, including neurodegenerative diseases (Alzheimer's, Parkinson's, Huntington's), motoneuron disorders (amyotrophic lateral sclerosis, type 2A Charcot-Marie-Tooth neuropathy), autosomal dominant optic atrophy, ischemia-reperfusion injury, diabetes, aging, and cancer.

Understanding how mitochondria can sense, handle, and decode vari-

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ous signals from the cytosol and other subcellular compartments represents a new exciting challenge in biomedical sciences.

Mitochondria: The Basics

The mitochondrion is a double membrane-bounded organelle thought to be derived from an a-proteobacterium-like ancestor, presumably due to a single ancient invasion occurring more than 1.5 billion years ago. The basic evidence of this endosymbiont theory [1] is the existence of the mitochondrial DNA (mtDNA), a 16.6-Kb circular, double-stranded DNA molecule with structural and functional analogies to bacterial genomes (gene structure, ribosome). This mitochondrial genome encodes only 13 proteins (in addition to 22 tRNAs and 2 rRNAs necessary for their translation), all of which are components of the electron transport chain (mETC) complexes (I, III, and IV), while the whole mitochondrial proteome consists of more than 1,000 gene products. Thus, one critical step in the transition from autonomous endosymbiont to organelle has been the transfer of genes from the mtDNA to the nuclear genome. At the same time, eukaryotes had to evolve an efficient transport system to deliver nuclear-encoded peptides inside mitochondria: TIM (Transporters of the Inner Membrane), TOM (Transporters of the Outer Membrane) and mitochondrial chaperones (such as hsp60 and mthsp70) build up the molecular machinery that allows the newly-synthesized unfolded proteins to enter mitochondrial matrix [2].

Mitochondria are defined by two structurally and functionally different membranes: the plain outer membrane, mostly soluble to ions and metabolites up to 5,000 Da, and the highly selective inner membrane, characterized by invaginations called cristae which enclose the mitochondria matrix. The space between these two structures is traditionally called intermembrane space (IMS), but recent advances in electron microscopy techniques shed new light on the complex topology of the inner membrane. Cristae indeed are not simply random folds but rather internal compartments formed by profound invaginations originating from very tiny "point-like structures" in the inner membrane [3]. These narrow tubular structures, called cristae junctions, can limit the diffusion of molecule from the intra-cristae space towards the IMS, thus creating a micro-environment where mETC complexes (as well as other proteins) are hosted and protected from random diffusion.

As mentioned before, mitochondria are the main site of ATP production. When glucose is converted to pyruvate by glycolysis, only a small fraction of the available chemical energy has been stored in ATP molecules: mitochondria can "release" the remaining amount of energy with an outstand-

ing efficiency (from a single glucose molecule mitochondria produce 15 times more ATP than glycolysis). The main enzymatic systems involved in this process are the tricarboxylic acid (TCA) cycle and the mETC. Products from glycolysis and fatty acid metabolism are converted to acetyl-CoA, which enters the TCA cycle where it is fully degraded to CO_2. More importantly, these enzymatic reactions generate NADH and $FADH_2$, which provide reducing equivalents and trigger the electron transport chain. mETC consists of five different protein complexes: complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (ubiquinol cytochrome c reductase), complex IV (cytochrome c oxidase), and complex V which constitutes the F1F0-ATP synthase. Electrons are transferred from NADH and $FADH_2$ through these complexes in a stepwise fashion: as electrons move along the respiratory chain, energy is stored as an electrochemical H+ gradient across the inner membrane, thus creating a negative mitochondrial membrane potential (estimated around -180 mV against the cytosol). H+ are forced to re-enter the matrix mainly through complex V, which couples this proton-driving force to the phosphorylation of ADP into ATP, according to the chemiosmotic principle. ATP is then released to IMS through the electrogenic Adenine Nucleotide Translocase (ANT), which exchange ATP with ADP to provide new substrate for ATP synthesis. Finally, ATP can easily escape the IMS thanks to the mitochondrial porin of the outer membrane, VDAC (voltage dependent anion channel) [4].

Mitochondrial Biogenesis

As mentioned before, mitochondrial proteins are encoded by two distinct genetic systems, the mtDNA and the nuclear DNA (nDNA). Thus, mitochondrial replication must be a highly coordinated process that combines mtDNA duplication with synthesis of gene products of both nuclear and mitochondrial genomes. Recently, new transcription factors that govern mitochondrial biogenesis have been identified, while the signaling pathway that leads to their activation is still debated. The master gene that coordinates mitochondrial biogenesis is peroxisome proliferator activated receptor γ coactivator 1α (PGC1α), originally identified as a cold-inducible coactivator in adaptative thermogenesis [5].

PGC1α gene is located on chromosome 4 and encodes a protein containing 798 amino acids placed in the cell nucleus. It is highly expressed in all the tissues where mitochondria are abundant and oxidative metabolism is active, such as brown adipose tissue (BAT), skeletal muscle and heart (but also in brain and kidney). PGC1α acts as a transcription factor which induces the expression of two other transcriptional regulators, NRF-1 and NRF-2 (nuclear respiratory factors 1 and 2), which in turn activate the synthesis

of nuclear encoded mitochondrial-targeted proteins. Moreover, PGC1α induces mtTFA (mitochondrial transcription factor A), a protein transferred to mitochondria where it promotes the expression of mitochondrial encoded proteins and mtDNA replication. By this way, PGC1α can guarantee the critical balance of mitochondrial and nuclear encoded proteins that is necessary for the correct assembling of respiratory complexes [6].

PGC1α regulation seems finely tuned to reflect cellular energy demands, with conditions of increased energy needs, such as cold, physical exercise, or fasting inducing its expression. Fasting induces hepatic PGC1α expression, which increases gluconeogenesis, while physical activity induces its expression in heart and skeletal muscle, thus increasing mitochondrial biogenesis and oxidative phosphorylation. As mentioned before, PGC1α was originally discovered as a cold inducible transcription factor in adaptative thermogenesis, the physiological process through which energy is dissipated as heat in response to environmental conditions such as cold or overfeeding. BAT in rodents and skeletal muscle in humans are the main sites involved in this process. Biochemically, the adaptive thermogenic process needs the stimulation of mitochondria (increasing nutrients catabolism) and the uncoupling of oxidative phosphorylation through the expression of the uncoupling protein UCP1. This protein dissipates the proton gradient created by mETC complexes by increasing mitochondrial inner membrane H+ permeability, thereby inducing energy dissipation as heat. This pivotal role of PGC1α in thermogenesis is lastly demonstrated by observing that knockout mice for this transcription factor are unable to face cold stress due to a continuous drop of the core body temperature [7, 8]. However, the physiological relevance of this protein goes far beyond the simple thermogenic program. Indeed, heart and skeletal muscle are profoundly remodeled upon PGC1α activation. Skeletal muscle fibers are classified in three main categories, showing different metabolic capabilities: slow-twitch type I and fast-twitch type IIa are rich in mitochondria and show a clear oxidative metabolism; fast-twitch type IIb fibers have instead a lower mitochondrial content, being mainly metabolically glycolytic. PGC1α expression is promptly activated by both short-term exercise and endurance training, resulting in the conversion of type IIb fibers into type IIa or type I fibers. Again, transgenic mouse models in which PGC1α is selectively overexpressed in muscle cells show a much higher resistance to fatigue, while null mice show reduced exercise capacities. The upstream signaling that activates PGC1α expression is not yet fully understood, but it seems to be mediated (at least in part) by CaMK pathway. Indeed, the increased contractile activity and neuromuscular stimulation induced by training cause the activation of CaMK and in turn of several transcription factors such as MEF2 that binds to the PGC1α promoter, thus inducing its expression [9, 10].

Mitochondria and Reactive Oxygen Species

The important role of redox signaling in the regulation of physiological responses is underscored by the apparent dysregulation of physiological responses in various disease-related oxidative stress conditions. Excessive levels of reactive oxygen species (ROS) may be generated by mechanisms that produce ROS "accidentally" in an unregulated fashion. This includes the production of ROS by the mitochondrial electron transfer chain, the quantitatively most important source of ROS in higher organisms. These chemical species are characterized by the presence of an unpaired electron on the oxygen atom that can promptly react with virtually any biomolecules. Thus, mitochondrial structures are particularly susceptible to oxidative damage as evidenced by lipid peroxidation, protein oxidation, and mitochondrial DNA mutations [11]. ROS have been implicated in many pathological conditions, in particular in the aging process. Indeed, the "free radical theory" of aging has a long history and it has been originally proposed in the 1950s [12]. This hypothesis was initially hotly debated, at least until the discovery of the first cellular enzyme involved in ROS metabolism, superoxide dismutase [13]. The existence of a protein whose unique function was to scavenge oxygen free radicals represented the first indirect but strong evidence that cells not only produce ROS but they also need systems to protect against them. ROS are generated by many enzymes, such as cyclo-oxygenases and NADPH oxidases, and in different subcellular compartments (i.e., they are generated by lipid metabolism within peroxisomes). However, the large majority of total ROS are undoubtedly produced by mitochondria, since they are a direct consequence of oxidative phosphorylation [14]. Indeed, at different sites along the mETC (in particular at complex I and III) electrons can "escape" and react directly with molecular oxygen, thus generating superoxide anions. ROS detoxifying enzymes represent the first line of defense against free radicals. Superoxide dismutase (SOD) is today known to exist in two different isoforms: while SOD1 is a copper-containing enzyme present is the cytosol, SOD2 (a manganese-containing protein) is located inside mitochondrial matrix where it converts superoxide anion to H2O2, which can be further degraded to water and oxygen by catalase. The number of known ROS detoxifying enzymes grew very fast in last few decades and includes the large family of glutathione peroxidases (GPx) and peroxiredoxins (Prx), which was recently reported to exist in mitochondrial matrix (Prx III) [15]. Mitochondria also have another mechanism to protect against ROS. Indeed, the uncoupling of oxidative phosphorilation through the action of UCP proteins and thus the decrease of mitochondrial membrane potential shortens the half-life of the most reactive steps in the electron

transport chain, thereby inhibiting ROS production [16]. Thus, given that prevention is better than the cure, mitochondria can "decide" to slow down their metabolism to prevent oxidative damage. This fact is shiningly demonstrated by observing that the above-mentioned PGC1α knockout mice show a much higher sensitivity to oxidative damage, especially in neurons [17]. This means that PGC1α not only promotes mitochondrial biogenesis and oxidative metabolism but it coincidentally takes care of the potentially harmful effect of ROS induction. This is achieved by the two described mechanisms: on one side by increasing ROS scavenging enzymes (SOD1, SOD2, catalase, and GPx) and on the other by decreasing ROS production (through the induction of UCPs).

By the way, cells have always been forced to cohabit with free radicals. Thus, it is not unworthy to wonder whether this harmful chemical species could also be exploited to participate in physiological regulation of normal cellular events. Indeed, one of the most fascinating hypotheses is that ROS, besides their obvious toxic effect, could even participate in signal transduction. This notion is supported by recent works on the role of p66shc, the first mammalian protein whose mutation was demonstrated to increase resistance to oxidative stress and to prolong life span [18]. Intriguingly, upon activation, including phosphorylation by PKCb and Pin1 recognition, p66shc translocates to mitochondria [19] where it exerts its own oxidoreductase activity [20]. Indeed, p66shc directly oxidizes cytochrome c (thus allowing electron to escape mETC) and generates H_2O_2, leading to mitochondrial permeability transition pore opening (mPTP) and in turn cell death. The existence of a protein that "steals" electrons from the mETC and produces reactive oxygen species represents the first molecular evidence of the role of reactive oxygen species in signal transduction, finally describing the biochemical basis of the free radical theory of aging.

Interestingly, other well-studied proteins such as p53, protein kinase C (PKC), and Apurinic-apyrimidinic endonuclease/Redox effector factor (Ape/Ref-1) play an important role in ROS-mediated pathways and translocate to mitochondria during redox stimulation [21-23].

Mitochondrial Dynamics

Mitochondrial shape appears very heterogeneous within different cell types and, in some cases, even in the same cell. Indeed, they can form either a short, rod-like structure or a continuous, elongated, tubular, highly dynamic and interconnected network. These differences in phenotype are the result of a complex equilibrium among mitochondrial motility, fusion, and fission rates. Since mitochondrial biogenesis occurs predominantly in the perinuclear region, eukaryotes had to evolve efficient systems to transport these

organelles where energy demands are higher or where their peculiar metabolic functions are required. This is of critical relevance in cells with complex topology such as neurons, where mitochondria are abundant in the synaptic region of the axon. This singular distribution most likely reflects the high-energy requirement of the synaptic transmission (ATP-driven release and recycling of vesicles, ATP-dependent pumps that control ions homeostasis, etc.) as well as the specific mitochondrial functions such as Ca^{2+} signaling regulation. Mitochondria exploit cytoskeletal elements as tracks for their directional movements by using specialized molecular machinery in a way we began to understand only in the last few years. These organelles have been shown to interact with every cytoskeletal element (microfilaments, microtubules, and intermediate filaments) in different species. However, while in budding yeast mitochondria seem to move predominantly through the actin network, in mammals mitochondrial movement is mainly a microtubules-driven process, with actin aiding in short-range mitochondrial positioning in microtubules-poor regions [24]. Recent studies suggest that the main microtubules-associated motors are kinesin-1 (for anterograde transport) and the cytoplasmic dynein (for retrograde movement). These motors are likely present in higher order molecular complexes that bind to mitochondria: for example, it has been demonstrated that the existence of a complex brings together the heavy chain of conventional kinesin-1 with the adaptor protein Milton and the mitochondrial protein Miro (mitochondrial Rho-like GTPase), and this complex is required for mitochondrial axonal transport [25]. Moreover, mitochondrial movement is also influenced by second messengers such as Ca^{2+}, and it actively participates in signaling cascades: for example Ca^{2+} release from endoplasmic reticulum transiently blocks mitochondrial movements. This inhibition in mitochondrial motility reflects an increased mitochondrial calcium uptake and thus enhances the local Ca^{2+} buffering capacities of mitochondria, with important consequences in signal transduction [26].

Apart from organelles movement along the cytoskeleton, mitochondria also continuously remodel their shape. In the early 1990s, genetic screens in yeast identified the first proteins involved in mitochondrial morphology and subsequent studies revealed that mitochondrial shape is determined by two dynamically opposed processes, fusion and fission. Indeed, genetic ablation of key regulators of the fusion machinery gives rise to cells with fragmented organelles because of unopposed ongoing fission, while the knockout of genes that mediate fission leads to the formation of an almost unique, deeply interconnected mitochondrial network. Interestingly, in yeast the coinciding ablation of both fusion and fission apparatus produces a wild-type mitochondrial morphology but also shows a high frequency of mtDNA loss, suggesting an essential role of fusion and fission in maintaining mitochondrial genome [27, 28]. Considering the structural complexity of mito-

chondria, it should be immediately clear that the molecular machinery mediating fusion and fission has to be a quite intricate mechanism, requiring the independent but coordinated processing of both outer and inner membranes. Proteins involved in mitochondrial dynamics have been originally identified in yeast but many of these genes have orthologs in mammals, mainly belonging to the large GTPase protein family. The molecular motors playing a pivotal role in outer membrane fusion are mitofusins (Mfn1 and Mfn2): they are characterized by the presence of a highly conserved GTPase domain, two transmembrane regions that enable the anchoring to OMM and two peculiar coiled coil structures, HR1 and HR2 (heptad repeat domain 1 and 2). During organelle fusion, mitofusins mediate the tethering of two adjacent mitochondria by forming trans homotypic (consisting of the same Mfn isotypes) or heterotypic (consisting of Mfn1 and Mfn2) complexes through the interaction of their C-terminal HR2 domains. Whether these two isoforms are functionally different or simply redundant remains to be clarified [29]. After that, mitochondrial inner membrane fusion is achieved through the activity of another protein, OPA1. Surprisingly, this protein has been recently shown to control also IMM ultrastructure: together with the rhomboid protease PARL (presenilin-associated rhomboid like), OPA1 forms oligomers essential for the maintenance of internal cristae structure, thereby controlling their remodeling during apoptotic cell death (see below). On the other hand, the master gene regulating mitochondrial fission is Drp1 (Dynamin-related protein 1). It shares high homology with dynamin, a mechanoenzyme involved in the excision of clathrin-coated endocytic vesicles, and is normally located in the bulk cytosol. Upon induction, Drp1 redistributes into punctuated foci colocalizing with mitochondria where it mediates organelle fission. Conversely, the deciphering of the molecular players mediating remains elusive both in mammals as well as in yeast. It has been proposed that IMM processing could also be a simple mechanical consequence of outer membrane constriction and cleavage induced by DRP1.

Mitochondria and Cell Death

Every multicellular organism has endogenous mechanisms for selectively killing their own cells. This process has a huge physiological relevance since it is necessary to eliminate damaged, superfluous, dangerous, or aged cells and is thus involved in many physiological and pathological processes, such as embryogenesis, development, differentiation, tissue homeostasis, tumorigenesis, neurodegeneration, and viral infections. The term "apoptosis" was originally coined by John Kerr in 1972, describing a peculiar cell death mechanism morphologically characterized by cell shrinkage, chro-

matin condensation, DNA fragmentation, plasma membrane blebbing, and formation of apoptotic bodies [30]. Biochemically, apoptosis is a highly regulated proteolytic event, achieved through the activation of a broad family of evolutionary conserved cysteine aspartate-specific proteases, caspases, which are usually present in cytoplasm as inactive enzymes (zymogens). Caspases, and consequently apoptosis, can be activated by two major pathways. First, the so-called extrinsic pathway triggered by plasmamembrane receptors such as TNFa (tumor necrosis factor a) or Fas (also known as Apo-1 or CD95): the activation of these receptors induces the assembly of a protein complex named DISC (death inducing signaling complex), which recruits and activates caspases cascade. On the other side, the intrinsic pathway relay is activated through the release of several mitochondrial proteins toward the cytosol. The main player in the finely tuned apoptotic activation process is undoubtedly cytochrome c. This protein is encoded by a nuclear gene and synthesized in the cytoplasm as a precursor; after being imported into mitochondria, it is refolded and bound to a hem prosthetic group, localizing in the IMS. The majority of cytochrome c is tightly bound to mitochondrial inner membrane thanks to its electrostatic interactions with acidic phospholipids, but a small fraction probably exists loosely attached to IMM and available for mobilization. This protein is an irreplaceable component of the mETC, shuttling electrons from complex III to complex IV, and it is thus essential to life: the disruption of its only gene is embryonically lethal [31]. Surprisingly, cytochrome c is also one of the pivotal players in the induction of cell death: once released in the cytoplasm, this protein drives the assembly of a caspases activating complex together with Apaf-1 (apoptosis-protease activating factor 1) and caspase 9, the so-called apoptosome. Apaf-1 consists of three functional domains: an N-terminal CARD (caspase-recruitment domain), a central nucleotide-binding domain, and twelve to thirteen WD-40 repeats at the C-terminus of the molecule. This protein is normally present in cytosol as an inactive monomer, where the WD-40 motifs self inhibit the CARD domain from recruiting caspase 9. Cytochrome c, once in the cytosol, induces the rearrangement and hepta-oligomerization of Apaf-1: each of these complexes can recruit up to seven caspase molecules, leading to their proteolytic self-processing and consequent activation [32].

Mitochondria contain many other proapoptotic, IMS-resident proteins, such as Smac/DIABLO, HtrA2/Omi, apoptosis inducing factor (AIF), and EndoG (Endonuclease G). DIABLO (direct inhibitor of apoptosis-binding protein with a low isoelectric point) and HtrA2 (high temperature requirement protein A2) both have an N-terminal domain that can interact and inhibit IAPs (inhibitor of apoptosis proteins). IAPs, such as XIAP, cIAP-1, and cIAP-2, are cytosolic soluble peptides that normally associate and stabilize procaspases, thus preventing their activation. Con-

versely, AIF and EndoG translocate from IMS to the nucleus upon treatment with several apoptotic stimuli where they seem to mediate chromatin condensation and DNA fragmentation [33].

The critical checkpoint in apoptotic intrinsic pathway induction is controlled by the BCL-2 protein family. The founding member, the anti-apoptotic BCL-2 proto-oncogene, was originally identified in human follicular B cell lymphoma and it represents the first oncogene acting as cell death inhibitor rather than as a promoter of cell proliferation. The BCL-2 family can be divided into three main categories according to the presence of the four conserved domains BH1-4 (BCL-2 homology). The anti-apoptotic members (BCL-2, BCL-XL, BCL-W, MCL-1) contain all four conserved domain BH1-4, while the proapoptotic multidomain proteins (BAX, BAK) possess only the BH1-3 amphipathic a-helices. Finally, the so-called BH3-only proteins such as BID or PUMA contain only one of these domains and display proapoptotic function by directly activating BAX and BAK. These two proteins exist as inactive monomers in viable cells with BAX localizing in the cytosol, loosely attached to membranes, and BAK residing in mitochondrial fraction. Upon apoptosis induction, BAX translocates to mitochondria where it homo-oligomerizes and inserts in the outer membrane; similarly, also BAK undergoes a conformational change which induces its oligomerization at the OMM level. Together these events trigger the mitochondrial outer membrane permeabilization (MOMP), the crucial process mediating the release of IMS-resident caspase cofactors into the cytoplasm [34]. Moreover, MOMP requires also the coincident remodeling of IMM structure through the widening of cristae junctions and the consequent mobilization of proteins entrapped in the intra-cristae space. As mentioned before, this process is under the control of the rhomboid protease PARL and the large GTPase OPA1. The latter is normally embedded in the IMM but it can be cleaved by the transmembrane protease PARL, thus originating a soluble pool of OPA1. These two versions of OPA1 (the longer membrane bound and the shorter IMS soluble) help the maintenance of the firm constriction of cristae junctions through their hetero-oligomerization. During apoptosis, the proapoptotic members of BCL-2 family disrupt these oligomers, thus aiding the mobilization of mitochondrial caspase cofactors [35, 36].

Apart from these ultrastructural changes, mitochondria also undergo more "macroscopic" remodeling of their shape during programmed cell death. Indeed, after apoptosis induction, mitochondria become largely fragmented, resulting in small, rounded, and numerous organelles. This process occurs quite early in cell death pathway, soon after BAX/BAK oligomerization, but prior to caspase activation. Interestingly, the perturbation of the equilibrium between fusion and fission rates seems to correlate with cell death sensitivity. In particular, conditions where mitochondrial fission is inhibited, such as

DRP1 downregulation or mitofusins overexpression, strongly delay caspase activation and cell death induced by numerous stimuli. Similarly, stimulation of organelle fission (by DRP1 overexpression or Mfn1/2 and OPA1 inhibition) promotes apoptosis by facilitating cytochrome c release and apoptosome assembly [37]. However, mitochondrial morphology is highly variable among different cell types and so are the signaling events leading to cell death, suggesting that mitochondrial fragmentation is not always a clear symptom of apoptosis. In some conditions, DRP1 overexpression has been reported to protect cells from apoptosis, such as in the case of apoptosis induced by mitochondrial Ca^{2+} overload [38].

Another hallmark in apoptosis is the loss of mitochondrial membrane potential, caused by the opening of the so-called mitochondrial permeability transition pore (mPTP). The mPTP is a large conductance channel presumably formed through a conformational change of several constituent mitochondrial proteins. Its opening can be triggered by different pathological conditions, such as Ca^{2+} overload, ATP depletion, oxidative stress, high inorganic phosphate (Pi), or fatty acid. The exact molecular structure of this pore is currently highly debated, but the main players in mPTP assembly seem to include the adenine nucleotide transporter (ANT) in the inner membrane, the voltage-dependent anion channel (VDAC), and the peripheral benzodiazepine receptor (PBR) of the outer membrane and cyclophilin D (CyP-D), a matrix protein [39]. The first obvious consequence of mPTP opening is mitochondrial depolarization followed by organelle swelling, cytochrome c release, caspase activation, and apoptotic cell death. The contribution of mPTP to normal cellular physiology in healthy cells (i.e., transient opening of this high conductance channel) is still a matter of debate. However, the availability of chemical mPTP inhibitors such as CsA (an immunosuppressant drug whose molecular target are cyclophilins) and the development of CyP-D knock-out mouse models clearly underline the huge relevance that permeability transition plays in pathological conditions, such as ischemia-reperfusion injury, liver diseases, neurodegenerative and muscle disorders [40-42].

Conclusions

Energy metabolism and the apoptotic program are the two major determinants of cell fate. Many growth factors increase glucose uptake and induce the translocation of hexokinase (the first limiting step of glycolysis) to mitochondria, thus promoting energy production. At the same time, mitochondria lay at the heart of programmed cell death regulation, representing an authentic “cellular poison cupboard” which takes care of the key components involved in apoptosis. Moreover, this ancient

endosymbiont fully integrated into its host, participating in many aspects of cell signaling (Ca^{2+} dynamics, steroid biosynthesis, ROS production etc.). All these observations lead to a really complex but fascinating picture, where mitochondria represent a sort of decoding station: they can sense different environmental conditions or receive diverse signals, integrate them all together, finally producing an outcome that can decide the fate of the cell. The understanding of these complex mechanisms is a hard and challenging task, but in the next few years it will provide new chances in the comprehension of numerous still poorly understood human pathologies.

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SECTION V

Nutrition, Physical Exercise and Obesity

Chapter 10

Evaluation of Nutritional State in Individuals that Practice Fitness

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Introduction

The nutritional assessment is a challenge that is best accomplished by having medical-, nutritional-, exercise- and sports-oriented professionals working together. It is also a key to determining the health and performance efficiency of individuals that practice sports [1].

One of the key nutritional principles for subjects that practice sport/fitness is maintenance of an optimal body mass and composition. Excessive loss of body mass may impair sport performance and may also have significant health consequences [2]. Excess fat impairs performance in sports in which the body must move efficiently. Thus, in order to achieve and maintain an optimal body weight/mass there are important nutritional and exercise considerations for individuals that practice sport [2]. In general, the diet that is optimal for health is also optimal for sport performance [1, 2]. Sport nutritionists indicate that a diet that is rich in fruits, vegetables, whole grains, lean meats, and low-fat diary products, and stresses variety, balance, and moderation, will provide the nutrients needed for individuals that practice fitness [2]. It is important to note that adequate energy intake derived from the macronutrients (carbohydrate, fat, and protein) and obtained from a wide variety of foods will provide adequate intake of the micronutrients (vitamins and minerals) [1, 2].

This chapter is broadly divided into three different parts. In the first part, body composition assessment is presented and discussed. In the second part, dietary assessment together with biochemical and clinical assessment are presented. Finally, the third part contains a general discussion and conclusions where body composition, dietary and clinical assessment are all considered as paramount for refining individualized health-related exercise prescription.

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Evaluation of Nutritional Status using Body Composition

The effects of exercise on body composition are diverse, in part because different assessment techniques of varying accuracy and precision are used to quantify exercise-related changes in body composition [3]. In addition, many exercise interventions are blended with other treatments, especially dietary modification, which further complicates the ability to determine the independent effects of exercise. It must be emphasized that there is a need to better understand the health benefits of exercise throughout the life span. The health benefits of regular physical activity and improved physical fitness are well documented [4], and many of the known health benefits of exercise result, either directly or indirectly, from the beneficial effects of exercise on body composition [5].

Body Weight and Height

The ability of exercise to influence body weight is governed by the first two laws of thermodynamics. According to the first law, energy is neither created nor destroyed; energy is converted from one form to another. In fact, during exercise the chemical energy from food intake is converted, in part, to the mechanical energy of human movement. This implies that the energy expended during and after physical activity is "vital" to balance the energy stored from food intake. The second law of thermodynamics states that biological energy conversions are inefficient. Thus, not all the energy expended during and after exercise contributes to movement. In addition, regular exercise help to preserve and increase Fat Free Mass (FFM), the metabolic active mass of the body [6-8].

Weight and height are the two most often used measurements of growth. Body weight is a measure of body mass; it is more appropriately called body mass and is a composite of independently varying tissues. Height (linear growth) in pediatric subjects provides insight into individual health and nutritional status [7, 9]. Appropriate linear growth reflects adequacy of energy intake for a particular training regimen in child athletes [10].

Body Mass Index

A more useful indicator of body mass in assessment of individuals that practice fitness is Body Mass Index (BMI), expressed as weight in kilograms

(kg) divided by height in meters (m) squared (kg/m^2). Unfortunately, BMI doesn't disentangle fat mass from FFM; in fact, annual BMI increases in children, unlike adults, cannot be automatically attributed to concurrent increases in fat tissue body mass [11]. BMI could be a suitable measure of adiposity in adults. However, cautious interpretation when comparing BMI across age groups or when predicting a specific individual's total body fat or percentage body fat [12].

Other Anthropometric Measurements

Anthropometry is a set of standardized techniques for systematically taking measurements of the body and parts of the body, more appropriately, dimensions of the individual.

Triceps Skinfold Thickness

Triceps thickness is an index of total body fatness [13], and it provides an indirect estimate of body fat percentage based on the size of the subcutaneous fat depot. This measurement may be a better indicator of fat tissue body mass than BMI both in children and in adults [14].

Circumferences

Circumferences and skeletal breadth are anthropometric measurements that are useful in determining body size and body proportions. Waist circumference and other circumferences are also useful important measures of cardiovascular risk [15]. The use of these anthropometric-based measurement methods are formulated on the concept that circumferences reflect fat mass and FFM and that skeletal size is associated with FFM [16]. Waist, hip, and thigh circumferences are used to predict body fat distribution in children, and waist and hip circumferences are both good predictors of intra-abdominal adipose tissue [17]. Waist circumference is associated with cardiovascular risk factors and with metabolic syndrome [18]. Recently, we pooled data from various investigators to evaluate the relationship between anthropometry and magnetic resonance imaging-derived abdominal fat measurements in children and we found that waist circumference can be considered a good predictor of visceral adipose tissue as well as BMI of subcutaneous adipose tissue [19].

Midarm Muscle Circumference

Midarm muscle circumference is an index of lean tissue body mass. It represents the circumference of muscle surrounding a central core of bone [20]. Midarm muscle circumference in athletes and in individuals that practice fitness serves as a marker of current lean tissue body status.

Midarm Muscle Area

Midarm muscle area is an index of lean tissue body mass [21]. It is derived from triceps skinfold thickness and midarm muscle circumference and reflects the true magnitude of any shift in lean tissue body mass [22]. This measurement needs to be done with extreme caution in obese individuals due to the fact that it overestimates the lean tissue body mass [23].

Bioelectrical Impedance Analysis

Using bioimpedance analysis methods (BIA) the electrical impedance of the body is measured by introducing a small alternating electrical current into the body and measuring the potential differences that result. The impedance magnitude (Z) is the ratio of the magnitude of the potential difference to the magnitude of the current. Alternating electrical current flows through the body by several different physical characteristics [24]. Tissues rich in water and electrolytes offer considerably less resistance to passage of an electrical current than do lipid-rich adipose tissue. Conceptually, a human devoid of adipose tissue would have minimum impedance, which would increase to a maximum when all lean tissue was replaced by fat-filled adipose tissue [25, 26]. A limitation of BIA is that it provides an estimate of total body water (TBW). Age- or pubertal-specific equations have been recommended, because age-related differences in electrolyte concentration in the extracellular space relative to the intracellular space may alter the relationship between bioelectrical resistance and TBW [24, 27, 28]. Also, race-specific prediction equations for FFM have been developed [29].

Until recently, body composition measurements using BIA have employed a single frequency of 50 kHz. In accordance with the axioms of impedance plethysmography, the total resistance measurement (R) is combined with stature as length of the conductor (S) to compute stature squared derived by resistance (S^2/R) as an index of the total conductive volume of the body. The ability of this impedance index to describe the volume of FFM is due to greater electrolyte content and measured conductivity of FFM compared

to adipose tissue or bone. Fat mass can then be calculated as the difference between body weight and FFM [24, 26, 27].

An important issue is that subject measurement conditions must be rigorously standardized in order to obtain accurate body composition estimates. Room and subject temperature, position of the patient, correct electrode placement, the use of appropriate equations, and several other factors (e.g., eating or drinking) influence measured impedance and must be standardized to the extent possible during BIA measurements [26].

Suggestions

BIA, skinfolds, and anthropometry are useful tools in subjects that practice fitness to design training programs intended to enhance physical performance and ultimately to control fat mass deposition. Anthropometric indicators of body composition are valuable for monitoring changes during the course of a season or from year to year in athletes and also in subjects that practice fitness [1-4]. Ultimately, the anthropometric indicators provide potentially useful information in monitoring individuals who might be at risk for disordered eating [1, 3, 4].

Evaluation of Nutritional Status using Dietary Assessment

Evaluation of food and nutrient intake in individuals, including those practicing fitness, is difficult.

Dietary assessment methods such as diet histories, food records, diet recalls, and food frequency questionnaires can be used to estimate dietary intake patterns and nutrient intake of subjects, and to determine relationships between diet and exercise [1-4]. However, those methods are not error proof and studies have shown that accuracy of reported dietary intake data is influenced by several factors such as age, gender, body weight, body composition, restrained eating habits, social class, and consumption of certain food groups [1-4, 30, 31]. This reporting bias can lead to misinterpretations of the nutrient adequacy of an individual's diet and nutritional status. Self-reported energy intake typically is underestimated, especially by adolescents and obese individuals, which can result in misinterpretation of individuals' nutritional status [1, 2, 30, 31]. Additionally, length of the dietary evaluation period also needs to be given attention due to day-to-day variations in intake. It is thus best to evaluate macro- and micronutrient contributions of the diets of subjects over several days rather than looking at a single day's intake [1, 2]. Thus, it is not only important to use the appropriate dietary assessment methods, but to use methods to improve the accuracy of the dietary assessment, given its influence on the reported nutrient intake data [1, 2].

Regarding dietary guidelines, it is fundamental to tell the subject a few "Key" points:

- Aim for fitness
  - Aim for a healthy weight and be physically active each day.
- Build a healthy base
  - Let the pyramid guide food choices. Keep food safe to eat, having a variety of fruits and vegetables daily.
- Choose sensibly
  - Choose a diet low in saturated fat and cholesterol and moderate in total fat. Choose beverages and foods to moderate intake of sugars and prepare foods with less salt.

Adequate nutrient intake by individuals that practice fitness is important to meet not only the demands of their increased physical activity and performance, but also the demands of their growth and development (young individuals) and for their short- (injury risk) and long-term (chronic disease risk) health [1, 2].

Eating disorders, in their many presentations, are a risk for individuals that practice fitness. Emphasizing the role of good nutrition and weight control in optimizing performance can reduce the risk of triggering an eating disorder. Education is a primary tool for reducing the risk of eating disorders.

Suggestions

Of course, regular assessments of dietary intake are required to identify potential problem areas, and a combination of methods should be used to assess adequacy of intake. Furthermore, these measures need to be validated against biochemical, anthropometric, and clinical parameters to determine the nutritional status and the adequacy of the dietary intake of persons that practice fitness.

Evaluation of Nutritional Status using Biochemical Assessment

Biochemical assessment of subjects that practice fitness provides information about how well their bodies are utilizing nutrients. Evidence exists that athletes need more protein than nonathletes [1]. It is well known that both men and women, athletes and nonathletes, frequently do not consume recommended amounts of calcium, folic acid, and vitamin E as well as iron [32].

Favorable changes in serum lipids usually occur in individuals as a result of moderate and vigorous activity/exercise. An increase in high-density lipoprotein cholesterol concentration and decrease in total cho-

lesterol, low-density lipoprotein cholesterol and triglyceride levels are generally observed in exercising adults as compared with values when they were more sedentary [1]. Possible lipid oxidation may also result from ultra-endurance activities [32]. Changes in cholesterol ester transfer protein and lecithin-cholesterol acyltransferase appear to be consistent with increased high-density lipoprotein cholesterol levels [1, 29]. Most of the studies were done in men; it's unclear whether exercise intensity affects the lipid changes in women [1, 3, 32].

The use of protein for energy formation by the bodies of persons that practice fitness is relatively small [1, 32]. Controversy exists as to whether aerobic or resistance exercises increase the need for dietary protein, and some evidence exists that aerobic exercise does [3]. It was also demonstrated that strength training results in reduced protein requirements [3].

Assessment of serum lipid is strongly recommended in subjects that practice fitness regularly [1, 32, 33]. Prior to assessment, it is important that subjects abstain from alcohol for a 48-h period and from food for 12 h, and it is suggested that they abstain from strenuous exercise in the 48 h preceding the measurement [1, 3, 32, 33]. Finally, factors that must be taken into consideration when interpreting the results include medication use, menopausal status, age, and smoking status [33].

Evaluation of Nutritional Status using Clinical Assessment

Many individuals of all ages participate in various athletic activities. Some of these individuals either may be at risk for a chronic disease, or may have already been diagnosed as having one. Of course, every person should have a medical and physical examination prior to initiating any exercise program [34]. Clinical assessment involves nutritional and medical histories, physical examination with biochemical tests in order to detect specific nutrient deficiencies, and identifying individuals at risk of future nutritional abnormalities [3, 32-34]. It is important that subjects who practice fitness be questioned about symptoms suggestive of angina, hypertension, diabetes, and renal disease, as well as previous exercise status and drug history before starting any activity.

Physical Activity Needs Assessment of Individuals that Practice Fitness

Dietary intake should provide sufficient energy to sustain life. The energy is measured in kilocalories (kcal) or kilojoules (kJ). The body can use carbohydrates, fat, and proteins to produce energy. The energy production from carbohydrates and proteins is about 4 kcal/g (17 kJ/g) and that from

fat about 9 kcal/g (37 kJ/g). Indirect calorimetry is used, usually, to estimate energy expenditure and is used in measuring the energy expended during a specific physical activity. The estimated total energy expenditure of a person who practices fitness is calculated by summing the resting energy expenditure of 24 h, the expenditure based on lifestyle (daily activity), and the expenditure related to exercise [1, 2].

Conclusions

Many people of all ages participate in various athletic activities. Some of these individuals either may be at risk for a chronic disease, or may have already been diagnosed as having one. Obese and sedentary elderly individuals should have a medical and physical examination prior to initiation of an exercise program. Also, a clinical assessment that involves nutritional and medical histories as well as a physical examination is fundamental to detect any nutrition deficiencies and identify individuals at risk for future nutritional abnormalities. Electrocardiographic examinations are frequently advised for the prescreening of individuals prior their initiating any training programs. Nutritional assessment, which is a key to determining and monitoring the health and physical performance of professional and recreational athletes, is best accomplished by a team of health professionals.

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Chapter 11

Physical Exercise for the Prevention and Treatment of Obesity

Edoardo Mannucci

Exercise in the Treatment of Obesity

Physical exercise is known to be an effective therapy for overweight and obesity [1]; in fact, exercise, when combined with dietary advice, enhances weight loss [2] and prevents weight regain [3, 4]. An increase of physical activity, which induces an increase of exercise-induced and resting energy expenditure, can effectively counterbalance the reduction of energy consumption determined by decreased food intake [1], while preventing the reduction of lean mass associated with weight loss [5]. Furthermore, the increase of activity level determines an improvement of insulin sensitivity, glucose tolerance, lipid profile, and blood pressure, as well as a reduction of several inflammatory markers, leading to a substantial decrease of long-term cardiovascular risk [1, 6]. In patients already affected by obesity-associated conditions, such as type 2 diabetes, hypertension, or dyslipidemia, physical activity is associated with an improvement of blood glucose, blood pressure, HDL cholesterol, and triglyceride [6]. For all these reasons, regular physical exercise is included in all recommendations for treatment of obesity issued by scientific societies and institutions [1].

Amount, Frequency, and Type of Physical Exercise

So far as the type of exercise is concerned, low-intensity and prolonged aerobic training is usually most recommended [1], although available evidence suggests that the amount and regularity of exercise could be more relevant than type of activity. Measures to obtain a modest increase in daily exercise (such as regular walking or cycling), or supervised programs with a more vigorous aerobic training, can both promote weight loss. In fact, mod-

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erate lifestyle activity has been reported to be similarly effective as structured aerobic exercise in obese patients [7]. Most authors agree that regularity is more important than type of exercise in promoting weight loss; standard recommendations require exercise at least three times a week, and possibly daily [1].

In clinical practice, when determining the exercise goals for individual patients, some points should be carefully be taken into account. First of all, obesity is an objective obstacle for many types of physical exercise; any recommendations should be based on actual capabilities of patients, particularly in those with severe obesity. Furthermore, most obese individuals have a long-standing history of sedentary behaviors, and they usually are unaccustomed to exercise. The increase of physical activity should therefore be very gradual; in some subjects, a preliminary intervention on lifestyle activities (regular walking, use of stairs instead of elevators, parking the car at some distance from workplace, etc.), can be followed by recommendations for a more structured and vigorous physical exercise after some time.

Another point to consider is that, obesity being a chronic condition, therapists should aim at a long-term (possibly lifetime) increase of physical exercise. For this reason, the type of exercise suggested should be compatible with the individual patient's lifestyle in the long term. For this reason, availability of facilities for exercise and compatibility of timing of exercise sessions with other activities (work schedules, family needs, etc.) can be crucial issues.

In order to obtain an increase of activity levels in the long term, the type of exercise should be pleasant for the individual patient. Although the theoretical superiority of some kinds of exercise can be discussed, the type of exercise which is preferred by the patient has a much greater chance to be maintained in the long term, leading to therapeutic success.

Ways to Promote Physical Activity in Obese Patients

Although the essential role of physical activity in the treatment of obesity is widely recognized, the simple prescription of exercise by health care providers does not seem to have any relevant effect on long-term behavior of overweight and obese individuals [8, 9]. In fact, the increase of activity levels requires a complex modification of lifestyle, which cannot be achieved through a simple prescription. Two different strategies can be implemented (and have been tested in clinical trials): supervised programs of aerobic training, or behavioral techniques either to increase lifestyle activity or to promote structured unsupervised exercise programs.

The approach through supervised programs has been used in several trials with good short-term results. It can be implemented either in the format

of group programs in dedicated facilities, or as an individual program in a home-based fashion. In either case, it is an expensive approach: general practices and specialist clinics are usually not equipped with facilities for group-supervised physical exercise; on the other hand, any individual, home-based program is remarkably time-consuming for exercise supervisors. In fact, supervised programs seem to be more appropriate for randomized trials than for routine clinical practice. Furthermore, it has been observed that, once that a supervised program has been terminated, its effects on levels of physical activity of participants tend to disappear with time [1].

Behavior therapy for obesity includes a variety of interventions specifically aimed at long-term modification of eating and exercise habits, which is obtained through a direct involvement of the patient in the management of diet and physical activity [10]. Although behavioral treatments can be very different one from another, typical features include:

1. Education and planning: the patient is provided with information about the benefits of exercise, and a training program is planned together by the patient and his/her health provider;
2. Self-monitoring: the patient is invited to record type, intensity, and duration of exercise, in order to monitor compliance to the planned exercise program. If impediments arise, these can be discussed with therapists. Self-monitoring sheets can also be used to monitor some positive effects of exercise, i.e., performance, for positive reinforcement (see below).
3. Positive reinforcement: it is essential that the patient receive some positive feedback from his/her training program, such as pleasure during exercise sessions, improvement of performance (in specific tasks or in activities of daily living), modifications of metabolic parameters, etc.

Behavioral techniques for increase of activity in obese patients can be applied in individual or group sessions, or with a combination of both. Group interventions, when feasible, seem to be more effective than the individual approach [10]. Most authors agree that, in order to obtain satisfactory long-term results, behavioral interventions should be completed by some kind of follow-up contact, even through telephone calls [11], internet or e-mail [12].

In most instances, behavioral interventions on physical activity in obese or overweight patients have been applied in association with supervised training, within more complex programs which included also dietary interventions. The two largest trials of this kind described so far, the Finnish Diabetes Prevention Study [13] and the Diabetes Prevention Program [14], both increased significantly activity levels in participants after a follow-up of 3-4 years [15, 16]. Although the results of these two trials are very relevant, it should be considered that the patients enrolled were aware of the fact that they were at risk for diabetes, and that physical exercise was effective

in the prevention of that disease; the long-term efficacy of this approach in the management of obesity uncomplicated by glucose intolerance needs further investigation. In obese schoolchildren, a program including both structured supervised physical exercise and behavioral techniques to reduce sedentary activities during leisure time, as well as appropriate dietary advice, produced encouraging results at 1 year, although a longer follow-up is needed to confirm the efficacy of this approach [17].

A relatively inexpensive and simple behavioral approach to the increase of physical activity, which was not associated with supervised training, nor with specific dietary intervention, was shown to be effective in enhancing activity levels, and ameliorating a number of metabolic parameters, in overweight type 2 diabetic patients [18]. Although obtained in a population which is in many ways different from that of obese nondiabetic individuals, this result is encouraging for simple, nonprescriptive approaches to exercise in the treatment of obesity.

In summary, although the central role of physical activity in the treatment of obesity is widely recognized, the simple prescription of exercise is usually ineffective. Individual or group behavioral programs designed to enhance activity levels in the long term are a much more promising approach.

A simple behavioral program for the increase of exercise in obese patients, which can be easily applied in clinical practice, should be introduced by proper information on the benefits of physical activity, in order to enhance motivation. An initial program of physical activity (which can be represented by lifestyle activities or by more structured exercise depending on the characteristics of the individual patient) should be agreed upon by the patient and the therapist. The implementation of this program can be assessed by regular self-monitoring (i.e., an exercise diary compiled by the patient). Some parameters of performance should be included in monitoring, to enhance motivation. In follow-up visits, the therapist can discuss with the patient impediments to the implementation of the program and determine new exercise goals, increasing gradually the levels of activity. In this process, the therapist should focus the attention of the patient on detected improvements in metabolic parameters determined by physical activity, and on the pleasant aspects of exercise. Physical activity should be perceived by the patient as a pleasure, and not as a punishment. The implementation of this kind of intervention requires some behavioral skills on the side of the health providers. Although a psychologist or a psychotherapist is not usually needed within the health care team, all professionals (physicians, dieticians, physical therapists and/or motor sciences specialists) should receive some behavioral training.

The use of structured supervised exercise sessions in combination with behavioral programs is likely to produce a relevant improvement in weight loss, at least in the short term. The main obstacle to this intervention is represented by the need for specific facilities within (or nearby) obesity clin-

ics, which require remarkable financial resources. Considering the heavy toll of obesity on the health status of the population of developed countries, and the resulting impact on public expenditure for the care of overweight-associated diseases, expenses for training facilities and specialized personnel for the implementation of supervised exercise programs for obesity should represent a good investment in the long term.

Another key point for the success of obesity programs is the quality of follow-up. Although behavioral interventions have been shown to be more effective than traditional dietary and exercise prescription in the medium term [10], their effects seem to disappear in the longer term. Regular follow-up session, mainly devoted to the increase of motivation, can be crucial for long-term success [11]. Such sessions can be implemented either in the format of individual visits, telephone interviews, or internet and/or e-mail contacts [11, 12]. The use of new communication technologies, whenever possible, could be very advantageous for the containment of costs.

Exercise in the Prevention of Obesity

Overweight and obesity are associated with a sedentary lifestyle in the general population. Prospective studies show that a lower level of physical activity predicts weight gain in the following years [19]. The increase of physical activity is thus a crucial target for prevention of obesity [1].

Promotion of Physical Activity in Adults

A number of community programs aimed at the increase of exercise levels in the general population have been developed over the years, although an appropriate assessment of efficacy was carried out only in a minority of cases [20]. Educational campaigns (through mass media and/or dedicated educators) targeted at adults usually fail to produce relevant results on long-term physical activity [1]. This is not surprising, considering that modifications in lifestyle, which are necessary to increase activity levels, need a sufficient motivation, which cannot be induced only by education on potential benefits of regular exercise.

Some greater improvement can be obtained through modifications of the environment, e.g., with exercise sessions and facilities for physical activity in the workplace [21]. Modifications of working schedules, in agreement with employers, could facilitate regular physical exercise in many sedentary young adults. It should be considered that behavioral programs produce a much greater increase of activity levels in older, retired persons, than in young working adults [16]. The development of adequate facilities for physical activity in different communities could also play a crucial role in the prevention of obesity. The investment

of resources by many governments in mass media-based campaigns for the promotion of physical exercise, while persons targeted by those campaigns are not given the actual possibility to exercise regularly, is somewhat paradoxical.

Some groups at higher risk for complications of obesity, who have a greater awareness of the potential benefits of weight control, can be more responsive to educational interventions; for example, first-degree relatives of type 2 diabetic patients have been shown to increase their activity levels 1 year after a brief educational intervention, with two group sessions and a follow-up by telephone interviews [22]. In fact, the awareness of the risk of medical complications of overweight such as diabetes, which has already been experienced by a family member, represent a powerful enhancer of motivation to control weight. For this reason, simple educational programs, which are unlikely to produce any results in the general population, can be effective in relatives of diabetic patients. It can be speculated that similar effects could be obtained in first-degree relatives of patients affected by other obesity-related conditions, such as cardiovascular disease.

In specific groups at risk for weight gain, more complex and expensive programs which include a behavioral intervention can effectively improve weight control [23]. Such programs, which involve the use of considerable financial and human resources, cannot be extended to the whole population, but they could represent a sound approach to some other groups at high risk for weight gain, e.g., patients treated with antipsychotic or antidepressant drugs, HIV-infected patients on antiretroviral therapy, etc.

Overall, available evidence discourages educational interventions on the general adult population to prevent obesity through the increase of physical activity. In fact, although several governments have launched mass media campaigns, it is unlikely that these will produce any relevant results. It would be more rational to invest available resources in community and workplace facilities for physical exercise, while concentrating educational interventions on specific groups with a greater awareness of benefits of weight control (e.g., first-degree relatives of patients with diabetes or cardiovascular disease). Some groups of subjects with a high risk for weight gain (e.g., menopausal women, or patients treated with antipsychotic drugs) could be the target of more complex, and expensive, programs for weight control.

Promotion of Physical Activity in Children

Community interventions on schoolchildren seem to be more promising than those targeted at adults. Although educational classes on the benefits of physical activity do not appear to produce any relevant result [24], supervised exercise programs have been shown to be effective in ameliorating weight control in several different settings [25-27].

In the last few decades, there has been a shift towards a more sedentary behavior during infancy and adolescence. In fact, in most developed countries, the majority of children spend a large part of their leisure time involved in sedentary activities, such as viewing TV, surfing the internet, or playing video games. The modification of these behaviors could have a notable impact on weight control. It is not surprising that some school-based behavioral programs aimed at the reduction of TV viewing, and encouraging a more active use of leisure time, have proven to be among the most effective interventions for the prevention of obesity in children [28-30].

In summary, the increase of supervised exercise classes and school-based behavioral programs aimed at a more active lifestyle are both effective means to prevent obesity in children, with a great potential benefit for public health. An appropriate intervention plan should include:

1. A relevant increase of the number of sessions dedicated to supervised physical exercise during routine school curriculum. These sessions, which allow children to familiarize themselves with one or more sports, induce a greater number of persons to practice regularly even after the end of their school years.
2. Behavioral programs aimed at contrasting sedentary behaviors (TV viewing, internet, etc.) and promoting diverse activities (dancing, playing football, etc.) during leisure time. Such programs can be delivered either by devoted personnel or by specifically trained teachers.

Further research is needed in order to assess the feasibility and efficacy of other factors possibly enhancing the effects of such a campaign (e.g., involvement of parents with specifically designed educational sessions). In any case, the design and implementation of programs focused on physical exercise to prevent obesity in children requires the participation of different professionals, including physicians, psychologists, educators, teachers, and motor sciences experts.

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SECTION VI

Physical Exercise - Health and Wellness: Sociological and Psychological Aspects

Chapter 12

Techniques for Assessing the Quality of Life with a Particular Emphasis on Physical Exercise

Giovanni Apolone and Paola Mosconi

Introduction

Measuring population health is important to evaluate the impact of interventions, to monitor the change in health status, and to predict the need for health care. Interest in measuring qualitative aspects of life that are most closely related to health, health care, and health policy has increased in recent years, and several questionnaires evaluating patients' subjective health status are now available. Examples in the literature of the use of these tools, now grouped into the umbrella term of Patients-Reported-Outcome (PRO), suggest that they may have an important role either in clinical studies or in the evaluation of samples from the general population [1, 2].

Quality of life is a broad and multidimensional concept related to personal satisfaction or happiness with life. Even if it is a concept apparently easy to be understood, actually the term quality of life is used with different meanings. There are several causes for this confusing situation. The most important are the high level of abstraction and complexity of "the qualitative" attribute of life and the interest of various disciplines in the efforts to define and to measure the quality of the life of citizens, patients, and customers in several contexts. Among the many definitions available, that of Campbell in 1976 [3] has the advantage being able to put in evidence the fact that quality of the life is a somewhat subjective concept, related to the well-being of an individual, including many factors (social, spiritual, economic, etc.) where only one of them is health, a "value" that is instead the principal object of interest of medicine and health care.

Health-related quality of life reflects an attempt to restrict the complex concept of quality of life to those aspects of life specifically related to a person's health that potentially respond to health care. The core of this definition is considered the World Health Organization definition of health that defines health *"…as a state of complete physical, mental and social well-being, and not merely the absence of disease…"* [4]. According to this definition, physical, emotional, social *functioning* and *well-being*, as well as indi-

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vidual evaluations of general health perceptions, are considered the principal components of the very concept of HRQOL, thus allowing one to "qualitatively" evaluate health, health status, and health outcomes [5-7].

Tools to Evaluate (Health-Related) Quality of Life

Over the last several years health care providers, the patient advocacy community, as well as the pharmaceutical industry have demonstrated increasing interest in health-related quality of life as an outcome measure either for clinical research or health care evaluation. Several approaches, where patients are the only source of information, reports, and ratings, have been implemented, most through standardized psychometric questionnaires [8], have the objective to document the yield of the physician-health care impact on perceived health, in the context of a more comprehensive evaluation that implies the use of other measures, including clinical and economic outcomes [9].

In accordance with the current taxonomy [10], the questionnaires now available as simple mono-item questions, health profiles, summary indexes, and utility measures may be classified as *generic,* not specific to any age, disease, or treatments; or *specific,* instruments that are conceptualized and developed to focus on symptoms and specific aspects of a given disease or treatment. Pro and cons of generic and specific questionnaire are summarized in Table 1.

Table 1 Classification of measures of health-related quality of life

Struments	PROS	CONS
Generic instruments	Single instrument	May not focus adequately on area of interest
	Detects differential effects on different aspects of health status	May not be responsive
	Comparison across interventions	
	Condition possible	
Disease, population, function or condition-specific instruments	Clinically sensible comparisons	Doesn't allow cross-condition
	May be more responsive	May be limited in terms of populations and interventions Restricted to domains of relevance to disease, population, function or problem; other domains that are important to overall HRQL not measured

Adapted from [9]

Between the plethora of the questionnaires available, some (like Health Survey SF-36 or SF-12, Psychological General Well Being Index) are known for their outstanding availability of data on validity, reliability, and added value. Examples of their utilization are present in various settings, including effectiveness, quality of care, health economics, and epidemiological studies. It is worth mentioning that the application of these approaches and instruments in retrospective and/or in noncontrolled studies is more critical because, in the absence of well-designed protocols and standardization of the clinical settings, the scores derived by the questionnaires are more susceptible to the effect of chance, bias, and confounders, making interpretation of results more difficult. In the same way, their use at the level of single clinical decisions in the context of normal clinical practice, remains an open and unsolved topic.

The principal characteristics of the questionnaire, together with definitions of principal attributes, are reported in Table 2.

Table 2 Main characteristics of quality of life instruments. The validity of an instrument is defined as the degree to which an instrument measures what it is intended to measure.

Face validity	A judgment-expressed from a group of researchers, experts and patients on the basis of their own experience-on the importance of the questions, domains, and dimensions evaluated by the questionnaire
Content validity	A judgment expressed from experts on the dimensions, representativeness, and completeness of the content of the questions (items) relative to each dimension/domain. For every question or dimension, the experts can provide a score through which it is possible to establish the degree of agreement or disagreement between the parts.
Construct validity	The most important aspect of the validation process in order to document if the theoretical assumptions on which the questionnaire has been constructed are satisfied, i.e., the correlation between the single question and the dimension to which, theoretically, every question belongs. The typical analyses to use are the factorial analysis and the Multi Trait analysis.
Criterion-related validity	Used to demonstrate the accuracy of a measure or procedure by comparing it with another measure or procedure which has been demonstrated to be valid.
Reliability	This characteristic is related to the precision of the instrument that must supply equivalent measurements in case of context stability. The most common method in order to estimate the reliability is the test-retest, i.e., the repeated administration, two or more times, to the same subject of the same questionnaire.

The Short Form 36 Items Health Survey (SF-36)

Among the generic questionnaires, one of the most widely used in industrialized countries, including Italy, is the SF-36 [11].

The Health Survey SF-36 was originally developed in the USA for use in large samples to monitor the yield of medical interventions on subjective aspects of health and quality of life. The SF-36 is a generic tool that measures two major health concepts: physical and mental health, with 36 items generating eight multi-item scales: Physical Functioning (PF), Physical Role limitation (RP), Bodily Pain (BP), General Health (GH), Vitality (VT), Social Functioning (SF), Emotional Role limitation (RE), and Mental Health (MH). A description of the contents of each scale and number of items for scale is reported in Table 3.

Table 3 SF-36 health status scales

Concepts	Summary of contents	No. of items
Physical		
Physical Functioning (PF)	Extent to which health limits physical activities such as self-care, walking, climbing stairs, bending, lifting, and moderate and vigorous exercise	10
Role Functioning-Physical (RP)	Extent to which physical health interferes with work of other daily activities, including accomplishing less than wanted, limitations in the kind of activities, or difficulty in performing activities	4
Bodily Pain (BP)	Intensity or pain and effect of pain on normal work, both inside and outside the home	2
General Health (GH)	Personal evaluation of health, including current health, health outlook, and resistance to illness	5
Mental		
Vitality (VT)	Feeling energetic and full of pep versus feeling tired and worn out	4
Social Functioning (SF)	Extent to which physical health or emotional problems interfere with normal social activities	2
Role Functioning-Emotional (RE)	Extent to which emotional problems interfere with work or other daily activities, including decreased time spent on activities accomplishing less, and not working as carefully as usual	3
Mental Health (MH)	General mental health, including depression, anxiety, behavioral-emotional control, general positive affect	5
Reported Health Perception (HP)	Evaluation of current health compared to one year ago	1

Adapted from [11, 16]

Because of its validity, strength in measuring health-related quality-of-life concepts, and brevity SF-36 has been the object of the IQOLA Project, established in 1991 to translate, adapt, and validate the SF-36 Health Survey [12] to other linguistic and cultural settings. The IQOLA group – including researchers from Australia, Canada, Europe, Japan, and the USA – developed protocols for translating, validating, and norming the SF-36 questionnaire in several languages [13]. Currently the questionnaire is available in more than 50 languages [14]. The SF-36 has been validated in Italy, and normative data from a large random sample of Italians are also available for historical comparison [15, 16].

For each patient, scores are assembled using the Likert method for summated ratings, and then the raw scores are linearly transformed to 0-100 scales, with 0 and 100 assigned to lowest and highest possible value, respectively. Higher transformed scores indicate better health [11]. The questionnaire has been developed to be self-administered, but interviewer-administered (by a trained interviewer in person or by telephone) or computerized administration is also feasible.

In 2000 an up-to-date version of SF-36 (SF36-II Version 2) was released in the USA and US normative data has also been published in the instrument manual [17]. The new version included wording changes of some items, the psychometric features of the role functioning scales, the reduction of the levels (from 6 to 5) of response options for a few scales, and an improvement of the survey's layout. These changes have led to a greater precision of measurement for Version 2 as compared with Version 1. In addition, they make the questionnaire more appropriate for use, particularly with such groups as the elderly. In Italy, the version 2 of SF-36 and new normative data is ongoing and results will be available by the end of 2006.

Quality of Life and Physical Activities

There is increasing evidence about the key role of exercise in increasing physical functioning, with beneficial effects on general health condition, the quality of life and life expectancy, as well as preventing the occurrence of new diseases or disease progression.

In cancer, for example, patients' positive attitude towards physical exercises/activities depends on some evidence that physical activities during and after the period of treatment improve some relevant outcomes, such as cardio-respiratory fitness, fatigue, body size and, in general, quality-of-life perception [18]. Exercise prescriptions for patients with congestive heart failure can have a significant impact on management of symptoms as well as exacerbation of further disease [19]. A meta-analysis con-

ducted on 16 studies reporting exercise and/or self-management interventions for patients with knee osteoarthritis showed that, in general, both patient education and exercise regimens had a modest, yet clinically important, influence on patients' well being [20]. A Cochrane review including thirteen studies, pointed out the evidence for the efficacy and effectiveness of physical training in patients with asthma. Authors, indeed, summarized their findings "…In people with asthma, physical training can improve cardiopulmonary fitness without changing lung function. It is comforting to know that physical training does not have an adverse effect on lung function and wheeze in patients with asthma. Therefore, there is no reason why patients with asthma should not participate in regular physical activity" [21].

Measuring Physical Health: An Example

To know the real impact of physical activity on health and quality of life implies the need to use validated tools that are able to measure the concepts under evaluation. It's unfortunate that not all the studies evaluating the impact of physical activities on well being and functioning have data derived by "formal" quality of life assessment. In addition, not all the available instruments are appropriate to measure dimensions of health and life relevant to the expected impact of physical exercise.

The SF-36, for example, has a latent structure that implies the presence of two independent concepts of self-perceived health (mental and physical) The way chosen to summarize its results (a general health profile with eight distinct scales and two summary indexes for the two major latent concepts), allows a straightforward evaluation of the impact of several factors (either at the individual or environmental level) on health perception that also facilitates the interpretation of results.

Figure 1, for example, shows the impact of ageing (classified in eight categories) on the physical summary score (data are from the Italian normative sample, 2,031 citizens randomly selected to be representative for age, sex, and geographical distribution of the Italian population). It can be noted that the scale is able to detect the effect of ageing on physical health, with higher scores in the younger groups (14-54 years old) where values always exceed the mean value of the entire Italian normative population, while the older groups had lower scores, reaching a difference of 10 points when compared to the mean sample value.

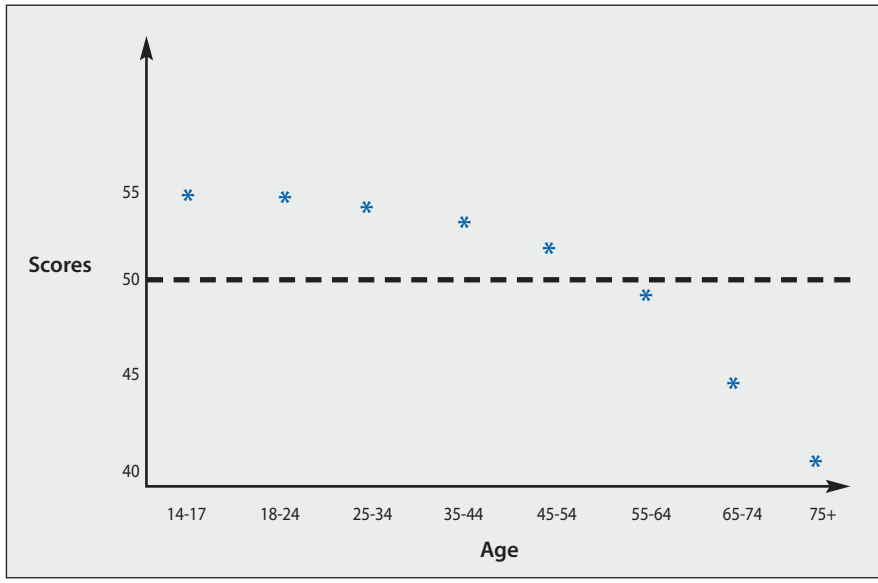

Fig. 1 Physical health and age, from normative Italian data

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Chapter 13

Effects of Physical Exercise on the Quality of Life of Individuals with Diabetes and Obesity

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Introduction

Health-related quality of life (HRQL) corresponds to a multidimensional concept, summarized as the satisfaction of the individuals with their life, specifically related to the individual's perception of his/her health status (somatic as well as mental) and the limitations to functioning related to health, independent of socioeconomic conditions. All aspects are considered as reported by patients. In metabolic diseases, as any chronic condition, HRQL has become a relevant target of interventions, and there is evidence that in diabetes and obesity the participation in programs of physical activity is significantly associated with better health status and HRQL, not limited to physical domains, but extending to mental health. All actors of the therapeutic process need to reconsider the importance of physical activity. It is a very demanding challenge for the coming years.

Quality of Life: a Significant Outcome in Chronic Diseases

The rising interest in quality of life in medicine represents an attempt to move from a disease- and physician-centered approach to a patient-centered assessment of the burden of diseases. Quality of life cannot be easily defined: it includes psychological as well as physical functions and the interrelations between individuals and society [1]. The overall concept can be summarized as the individuals' satisfaction with their life, in the broadest possible sense. HRQL represents a part of quality of life specifically related to the individual's perception of his/her health status (somatic as well as mental) and the limitations to functioning related to health,

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independent of socioeconomic conditions. Any experience of illness, not limited to pain, fatigue, and disability, but extending to social and emotional well-being, is part of HRQL. All aspects are considered as reported by patients, and form the basis of new outcome measures [2].

The needs of individual patients and the benefits of specific treatments will be more and more assessed on the basis of how much the changes in patients' well-being will correspond to their perspectives. Accordingly, regulatory agencies recommend the use of HRQL as specific outcome for several chronic conditions, where life is not immediately at risk, but complete recovery cannot be achieved and the patients have to learn how to cope with the burden of disease [3].

Given the multidimensional concept of HRQL, several questionnaires have been developed to measure, simultaneously, the various aspects of perceived health status. The Medical Outcome Survey Short-Form 36 (SF-36) [4] and the Psychological General Well-Being Inventory (PGWBI) [5] are among the most widely used tests, for their easy and rapid use and the existence of normative values of the general population (Table 1). The SF-36 was progressively simplified to produce comprehensive indices of the two fundamental aspects (the physical and mental well-being), widely used in clinical research.

Table 1 Domains of the Medical Outcome Survey Short-Form 36 and Psychological General Well-Being Inventory

Medical Outcome Survey Short-Form 36 [4]	**Psychological General Well-Being [5]**
Physical domains	**Affective states**
Physical functioning	Anxiety
Role-limitation, physical	Depressed mood
Bodily pain	Positive well-being
General health	Self-control
	General health
Mental domains	Vitality
Role-limitation, emotional	
Vitality	
Social functioning	
Mental health	

The physical domains and the mental domains of SF-36 may be combined to produce a Physical Component Summary and a Mental Component Summary, respectively.
The affective states of PGWBI may be combined unto a Global Severity Index

Physical Activity and HRQL

The beneficial effects of a healthy lifestyle and physical activity on perceived health status are well recognized, and medical societies support physical exercise as an important aspect of prevention and therapeutic measures [6]. A few review articles have pointed out that exercise benefits are not limited to somatic diseases, but also extend to mental health. A lot of studies support the use of physical activity as a means of improving HRQL through enhanced self-esteem, improved mood states, body image and stress responsiveness, reduced state and trait anxiety and depression [7-9]. Also the stage of change for regular exercise is associated with self-perceived quality of life [10]. Subjects who are least motivated to adopt regular exercise report the lowest levels of HRQL, suggesting that cognitive-motivational messages designed to emphasize the benefits associated with exercise may be helpful to move people along the stages of change.

Particularly in young people, physical activity favors social life and is associated with improved mental health, adding to the positive effects on physical health and reduced cardiovascular risk factors. Data from a cohort of nearly 5,000 adolescents in the UK found that the participation in sport and vigorous recreational activity was positively associated with emotional well-being, independently of sex, socio-economic class, and health status [11]. Although causal associations cannot be derived in cross-sectional analyses, these results are consistent with the experimental evidence suggesting that exercise has favorable effects on the emotional state. Using data from the 175,850 adults of the 2001 Behavioral Risk Factor Surveillance System survey, Brown et al. [12] reported that the relative odds of 14 or more unhealthy days (physical or mental) in those with the recommended level of physical activity compared to physically inactive adults were reduced by 33% or more, and the results persisted even among adults with chronic conditions. Persons exercising at levels of physical activity recommended by international agencies were more likely to report fewer unhealthy days compared with inactive and insufficiently active persons [13]. Finally, physical activity, as part of a healthy lifestyle, is a pivotal component of the prevention of obesity and type 2 diabetes [14].

The effects of physical activity on HRQL, measured by standard questionnaires, are definitely proven. In the cross-sectional analysis of the Dutch MORGEN project, an association between moderately intense leisure time physical activity and general health perception was demonstrated, as well as an association between changes in leisure time physical activity and changes in social functioning in men and women, irrespective of the

intensity of physical activity. The physical components of health-related quality of life were associated with physical activity in the cross-sectional analysis, whereas the mental components were more closely associated in the longitudinal analysis [15].

The effects of physical activity on mood states are particularly relevant, considering that depression may be observed in subjects with obesity [16] and diabetes [17, 18]. Leisure time physical activity, mental health, and depression are significantly related; physically active women experience better mental health and less depression in two large surveys carried out by means of the Beck Depression Inventory and State-Trait Anger Scale [19, 20], and even a low level of physical activity (1-2 times per week) had positive effects on women's mental health [19].

Physical Activity and HRQL in Obesity

The negative effects of obesity on HRQL are clearly demonstrated. Both physical and mental components of HRQL are remarkably impaired when compared with population norms, particularly in subjects seeking treatment [21] and in those with psychological or psychiatric distress [22, 23]. Also in subjects where obesity is superimposed on other chronic illnesses, a further deleterious impact is observed [24], limited to physical components. Behavior therapy produces a systematic improvement in all scales of HRQL, largely outweighing the effects on body weight and resulting in a significant change in self-perceived health status [25]. Several uncontrolled studies have consistently demonstrated that physical exercise can positively influence the quality of life in obese adolescents, at risk of psychological distress for the stigma of obesity. Walker et al. investigated the change in body image, self-esteem, and worries in 57 obese adolescents attending a residential, weight-loss camp [26]. Obese adolescents had low self-worth and great body dissatisfaction at the start of the camp, and the intervention improved athletic competence and physical appearance, as well as psychological state, in strict correlation with weight loss.

Comprehensive treatments, including diet and exercise, are also effective. In a 15-month randomized study [27], the intervention SHAPEDOWN program of adolescent obesity, employing a variety of cognitive, behavioral, and affective techniques adapted to make successive small modifications in diet, exercise, communication, proved effective in improving weight, weight- related behavior, and depression. Self-esteem also increased significantly.

Similar data are available in adults. A higher level of physical activity in an obese female clinical population was positively associated with several dimensions of HRQL, although no cause-effect relationship can be derived from cross-sectional analysis [28].

In the Italian QUOVADIS study on nearly 2,000 obese subjects seeking treatment at medical centers, regular physical activity was associated with a lower prevalence of diabetes, hypertension, and metabolic syndrome [29, 30]. The study was specifically aimed at measuring HRQL in obesity, and the results of the SF-36 and PGWBI questionnaires have recently been analyzed (G Marchesini, personal communication). Both questionnaires confirmed that HRQL was significantly impaired when compared to the normative values of the Italian population by the use of Z- score [31, 32], with effect sizes larger for the physical domains of SF-36 (Figs. 1, 2). In subjects exercising regularly, at minimum levels of 1h per week, HRQL was systematically better than in sedentary persons, and significantly so in the four physical and two mental domains of SF-36, as well as in two domains of General Health and Vitality of PGWBI.

Physical activity is an essential component of the behavioral treatment of obesity, and is pivotal in weight loss maintenance [33]. A specific program to implement physical activity, set at a light-to-moderate daily physical activity (brisk walking), markedly increases the probability of losing weight, contributing to the long-term control of obesity [34]. In particular, the probability of losing from 5% to 10% of initial body weight increased by 20% for any 1,000 steps/day (OR, 1.20; 95% CI (confidence interval), 1.07-1.35), and that of losing more than 10% by over 30% (OR, 1.33; 95% CI, 1.19-1.49).

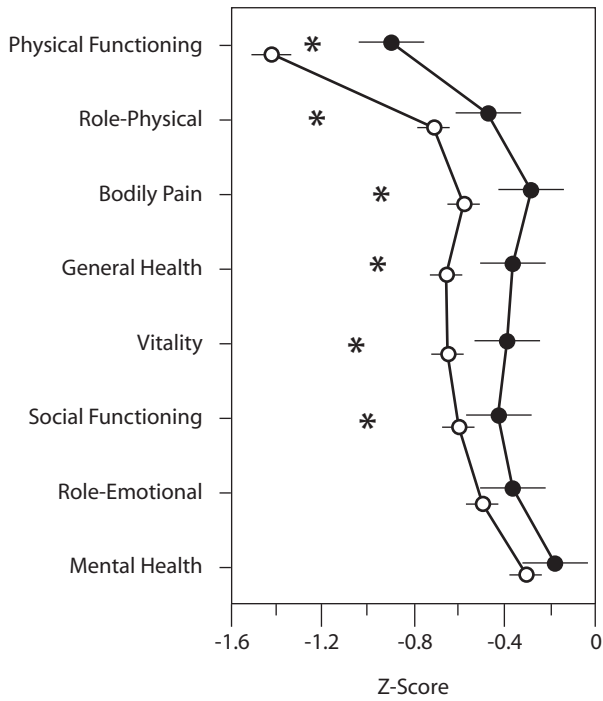

Fig. 1 Values of individual domains of the SF-36 questionnaire in the obese population of the QUOVADIS study [36], in relation to regular exercise. The results are presented as mean and 95% confidence interval of Z-score, calculated on the basis of the Italian normative sample [32], corrected for age and gender. All values are significantly different from population norm. *Closed circles* represent subjects involved in regular exercise (n = 274), *open circles* are sedentary people (n = 1,601). *Asterisks* indicate a significant difference between active and sedentary obese subjects

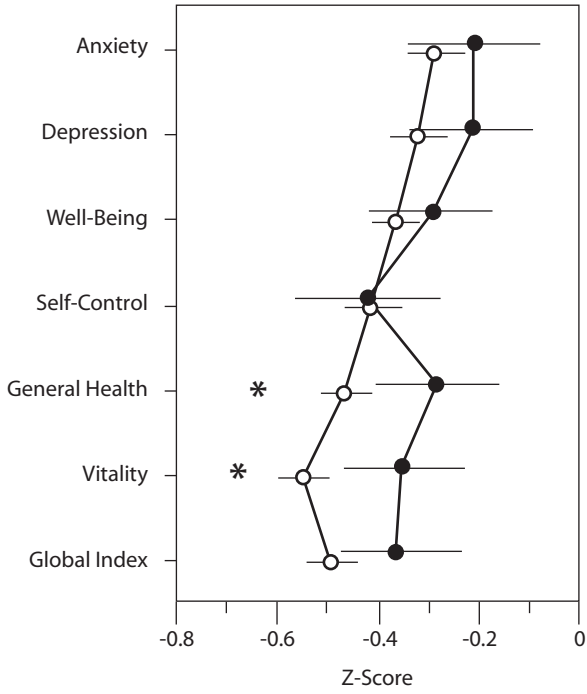

Fig. 2 Values of individual domains and global index of the PGWB inventory in the obese population of the QUOVADIS study [36], in relation to regular exercise. The results are presented as mean and 95% confidence interval of Z-score, calculated on the basis of the Italian normative sample [31], corrected for age and gender. All values are significantly different from population norm. *Closed circles* represent subjects involved in regular exercise (n = 274), *open circles* are sedentary people (n = 1,601). *Asterisks* indicate a significant difference between active and sedentary obese subjects

The benefits on weight loss maintenance are accompanied by a remarkable reduction of laboratory and clinical features of the metabolic syndrome.

Drop-out from weight loss programs is almost invariably associated with weight regain, and weight cycling is associated with reduced self-esteem and self-efficacy [35], leading to poor HRQL [36]. Specific studies addressing the relationship between physical activity, long-term weight loss maintenance, and HRQL are eagerly needed in obesity.

Physical Activity and HRQL in Diabetes

The levels of physical activity of patients with various chronic diseases, including diabetes, hypertension, congestive heart failure, recent myocardial infarction, depressive symptoms, or current depressive disorder, are associated with subsequent functioning and well-being [37]. Greater levels of exercise were also associated with better functioning for patients with chronic conditions over a 2-year period. Physical activity has become an essential component of prevention and treatment of type 2 diabetes, and very recent recommendations of the American Diabetes Association classified as level of evidence "A" the benefits achieved by 30 min physical activity per day in subjects with impaired glucose tolerance or diabetes [38].

In both type 1 and type 2 diabetes mellitus, HRQL is remarkably impaired when tested with generic and disease-specific questionnaires [39], with type I diabetes having the greater negative effect [40]. In a large US sample of adults with diabetes, the respondents reported a moderate-to-low quality of life, and the factors associated with lower HRQL included lower levels of physical activity [41]. Multiple regression analyses revealed that the intensity of self-reported exercise was the only significant self-management behavior associated with HRQL, after controlling for demographic and medical variables. Also in subjects with diabetes complications, physical inactivity remains an independent predictor of poor HRQL [42].

Notably, diabetes patients undergoing intensive diabetes treatment do not face deterioration of their HRQL and psychopathology, assessed by generic and disease-related HRQL questionnaire. A questionnaire of general psychiatric distress (Symptom Checklist-90R [43]) in the Diabetes Control and Complications Trial [44] and intensive treatment for type I diabetes, coupled with education, are reported to improve HRQL [45]. The quality of life and the psychological well-being in patients with type 1 diabetes participating in an empowerment program improves significantly when compared with the scores measured in patients who refuse participation [45]. In particular, the Vitality and Social Functioning scales of SF-36 are no longer different from population norm after intensive education. Similarly, the Symptoms, Discomfort and Impact scales of the Well-Being Enquiry for Diabetics [46], reflecting physical functioning, diabetes-related worries and familiar relationships, role functioning and social network, improve significantly in treated patients. In this experience, the education program remarkably addressed the problems related to physical activity, favoring exercise without the risk of hypoglycemia.

When diet is coupled with exercise in a behavioral approach of a non-diabetic population at risk of type 2 diabetes, positive changes in lifestyle, blood lipids, and fasting insulin can be achieved and maintained after 2 years, and the results are better than diet alone [47].

Conclusions

There is a lot of evidence supporting physical activity as an essential component of the prevention and treatment of obesity and diabetes. In obesity, physical activity is pivotal to prevent weight regain, associated with poor HRQL. In type 2 diabetes, the participation in programs of physical activity is mandatory to achieve a better metabolic control [48], and may be extremely cost effective [49]. Schultze and Hu recently wrote that "a healthy diet, together with regular physical activity, maintenance of a healthy weight, moderate alcohol consumption, and avoidance of sedentary behaviors and

smoking, could nearly eliminate type 2 diabetes" [50]. However, subjects at risk are frequently in the precontemplation or in the contemplation stage of change [51], and this is particularly true for the propensity to consider physical activity in the therapeutic program [52]. In type 1 diabetes, regular physical exercise is part of any empowerment program to help patients experience a normal way of living [45]. The benefits of exercise far outweigh the metabolic effects and extend to psychological aspects of diseases, where improved HRQL adds significantly to the physical fitness. All actors of the therapeutic process need to reconsider the importance of physical activity. Patients have to change their disbelief of exercise as a low-grade intervention, compared with drug treatment. Physicians need to reconsider their standard of care in a more patient-centered approach, and promote physical activity in order to prevent disease progression as well as to improve HRQL in metabolic diseases. Health care providers need to make any effort to facilitate education. It is a very demanding challenge for the coming years.

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Chapter 14

A Longitudinal Investigation of Physical Activity and Health Behaviors in Italian University Students

Arnaldo Zelli, Simona K. Reichmann, Fabio Lucidi and Caterina Grano

Introduction

Sedentary lifestyle is one of the ten leading causes of death and disability in the world [1]. Physical inactivity increases all causes mortality and the risk of cardiovascular disease, hypertension, type II diabetes, obesity, osteoporosis, colon and breast cancer, depression, and anxiety [2]. Yet, around the world, physical activity levels are decreasing, particularly among young people. It is estimated that less than 35% of young people are sufficiently active to benefit their present and future health and well-being [1]. This is discouraging for a number of reasons. First, research suggests that patterns of physical activity adopted at a young age are likely to persist into adulthood [3, 4]. Second, involvement in physical activity and sports may encourage the adoption of other health behaviors such as a healthy diet, better safety practices such as seatbelt use, and the avoidance of health risk behaviors such as tobacco and alcohol use [5, 6]. Thus, physical activity may influence health outcomes both directly and indirectly through the encouragement of other behaviors that promote health and reduce the risk of accident and injury.

These general considerations have important implications for a psychological analysis of the processes regulating individuals' adoption of health behaviors, including physical activity. Such an analysis should contemplate a longitudinal assessment of individual differences in these behaviors in order to estimate how behavioral changes across dimensions are linked over time. Furthermore, a longitudinal analysis may move us beyond mere description by considering psychological constructs that may provide insights about the processes intervening in the relations linking physical activity and health behaviors. Relatedly, this analysis can evaluate the generalizability of the relations linking physical activity and health behaviors by focusing on psychological characteristics that may qualify these relations.

As to the first objective, it is empirically important to determine the rel-

V. Stocchi (ed), *Role of Physical Exercise in Preventing Disease and Improving the Quality of Life*

ative stability of physical activity and health behaviors, that is, to measure the extent to which the adoption of any health behavior varies across individuals and the extent to which this variation remains relatively unchanged over time. Since behavioral stability is plausibly the expression of personality processes at work [7, 8], its assessment is an important prerequisite for initiating a psychological analysis of health behaviors. Furthermore, by estimating and controlling statistically for the stability in physical activity and health behaviors, the assessment of how these behaviors are related over time becomes more precise as one can estimate how one behavior affects *changes* in another behavior over time, thus approaching a cause-effect analysis.

As to the second objective, it is quite well established that physical activity in particular, and health behaviors in general, are regulated by psychological mechanisms that primarily call upon the motivational and personal control capacities of the individual [9]. Under this rubric, many psychological constructs have been proposed and examined to understand how people adopt or persist in enacting certain behaviors. Constructs such as people's health beliefs, motivational readiness, attitudes toward physical activity and other health behaviors, social pressure from significant others, and personal confidence in adopting specific courses of health-promoting actions are only some examples of the psychological constructs of current interest in the study of physical activity and health behaviors [10-12]. Despite this research tradition, physical activity and other health behaviors have typically been examined separately. Rigorous, integrated psychological analyses are needed to determine how the different health behaviors are related, and whether some health behaviors directly facilitate improvements in others [13].

Finally, an analysis of the psychological processes linked to physical activity and health behaviors can not only identify key constructs (e.g., motivational factors) these behaviors have in common, but can also assess how these processes intervene in regulating or moderating the relations between physical activity and other health behaviors. Thus, for instance, it may be critical not only to ascertain the ways in which individual differences in physical activity are related to differences in individuals' personal values about health, but also to understand whether physical activity and other health behaviors are more likely to co-occur in individuals endorsing these values highly, as compared to individuals who do not.

The present study is part of a program of research designed with these general research objectives in mind. To our knowledge, research of this kind is in its infancy in Italy, and the data reported in this chapter are the first to comply with these research objectives. In particular, we examined group and gender differences in physical activity levels and health behaviors as well as the relative stability of these behaviors over time, and initiated a

longitudinal analysis of the possible relations linking physical activity and health behaviors by focusing on a set of self-evaluations and personal values concerning physical appearance, fitness, and health. The study employed two groups of Italian university students, namely, students enrolled in academic programs related to sport sciences (e.g., physical education, exercise physiology) and students enrolled in programs unrelated to sports (e.g., psychology, law, medicine).

Method

Participants and Procedures

We collected longitudinal data across two assessment waves, approximately 9 months apart, from students enrolled in the University of Sport Sciences in Rome, Italy, and students enrolled in non-sport programs offered at the University of Rome "La Sapienza." Sport science students were presumed to be active due to the nature of their academic programs, whereas the students from the University of Rome were considered to be representative of the general population of university students in terms of physical activity levels. All students filled out a series of questionnaires during group sessions of about 25-30 students.

The first wave of the study was conducted in the spring of 2003, and 596 students participated. Of these, 286 (45% females) were sport science students and 310 (63% females) were students enrolled in other programs. Wave two was conducted in the winter of 2004, and 125 students from the original sample completed the questionnaires a second time. Of these, 65 were sport science students (49% females) and 60 (65% females) were students from other programs. The wave 2 sample did not differ in significant ways, on wave 1 data, from the sample of students who did not participate in the wave 2 assessment, thus excluding the possibility of sample biasing. At the time of the first assessment, the majority of the students were between 19 and 29 years old, with the average age being 23 years.

Measures

At each assessment, study participants completed a series of self-report questionnaires including the "International Physical Activity Questionnaire," a "Health Behavior Questionnaire," and the "Multi-Dimensional Body-Self Relations Questionnaire."

The International Physical Activity Questionnaire [14] is designed to measure the frequency of three types of physical activity during the "last

7 days": (1) Vigorous physical activity, defined as activities that take considerable physical effort and make one breathe much harder than normal such as heavy lifting, aerobics, or fast bicycling; (2) moderate physical activity, defined as activities that take moderate physical effort and make one breathe somewhat harder than normal such as bicycling at a regular pace or doubles tennis; and (3) light physical, defined as walking done to move from place to place or walking done for recreation or sport. For each type of physical activity, students reported the number of days per week and the number of hours per day they engaged in the activity. A frequency score was then calculated as the number of hours per week participants engaged in each of the three intensities of physical activity, by multiplying the two pieces of data obtained by the students.

An 18-item Health Questionnaire was created for this study based on questionnaire items assessing health behaviors found on university websites [15, 16] and in the literature [17]. In addition to collecting basic demographic information, the questionnaire assessed the frequency with which participants engaged in 13 diverse health and health-risk behaviors. Of the 13 items, four assessed nutrition related behaviors (i.e., eating breakfast, low-fat diet, fruit and vegetable consumption, and intake of sugary snacks), seven assessed preventive health behaviors (i.e., doctor visits, dental visits, dental hygiene, sleep time, seatbelt use, helmet use, and sunscreen use), and two assessed health risk behaviors (i.e., cigarette smoking and alcohol consumption).

Because the response scales for the nutrition and prevention behavior items were so diverse, for each assessment wave we transformed responses to each item into binary categorical outcomes, with healthy options being coded 1 and unhealthy options being coded 0, and then created nutrition and prevention behavior index scores which consisted of the count of healthy outcomes for each item in the index.

For breakfast, participants were classified according to whether they ate breakfast 5 to 7 days a week (coded 1), or 0 to 4 days a week (coded 0). For low-fat diet, responses indicating a diet low or very low in fat were coded 1, while those indicating a diet high or very high in fat were coded 0. For fruit and vegetable consumption, a division was made on whether respondents ate fruits or vegetables two or more times a day (coded 1), or one or fewer times per day (coded 0). Finally, with respect to the intake of sugary snacks and sodas, responses indicating an intake of less than once a day were coded 1, while responses indicating an intake from once a day to more than once a day were coded 0. Thus, for the nutrition index, consisting of these 4 individual items, scores could range from 0 to 4.

Similarly, for doctor visits, a visit once every year or two was coded 1, while visits once every 3 years or more was coded 0. Dental visits once or twice a year were coded 1, while less frequent visits were coded 0. Dental hygiene was coded 1 if respondents brushed their teeth at least once a day.

For sleep time, at least 8 hours a night received a code of 1, while 7 or less hours per night received a code of 0. For seatbelt, helmet, and sunscreen use, participants were classified according to whether they used these items often to always (coded 1) or almost never or never (coded 0). Thus, index scores for the prevention behavior index could range from 0 to 7. However, since the wave 2 assessment occurred within a year of the first assessment, the two questions concerning doctor and dental visits were not included in the wave 2 health behavior questionnaire; therefore, for wave 2, the prevention behavior index scores ranged from 0 to 5. As one would expect, the relations among the binary outcomes used to create the two index scales were very modest, with r=.21 being the highest correlation across both nutrition index items and the items used for the prevention behavior index.

In contrast to the nutrition and prevention behavior items, the response scales of the two risk behavior items (i.e., cigarette smoking and drinking) were similar enough to create a risk index based on the sum of the 2 item scores, with higher scores reflecting greater frequency/quantity of use.

The Multi-Dimensional Body-Self Relations Questionnaire [18, 19] is a 69-item inventory that assesses self-evaluation and orientation towards appearance, fitness, and health. In particular, the self-evaluation items measure one's overall judgment of appearance, fitness, and health (e.g., "I like my looks just the way they are;" "I am very well coordinated;" "I am a physically healthy person"), while orientation items measure one's psychological investment in, or the degree of importance of, appearance, fitness, and health (e.g., "It is important that I always look good;" "I try to be physically active;" "I have deliberately developed a healthy lifestyle"). Individual items are rated on a 5-point scale (from definitely disagree to definitely agree), and responses are then averaged for each of the six scales, with higher scores indicating greater endorsement (i.e., more positive self-evaluation and higher importance ratings for appearance, fitness, and health dimensions). Item reliability analyses on these scales suggested that there were reliable individual differences in both self-evaluation and orientation ratings of appearance, fitness, and health (i.e., all alpha coefficients were adequate and superior to .70).

Results

Group and Gender Differences in Physical Activity and Health Behaviors

We first examined students' activity levels and their frequency of engaging in health behaviors, indexed separately in terms of nutrition, prevention, and risk behaviors. All analyses were performed taking into account possible differences due to time, gender and program of study. Table 1 shows, sep-

arately for the two assessments, the means and standard deviations of vigorous, moderate, and light physical activity for the entire sample, for males and females, and for sport and non-sport students.

Overall, in terms of average hours per week, students showed a high level of involvement in physical activity, meeting current recommendations for at least thirty minutes of moderate intensity physical activity on most, preferably all, days of the week [20]. In fact, at each assessment, students were, on average, involved in about 16-17h of physical activity per week, aggregating data across types of physical activity. Light physical activity was the most frequent, even though students in general reported, at each assessment, to also engage in about 5h of vigorous physical activity per week.

At each assessment, males and sport science students spent significantly more time in practicing vigorous and moderate physical activity than did females and non-sport students, respectively, as traditional findings and general expectations on study program effects would suggest [2, 21]. Additional analyses also suggested that the differences in physical activity levels between sport and non-sport students were significantly more pronounced among female than male participants, especially for vigorous and moderate physical activity, with sport science females engaging in more vigorous and moderate level physical activity than their non-sport counterparts.

Table 2 shows, separately for the two assessments, the means and standard deviations of the three indices of nutrition, prevention, and risk behaviors for the entire sample, for males and females, and for sport and non-sport students. It is important to note that the first two indices reflect the extent to which students adopt healthy habits across a number of behaviors (i.e., four nutritional behaviors and seven prevention behaviors). Overall, across both measurement waves, students had, for the most part, healthy habits. Statistical analyses further indicated that female students had, on average, significantly healthier nutrition and prevention practices than male students, a difference that was particularly pronounced at the time of the first assessment. Similarly, sport students were significantly healthier than non-sport students with respect to nutrition habits. Finally, Table 2 also shows, and analyses confirmed, that sport students and females were, on average, significantly less likely to engage in health-risk behaviors such as smoking and drinking compared to their respective counterparts.

Table 1 Means and standard deviations for levels of physical activity for the entire sample, male and female students, and sport and non-sport students

Wave 1	Entire sample		Males		Females		Sport		Non-sport	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Vigorous	4.95	5.85	5.66	5.77	4.35	5.86	6.80	5.70	3.30	5.49
Moderate	3.78	6.54	4.34	6.95	3.30	6.13	5.35	7.75	2.38	4.82
Light	6.99	10.75	6.52	11.32	7.40	10.22	7.05	11.50	6.93	10.05
Wave 2										
Vigorous	5.08	6.23	5.73	6.27	4.60	6.20	6.85	6.17	3.12	5.74
Moderate	4.40	6.55	4.87	8.14	4.08	5.25	5.08	5.02	3.64	7.93
Light	8.59	15.32	8.72	21.78	8.50	9.17	9.00	17.14	8.11	13.05

Table 2 Means and standard deviations for health and health-risk behaviors for the entire sample, male and female students, and sport and non-sport students

Wave 1	Entire sample		Males		Females		Sport		Non-sport	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Nutrition	2.84	1.02	2.67	1.01	2.98	1.00	2.95	0.92	2.74	1.09
Prevention	5.18	1.20	4.88	1.29	5.44	1.05	5.07	1.15	5.29	1.23
Risk	5.61	2.50	5.83	2.57	5.42	2.43	5.18	2.33	6.00	2.59
Wave 2										
Nutrition	2.78	0.98	2.77	1.04	2.79	0.94	2.86	0.99	2.70	0.97
Prevention	4.24	0.67	4.09	0.79	4.36	0.56	4.26	0.69	4.23	0.67
Risk	5.40	2.61	5.81	2.61	5.10	2.58	4.78	2.15	6.10	2.90

Stability of Physical Activity and Health Behaviors

Thus far, we have discussed averaged trends and group differences for physical activity and health behaviors as they can be documented from the participating students' responses across two measurement waves.

A further purpose of the study was an assessment of the extent to which individual differences in levels of physical activity and health behaviors are a systematic phenomenon, that is, the extent to which they are stable over time. Table 3 shows the within-construct correlations that the physical activity dimensions and health behaviors showed over time. The table shows, for instance, the extent to which initial levels of physical activity were correlated with levels of physical activity 9 months later. One can see that, for most of the dimensions of physical activity and health behaviors, there existed substantial or moderate temporal stability.

Table 3 Within- and across-construct longitudinal correlations, linking levels of physical activity and levels of health and health-risk behaviors

Wave 1						
Wave 2	Vigorous	Moderate	Light	Nutrition	Prevention	Risk
Vigorous	.54**	.39**	.06	.13	.01	-.23*
Moderate	.37**	.46**	.15	.04	-.04	-.07
Light	.22*	.26**	.30**	.15	.10	-.19*
Nutrition	.05	-.02	-.03	.57**	.07	-.04
Prevention	.12	-.08	.05	.12	.15	.01
Risk	-.03	-.10	.03	-.29**	-.10	.82**

* p<.05, ** p<.01

This stability was estimated more formally by performing, only for the sample of students who provided data on both assessments, a series of hierarchical regressions in which scores for each wave 2 measure were predicted by the respective wave 1 scores after controlling statistically for the gender and group differences discussed above (i.e., after inserting gender and group factors in a step 1 of the regression model). The regression estimates obtained for wave 1 scores provided a measure of relative stability in physical activity, healthy nutrition, and preventive health behaviors (i.e., stability in behavioral individual differences). Illustratively, one regression model examined whether students who reported greater levels of vigorous physical activity early on were also likely to report greater levels of vigorous activity 9 months later, relative to what others reported.

Overall, the results of these analyses confirmed the correlational patterns of Table 3, indicating substantial 9-month stability in physical activity, healthy nutrition, and health-risk behaviors. This relative stability was particularly high for vigorous physical activity (Pearson r=.54), and still substantial for moderate and light physical activity (r=.46 and r=.30, respectively). Furthermore, this longitudinal stability accounted for most of the variance in wave 2 scores, with a minimum of 60% of the variance in levels of vigorous physical activity and a maximum of 90% of the variance in levels of moderate physical activity.

Similarly, differences in healthy nutrition habits (r=.56) and frequency/quantity of smoking or drinking (r=.82) were quite stable over time, and this stability explained most of the variance in the wave 2 scores of these two indices. Finally, hierarchical regressions showed no significant stability in differences in preventive health behaviors (e.g., sleep time, seatbelt use), suggesting that these behaviors might be more sensitive to changes in the circumstances, situations, or life events students experienced over the 9-month period that elapsed between assessments.

Longitudinal Effects Linking Physical Activity and Health Behaviors

The assessment of stability in individual differences provided an opportunity to evaluate more rigorously a key topic of the study, that is, the longitudinal effect that physical activity may have on the adoption of health behaviors or the longitudinal effects the latter behaviors may have on physical activity.

For longitudinal effects to exist, physical activity and health behaviors needed to be correlated over time. Table 3 also shows the longitudinal bivariate correlations linking vigorous, moderate, and light physical activity to the nutrition, prevention, and health-risk behavior indices. Overall, early physical activity was not correlated with later health behaviors, whereas there existed some evidence of the opposite pattern, especially for the negative correlation between early health-risk behaviors and later physical activity.

These correlations were examined more thoroughly by performing another series of hierarchical regressions in which, for example, the longitudinal effects of vigorous physical activity on healthy nutrition habits were estimated after controlling statistically for possible gender or group differences and stability in nutrition scores. In this case, this analysis allowed us to examine whether, after taking into account average differences across groups or stability in nutrition over time, levels of vigorous physical activity influenced changes in nutrition behaviors, thus providing a more rigorous test of longitudinal effects.

Even though physical activity did not predict changes in nutrition or prevention behaviors over time, higher levels of vigorous and light physical activity early on significantly predicted larger reductions in smoking/drinking habits 9 months later, especially among male students. Instead, healthier nutrition habits early on predicted significant increases in the subsequent level of vigorous and light physical activity, especially among females and males, respectively. The details of these regression analyses in terms of coefficients of the estimated effects and model characteristics are available from the first author upon request.

Thus, physical activity did not contribute to positive changes in health behaviors, although it seemed to represent an important protective factor against the adoption of health-risk behaviors, especially in males. Interestingly, healthy eating habits contributed to positive changes in physical activity levels. However, it is not known if this was due to deliberate attempts to lose weight.

Group and Gender Differences in Appearance, Fitness, and Health Beliefs

At each assessment, students, on average, expressed positive evaluations about themselves in terms of physical appearance, fitness, and health, and considered these dimensions to be quite relevant and important to them. For all 5-point judgments, students' mean scale scores were at or greater than 3.6 (and standard deviations smaller than one unit), thus suggesting homogeneous positive endorsement.

These trends varied somewhat across gender. Male students reported significantly more positive evaluations about their appearance and their perceived fitness and health than the female students. They also assigned relatively more importance to fitness than the female students, whereas the female students rated appearance and health as relatively more important than the males. Descriptively, all these differences were not greater than two thirds of a point (on a 5-point scale), thus indicating important but not dramatic gender differences. Similarly, students enrolled in sport programs evaluated themselves more positively and considered it significantly more important to stay fit and healthy than did their counterparts. Again, these differences were statistically significant but not so pronounced in terms of absolute value.

Value Systems in the Relation Between Physical Activity and Health Behaviors

In lieu of these differences in the value and self-evaluation ratings, we examined whether they could qualify the patterns of longitudinal relations we observed between physical activity and health behaviors. In other words, we evaluated whether the strength of these relations would change depending upon the degree of participants' endorsement of value systems conceptually related to physical activity and health.

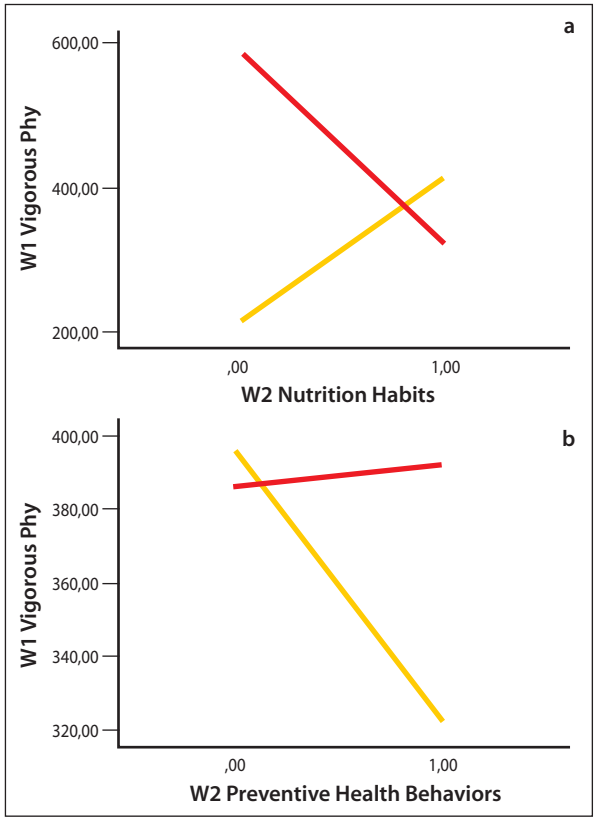
For instance, we wondered whether any effect of early physical activity on later health behaviors would emerge among those students who endorsed certain health-related values. Technically, this possibility can be evaluated by including a product term linking early physical activity and values' endorsement as a predictor in a regression model predicting wave 2 health behaviors, after inserting into the model, and controlling for, the main effects of students' early values and physical activity.

We performed this type of analyses for each type of self-evaluation and importance ratings obtained by the students in the first assessment. The only dimension that made a statistically significant difference in the patterns of relations between physical activity and health behaviors was the importance that students assigned to physical appearance.

As one can see from the Figure 1, the null findings discussed earlier about

any longitudinal effect of physical activity on changes in later health behaviors can be reconsidered with respect to people's values.

Among those who assigned relatively little importance to physical appearance, those who practiced more vigorous physical activity were more likely to have healthier nutrition habits 9 months later. This pattern corresponded to a statistically significant longitudinal correlation (r=.28). An opposite pattern characterized those who assigned more importance to physical appearance, although this pattern corresponded to a statistically nonsignificant longitudinal correlation between initial physical activity and subsequent healthy nutrition (r=-.18). Only for illustrative purposes, these two patterns are diagrammed in Figure 1a in terms of mean levels of initial physical activity across varying degrees of nutrition habits determined by a median-split. Likewise, among those students who considered physical appearance important, those who practiced more vigorous physical activity were significantly more likely to show preventive health behaviors 9 months later (r=.26). Again, this pattern was utterly absent in the counterpart group. Figure 1b shows these differences in patterns by plotting levels of early physical activity for varying frequencies of preventive health behaviors as reported by students 9 months later.



Legend (for both figures)
Importance of Physical Appearance
not so important
important

0 = below-the-median W2 scores
1 = above-the-median W2 scores
Scores on the Y-axis represent average minutes of physical activity during last 7 days

Fig. 1 The relation between W1 physical activity and W2 nutrition habits moderated by the level of importance assigned to physical appearance (**a**). The relation between W1 physical activity and W2 preventive health behaviors moderated by the level of importance assigned to physical appearance (**b**)

Conclusions

It is common to view physical activity as an important marker of a person's consideration and pursuit of a healthy life style. This view partly implies that the individual who practices physical activity chooses courses of action favoring health-promoting behaviors and assigns particular importance to health and health-related values. Finally, the same view also underlies the notion that a person's behavior does not change easily in response to circumstances and situations the person experiences but, rather, is relatively stable over time and influenced and regulated by personality factors and processes.

Our study examined the validity of these notions in a sample of nearly 600 Italian university students who were enrolled in either sport-related or other study programs. Some of them were interviewed a second time, about 9 months after the first assessment. In both assessments, we measured students' frequency of participating in three types of physical activity, the extent to which they engaged in health and health risk behaviors, and their beliefs about appearance, fitness, and health.

Overall, Italian university students engage in both regular physical activity and in healthy behaviors, at least if one considers averaged trends. Group differences also exist indicating that females engage in more healthy habits than males, whereas the latter are more physically active, especially when we consider vigorous physical activity. Furthermore, it clearly appears that sport students are more likely than other students to engage in health-promoting behaviors and to avoid health-risk behaviors.

In addition to group differences, our analyses also clearly indicated that there exist reliable individual differences both in levels of physical activity and frequency of health-promoting and health-risk behaviors. That is, the differences that emerged among students in the first assessment emerged with a high degree of stability 9 months later, and this finding characterized quite well, and substantially, both students' type of physical activity, their nutrition habits, and frequency of smoking and drinking.

These trends do not necessarily indicate that physical activity and health behaviors converge meaningfully in a person's behavioral patterns. When we analyzed the relations linking physical activity and health behaviors, we found that these behaviors did not show evident linkages. That is to say that a student's high level of physical activity does not necessarily imply that he or she also will show healthy habits concerning nutrition or prevention behaviors.

However, other careful analyses of how physical activity is related to health behaviors over time indicated that, while physical activity did not influence changes in healthy habits, nutrition and eating habits influenced

positive changes in students' physical activity. Furthermore, physical activity influenced positive changes in students' health-risk behaviors such as smoking and drinking.

Furthermore, and perhaps more importantly, the relations linking physical activity and health behaviors over time varied with the degree of importance students' assigned to physical appearance. Over time, initial physical activity predicted healthier eating habits later on among those who assigned little importance to physical appearance, whereas it predicted more preventive health behaviors among those who assigned greater importance to physical appearance.

Some final considerations seem warranted, albeit they only can be advanced with some caution given the preliminary nature of the study and its limited sampling data. The data we presented stress the importance of engaging in physical activity. University students who practiced sport-related activities are more likely to have healthy habits, at least when they are compared to the averaged trends of students who are not enrolled in sport-related programs. Students who initially show relatively high levels of physical activity are more likely, later on, to positively diminish their health-risk behaviors more than students who are less physically active. Thus, physical activity seems to represent an important protective factor against health risk behaviors. Finally, more physical activity is also linked to better eating habits, even though the ways this relation unfolds over time in our data suggest that better eating habits may elicit positive changes in physical activity, rather than the opposite. This conclusion suggests that intervention programs focused on promoting healthy lifestyles may have a better chance to succeed than programs which strictly focus on increasing physical activity levels. Furthermore, the promotion of healthier eating habits could be a means for increasing physical activity among late adolescents.

The findings of the study also provided some initial and encouraging insights about the importance of examining physical activity and health behaviors in the context of psychological analyses that may help to understand descriptive findings and embed them into a coherent framework of a working hypotheses. In our data, the relations between physical activity and health behaviors clearly varied with the value students' assigned to physical appearance. This general finding suggests that people's values about personal matters represent an important psychological construct for examining and understanding the relations between physical activity and health behaviors. These relations cannot be necessarily subsumed to general patterns in the populations but, rather, depend in important ways on personal factors that may motivate and determine personal courses of action. The core challenge is to pinpoint and understand these personal courses of action without necessarily viewing behavior as being regulated by general trait-like health styles but, rather, by factors and processes that encompass

the ways people feel and think about their experiences and what they consider important and valuable for their health and life. In this sense, physical activity and other health behaviors do not necessarily need to create a cohesive pattern, and a key scientific task is to understand when and how this possibility, which is of course auspicious, materializes.

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Subject Index

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