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LUMME KADAJA

TARTU UNIVERSITY
PRESS

Department of Pathophysiology, University of Tartu, Tartu, Estonia

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Opponent: Professor Kent Sahlin, Ph.D.
Institute of Sports Science and Clinical Biomechanics,
University of Southern Denmark, Odense, Denmark

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To my children with love

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LIST OF ORIGINAL PUBLICATIONS

The work is based on the following publications:

I. Kuznetsov, V., Tiivel, T., Sikk, P., Käämbre, T., Kay, L., Daneshrad, Z., Rossi, A., Kadaja, L., Peet, N., Seppet, E., Saks, V. Striking difference between the kinetics of regulation of respiration by ADP in slow-twitch and fast-twitch muscles *in vivo*. European. Journal of Biochemistry 241: 909–915, 1996.

II. Tiivel, T., Kadaja, L., Kuznetsov, A., Käämbre, T., Peet, N., Sikk, P., Braun, U., Ventura-Clapier, R., Saks, V., Seppet, E. K. Developmental changes in regulation of mitochondrial respiration by ADP and creatine in rat heart *in vivo*. Molecular and Cellular Biochemistry 208: 119–128, 2000.

III. Braun, U., Paju, K., Eimre, M., Seppet, E., Orlova, E., Kadaja, L., Trumbeckaite, S., Gellerich, F. N., Zierz, S., Jockusch, H., Seppet E. K. Lack of dystrophin is associated with altered integration of the mitochondria and ATPases in slow-twitch muscle cells of MDX mice. Biochim. Biophys. Acta 1505: 258–270, 2001.

IV. Puurand, Ü., Kadaja, L., Seppet, E. K. Kindred DNA amplification from two distinct populations of cDNA fragments. BioTechniques, 34: 994-1000, 2003.

V. Kadaja, L., Kisand, K. E., Peet, N., Braun, U., Metsküla, K., Teesalu, K., Vibo, R., Kisand, K.V., Uibo, R., Saks, V. A., Jockusch, H., Seppet, E. K. IgG from patients with liver diseases inhibit mitochondrial respiration in permeabilized oxidative muscle cells: Impaired function of intracellular energetic units? Molecular and Cellular Biochemistry 000: 000-000, 2003. In press.

ABBREVIATIONS

ADP	adenosine diphosphate
AIH	autoimmune hepatitis
AK	adenylate kinase
ANT	adenine nucleotide translocase
ATP	adenosine triphosphate
ATR	atractyloside
BW	body weight
cDNA	complementary deoxyribonucleic acid
CH	chronic hepatitis
CK	creatine kinase
DW	dry weight
EDL	*m. extensor digitorum longus*
HE	subtracted heart-specific cDNA library
HW	heart weight
ICEU	intracellular energetic units
IF	intermediary filaments
IgG	immunoglobulin G
KDA	kindred DNA amplification
K_m	Michaelis's constant
LDH	lactate dehydrogenase
MAP	microtubule-associated protein
MDX	X-chromosome-linked muscular dystrophy
MHC	myosin heavy chain
mi-CK	mitochondrial creatine kinase
mRNA	messenger ribonucleic acid
MT	microtubule
NRF	nuclear respiratory factor
OM	outer membrane
PBC	primary biliary cirrhosis
PBS	phosphate buffered saline
PCr	phosphocreatine
PCR	polymerase chain reaction
PK	pyruvate kinase
RCI	respiratory control index
RT	reverse transcription
SHE	*m. soleus*- and heart-specific cDNA library
SSH	suppression subtractive hybridization
V_0	basal respiration
V_{max}	maximal rate of oxidative phosphorylation
WW	wet weight

INTRODUCTION

Muscles of humans and animal species are characterized by a large diversity of contractile performance, which mostly depends on fiber type (Pette and Staron, 1990; Schiffiano and Reggiani, 1994, 1996; Moss *et al.*, 1995). Based on their contractile parameters, myosin ATPase (adenosine triphosphatase) activities and respiratory capacity the muscles can be divided into two major classes: the slow-twitch oxidative muscles and fast-twitch glycolytic muscles, with a variety of subtypes between these two types (Pette and Staron, 1990; Schiffiano and Reggiani, 1994, 1996; Moss *et. al.*, 1995). Recent studies on skinned muscle fibers have revealed that the mitochondria in glycolytic skeletal muscle cells express much higher mitochondrial affinity to adenosine diphosphate (ADP) compared to oxidative ones. At the same time the mitochondria isolated from both types of muscles exhibit similarly high affinity to ADP, comparable to that in skinned fibers of glycolytic muscles (Veksler *et al.*, 1995, Kuznetsov *et al.*, 1996). These experiments point to principal differences in the regulation of mitochondrial function in different types of muscle cells. At the same time, the mechanisms underlying distinct regulatory properties have remained largely unclear. Ultrastructural and biochemical studies demonstrate tight connections between mitochondria and cytoskeletal structures, particulary with annexin V and desmin (Penman, 1995; Leterrier *et al.*, 1994; Sun *et al.*, 1997; Kay *et al.*, 1997). Based on these data, it is hypothetized that in oxidative muscle cells the mitochondria and ATPases are compartmentalized into the complexes of organized metabolic pathways — intracellular energetic units (ICEUs). Within these units the mitochondria are integrated with ATPases by means of specific energy transfer systems mediated by creatine kinase (CK) and adenylate kinase (AK) or direct transfer of adenine nucleotides, without equilibration of them in the cytoplasmic space (Seppet *et al.*, 2001; Saks *et al.*, 2001). It is likely that cytoplasmic proteins are responsible for organization of ICEUs in oxidative cardiac muscles (Seppet *et al.*, 2001; Saks *et al.*, 2001). In glycolytic muscles these units apparently do not exist, and therefore, mitochondria and ATPases are linked to each other via simple diffusion of adenine nucleotides in the cytoplasm (Seppet *et al.*, 2001; Saks *et al.*, 2001).

In the present study the functional properties of mitochondria in oxidative and glycolytic muscles were detailly characterized *in situ*, by utilizing the skinned fiber technique. Also, the developmental changes in regulation of mitochondrial respiration in rat myocardium were assessed. It was hypothetized that if both oxidative muscle cells, myocardium and *m. soleus*, possess the ICEUs, the proteins that participate in formation of these units must be expressed in both of these muscles. Therefore, tissue-specific library consisting of the complementary deoxyribonucleic acids (cDNAs) common in mouse myocardium and *m. soleus* (oxidative) but not expressed in *m. extensor digitorum longus* (glycolytic) was constructed. For creation of the library, a

novel method for isolation of kindred DNA from two distinct populations of cDNA fragments was developed and employed.

The alternative strategy of the study was based on the assumption that the circulatory autoantibodies may recognize the structures belonging to ICEU. Therefore, the effects of autoantibodies developed in healthy persons and patiens with different liver diseases on mitochondrial respiration *in situ* and their binding to the intracellular structures in cardiac muscle were studied. The results of the study open new perspectives for molecular approach to identifying the proteins responsible for regulation of mitochondrial function in the oxidative muscle cells.

REVIEW OF LITERATURE

1. Classifications of muscles

The generation of muscle fiber heterogeneity is based on gene regulation through two main mechanisms reviewed for human fibers by Bottinelli and Reggiani (2000):

- *Qualitative mechanism:* muscle proteins (like myosin) may exist in forms, which are similar but not identical (isoforms). Isoforms can be derived from the same gene through alternative splicing (pre-mRNA is spliced, to produce different versions of the mRNA and ultimately, different proteins) or from different genes of the same family (isogens). Replacement of isoforms represents the first mechanism generating diversity among muscle fibers.
- *Quantitative mechanism*: differential expression of the same gene. Lot of genes can be up- and downregulated independently of each other on the basis of factors such as mechanical load, hormones *etc.* The proportion between the products of these genes will therefore be modified and new functional or structural features will appear.

Thus, muscles can be grouped according to myosin heavy chain (MHC) isoform profile, and also according to a variety of other criteria and parameters, such as contractile properties and metabolic enzyme activities (Fink *et al.*, 1990; Ruff and Whittlesey, 1991; Larsson and Moss, 1993; Lynch *et al.,* 1994; Bottinelli *et al.*, 1996; Widrick *et al.*, 1996; Stienen *et al.*, 1996; Schiaffino and Reggiani, 1996; Harridge *et al.*, 1996).

1.1. Delineation of main fiber types on the basis of myosin heavy chain isoform profile

The method based on electrophoretic mobility yields important quantitative information for muscle typing. By silver-stained polyacrylamide gel electrophoresis (PAGE) of human mixed *m. pectoralis* the three MHC bands (Hilber *et al.*, 1999) can be separated. The fastest migrating MHCI is expressed in type I fibers, the slowest migrating MHCIIb is expressed accordingly in type IIB and MHCIIa moving slightly faster than MHC is expressed in IIA fibers (Hilber *et al.*, 1999). Electrophoretic separation of MHC on polyacrylamide gel, from rat *m. extensor digitorum longus* (EDL) and *m. soleus*, exhibit dependently from muscle type MHCIIb or MHCI dominating band (Vescovo, *et al.*, 1998). These isoforms have been assigned to the myofibrillar myosin ATPase activity based fast fiber types IIB and IIA and slow fiber type I (Brooke and Kaiser, 1970; Pette and Staron, 1990; Staron, 1997). The MHC represents the essential component of the force-generating system of the muscle and by virtue of its ATPase activity determines the velocity of contraction (Huxley, 1969; Huxley

and Simmons, 1971; Kishino and Yanagida, 1988). Therefore, the contractile speed strongly correlates with the myosin isoform profile (Bàràny, 1967; Close, 1967; Reiser *et al.*, 1985,; Sweeney *et al.*, 1986; Bottinelli *et al.*, 1991; Galler *et al.*, 1994, Larsson and Moss, 1993).

1.2. Differentiation on the basis of the metabolic profile

Metabolic differentiation is based on determination of the activities of different respiratory (succinate dehydrogenase, cytochrome oxidase, NADH dehydrogenase) and glycolytic (lactate dehydrogenase, pyruvate kinase, phosphofructokinase, citrate synthase *etc.*) enzymes (Ogata and Mory, 1964; Padykula and Gauthier, 1967; Peter *et al.*, 1972, Lowry *et al.*, 1978). Accordingly, the muscles with high activity of glycolytic enzymes, large glycogen deposition (Greenhaff *et al.*, 1993) and low lipid content are considered to be the glycolytic fibers (e.g. EDL) (Gollnick *et al.*, 1984, Howald *et al.*, 1985; Peter *et al.*, 1972; Dubowitz, 1985). The muscles rich of capillaries and mitochondria and expressing high respiratory activity belong to the group of oxidative muscles as heart and *m. soleus* (Kiessling, 1974; Andersen, 1975; Ingjer, 1979; Nemeth and Pette, 1981; Jansson *et al.*, 1982; Svedenhag, 1983; Nemeth and Lowry, 1984; Howald *et al.*, 1985; Ogata and Yamasaki, 1997).

Skeletal muscles, which have a high oxidative potential, are less sensitive to the catabolic action of dexamethasone. It has been proposed that the glucocorticoid-caused myopathy is a result of elevated degradation of contractile proteins. This process of degradation of contractile proteins begins in the myosin filaments and then spreads to the thin filaments and the Z-line (Seene *et al.*, 1988). On the other hand, the catabolic action of glucocorticoids on the molecular level of the MHC and actin was found to be similar in all muscle types while the synthesis rate of actin and myosin heavy chain was decreased in all muscles studied (Seene and Alev, 1985).

Muscle fibers also differ in their content of high energy phosphates. In human muscles the resting phosphocreatine (PCr) content is higher is fast (83–86 mmol/kg DW) than in slow fibers (66–72 mmol/kg DW (Söderlund and Hultman, 1991; Sant'ana Pereira *et al.*, 1996; Sahlin *et al.*, 1997). Upon exercise the PCr content decreases to similar values both in fast-twitch and slow-twitch fibers (Söderlund and Hultman, 1991; Sahlin *et al.*, 1997). By contrast, resting ATP (adenosine triphosphate) content is rather similar in slow and fast fibers (22–27 mmol/kg DW) (Söderlund and Hultman, 1991; Greenhaff *et al.*, 1993; Sant'ana Pereira *et al.*, 1996). The ATP concentration is maintained relatively constant in both fiber types also during exercise (Sahlin *et al.*, 1997). Only after contractile activity under extreme conditions (for example maximal electrical stimulation with occluded circulation) has a significant decrease been observed (Söderlund and Hultman, 1991).

ADP content in human muscle fibers has been estimated around 2–3 mmol/kg DW (Söderlund and Hultman, 1991; Sahlin *et al.*, 1997). ADP content can increase up to 4 mmol/kg DW after heavy exercise due to imbalance between ATP hydrolysis and ATP resynthesis (Söderlund and Hultman, 1991). From the viewpoint of metabolic regulation, it is important to consider that because most of the cellular ADP is tightly bound to different structures, only a small fraction of its intracellular content (free ADP) can be metabolically active, e.g. in regulation of mitochondrial respiration, or in reactions with phosphotransferases. The free ADP content cannot be directly determined, and is calculated from the CK equilibrium (Chance *et al.*, 1985). It is generally found that increased contractile activity of glycolytic muscle is associated with augmented increase in the cytoplasmic ADP concentration, whereas the level of that parameter does not change in slow-twitch muscles (Kushmerick, 1992b). This observation can be explained by higher rates of ATP consumption in fast-twitch muscles based on the higher myosin ATPase activity compared to slow-twitch muscle as shown in rat muscle fibers (Bottinelli *et al.*, 1994; Reggiani *et al.*, 1997) and in human fibers (Stienen *et al.*, 1996; Sahlin *et al.*, 1998). It also leads to an important conclusion, again suggesting the principal differences in different muscles in terms of regulation of mitochondrial respiration: While in fast-twitch glycolytic muscles activation of mitochondrial respiration can be attributed to large increase in cytoplasmic ADP concentration, this process must be controlled by other factors in slow-twitch muscles.

The Pi content in slow fibers is higher than in fast fibers. In small mammals the Pi content ranges between 0–2 mmol/kg in fast-twitch muscles and 3.6–6.7 mmol/kg in slow-twitch muscles (Kushmerick, 1992a). In human muscles at rest Pi content varies between 1.5 and 3 mM and increases in direct relation to the percentage of slow-twitch fibers (Vanderborne *et al.*, 1995). Thus the Pi/PCr ratio in slow-twitch muscle fibers is higher than in fast-twitch muscle fibers, in human *m. soleus* 0.15±0.01, in medial and lateral human *m. gastrocnemius* 0.12±0.01 or 0.10±0.01, respectively (Vanderborne *et al.*, 1995).

Slow-twitch fibers appear to be particularly suited for isometric and tonic contractions as they develop almost the same isometric force as fast-twitch fibers, but consume much less ATP and do not tend to fatigue due to their mostly aerobic metabolism performance. Because they contain more myoglobin than fast-twitch muscles, they have also been traditionally characterized as the red muscles. Muscle performance *in vivo*, in fact, is determined not only by fiber type composition, but also by neural control and by other factors, such as muscle architecture (Narici *et al.*, 1996).

Table 1 summarizes the basic parameters used in differentiation of the muscle types. It can be seen that two muscles used in our study i.e. myocardium and *m. soleus*, represent the oxidative and slow-twitch muscles, whereas EDL belongs to the glycolytic fast-twitch muscle group.

Table 1. The basic properties of different fiber types in rat and mouse.

Muscle fiber typing	MHC iso-forms	ATPase activity	Glycolytic capacity	Oxidative capacity	Myo-globin content	pH dependence	Examples of muscles
Type I, slow-twitch, oxidative, red, fatigue resistant	MHC I	Low	Low glycogen content and glycolytic enzyme activities	High oxidative enzyme activities, a large content of mitochondria and cytochromes	High	Acidic stable	heart *m. soleus*
Type IIA, fast-twitch, oxidative-glycolytic, red, fatigue resistant	MHC IIa	High	Intermediate or high glycogen content, intermediate glycolytic enzyme activities	Intermediate content of mitochondrial protein and high cytochrome content	High	Intermediate stability in alkaline and acidic medium	*m. quadriceps*
Type II B, fast-twitch, glycolytic, white, fast fatiguable	MHC IIb	High	Limited glycogen content, high glycolytic enzyme activities	A small content of mitochondria, low capacity of oxidative metabolism: a small cytochrome content and low activity of glycolytic enzymes	Low	Alkaline stable	*m. extensor digitorum longus*

2. Creatine Kinase system in muscle cells

Several muscle proteins (creatine kinase *etc.*) exist in forms of isoenzymes, which are derived from different genes of the same family. The creatine kinases comprise multigene family. Three cytosolic creatine kinase (CK) isoenzymes MM, MB and BB are dimers composed of M (muscle-type) and B (brain-type) subunits encoded by two separate nuclear genes (Payne *et al.*, 1991). Native mitochondrial creatine kinase (mi-CK) exists as an octamer composed of four homodimers (Schlegel *et al.*, 1988). Two distinct isoenzymes of mi-CK, differing in molecular weights, NH_2 terminal amino-acid sequences, and isoelectric points have been revealed (Hossle *et al.*, 1988; Schlegel *et al.*, 1988; Haas *et al.*, 1989). By cloning cDNAs encoding cardiac mi-CK it has been proved that these proteins are the products of separate nuclear genes (Haas *et al.*, 1989). One of the enzymes (ubiquitous mi-CK) is present in many tissues, with highest level in brain and kidney. Sarcomeric type of mi-CK is present only in heart and skeletal muscle. Expression of both types of mi-CKs parallels the expression of the cytosolic (M-type and B-type) CKs (Payne *et al.*, 1991).

Mi-CK appears to be accumulated in contact sites between the inner membrane and OM (Scholte *et al.*, 1973; Wyss *et al.*, 1992). Mi-CK is located on the outer surface of the inner membrane in the structural closeness of adenine nucleotide translocase (ANT) (Figure 13). There exists experimental evidence that mi-CK and ANT are functionally coupled (Saks *et al.*, 1980; Wyss *et al.*, 1992).

In muscle cell, a portion of MM-CK is associated with the myofibrils (Figure 13) as an integral component of M-band together with M protein and myomesin. The role of this CK is to rephosphorylate ADP generated by the actin-activated myosin ATPase (Saks *et al.*, 1978; Bessmann and Carpenter, 1985; Walliman and Eppenberger, 1985).

Determination of the intracellular distribution of CK have shown that 40–50% of the total cellular activity of CK is localized in the cytoplasm (MM and MB isoenzymes), 30-40% in the mitochondria and 20% are bound to myofibrils, sarcoplasmatic reticulum and sarcolemma (Saks *et al.*,1976, Sharov *et al.*, 1977; Levitsky *et al.*,1978). During development of mammalian heart, the change in CK isoenzyme profile occurs: the amount of BB-CK decreases and MM-CK increases (Eppenberger *et al.*, 1964).

Recent studies have opened the new aspects of the metabolic regulation *in vivo*, i.e. under conditions of natural intracellular environment of mitochondria. It was shown, by using saponin-skinned fibers of rabbit myocardium, that early postnatal development is characterized by binding of mi-CK to the inner membrane of mitochondria due to which the respiration becomes stimulated by creatine (Hoerter *et al.*, 1991). Deprivation of CK system function by feeding the developing rats with β-guanodinopropionic

acid (Pelouch *et al.*, 1996) has been shown to significantly impair the contractile function of developing rat heart. These experiments strongly suggest that compartmentation of different CK isoenzymes to intracellular organelles is an important part of the postnatal cardiac maturation (Hoerter *et al.*, 1991; Pelouch *et al.*, 1996). However, this matter is still open for discussions, since recently no functional coupling between mi-CK and ANT was found in 1–2 weeks old rats (Vannier *et al.* 1996).

3. Regulation of mitochondrial function in different muscles *in vivo* — a new challenge in muscle bioenergetics

Regulation of mitochondrial respiration and oxidative phosphorylation *in vitro*, in isolated mitochondria, is well described since the classical work of Chance (Chance and Williams, 1956) and explained in molecular terms in the framework of chemiosmotic theory of Mitchell (1961). The oxidative phosphorylation itself — the ATP synthesis in mitochondria — is explained by a rotary mechanism discovered by Boyer and Walker's groups (Boyer, 1997; Abrahams *et al.*, 1994).

The basic mechanisms of mitochondrial oxygen consumption, its regulation and ATP synthesis in the processes of oxidative phosphorylation have been described in *in vitro* studies of isolated mitochondria or their components (Skulachev, 1998; Boyer, 1997). It has been found that these mechanisms operate also in mitochondria *in vivo*, where the organelles are integrated into cellular metabolic systems. However, the nature of feedback signal from ATPases in myofibrils to mitochondria *in vivo,* responsible for effective feedback between energy production and its demand is still unclear. In the heart the workload and respiration rate may be increased by more than an order of magnitude at practically constant levels of PCr, creatine, ATP and ADP (Balaban *et al.*, 1986; Williamson *et al.*, 1976). This observation has led to the conclusion that there is no metabolic feedback regulation of mitochondrial activity in the heart by ADP fluxes, as originally proposed by Chance and Mitchell. Thus, alternative mechanism of regulation of respiration was suggested according to which the transient rise in the cytoplasmic Ca^{2+} concentration before the contraction could activate in parallel both the contraction and mitochondrial respiration, thus enabling effective link between these two processes (McCormack *et al.*, 1990; Korzeniewsky *et al.*, 1998). However, the observation that there is still a linear relationship between workload and oxygen uptake after inhibition of Ca^{2+} uptake into mitochondria (Katz *et al.*, 1988; Khuchua *et al.*, 1994) disagrees with the Ca^{2+}-theory proposed. Thus, the problem of how the mitochondria know how much they should produce ATP still awaits to be solved.

A major breakthrough in this issue was made by introducing the skinned fiber technique (Veksler, *et al.*, 1987) that allowed to analyse the function of mitochondria in living cells. This method is based on the use of saponin, which dissolves the cell membrane by specific interaction with cholesterol. Because the sarcolemma contains much more cholesterol than other intracellular membrane structures, the sarcolemma is selectively permeabilized or even removed, whereas other structures (sarcoplasmic reticulum, mitochondria and myofibrils) remain intact (Daum, 1985, Veksler *et. al.*, 1987, Endo and Kitazawa, 1978). The medium for assesment must closely simulate the composition of muscle cell cytoplasm. In the presence of respiratory substrates the basal respiration occurs (state 2 or V_0), whereas addition of 1mM ADP results in maximal rate of respiration (state 3). In contrast to isolated mitochondria where the state 3 transition to state 4 is observed due to full phosphorylation of ADP, the respiration in skinned fibers remains constantly activated in state 3 because the endogeneous ATPases continuously regenerate ADP.

These fibers can be easily used for determination of respiratory parameters of mitochondria in small amounts of biopsy material allowing to detect the changes in different pathological conditions. The effects of ischemia on the functional properties of cardiac mitochondria in skinned muscle fibers have been studied. The results show that ischemia affects mitochondrial function and decreases the creatine-stimulated respiration most significantly (Veksler *et al.*, 1987). The results of Seppet *et al.* (1991) show the hyperthyroidism-induced imbalance between PCr synthesis and respiration in cardiac skinned muscle fibers (Seppet *et al.*, 1991).

Saponin-permeabilized fibers also allow to get information about the entire intracellular population of mitochondria, in their natural interaction with other cellular structures in living cells, which is impossible when only the isolated mitochondria are analysed.

4. Functional complexes of mitochondria with ATPases of myofibrils and sarcoplasmic reticulum in muscle cells — intracellular energetic units

Since the first application of the skinned fiber techniques in the studies of respiratory regulation (Veksler *et al.*, 1987; Kümmel, 1988; Seppet *et al.*, 1991; Saks *et al.*, 1995), it became clear that mitochondria in cardiac muscle and other slow-twitch oxidative muscles exhibit much lower affinity to exogenously added ADP than isolated mitochondria. Moreover, the affinity of mitochondria in glycolytic muscles was found to be comparable with that parameter for isolated mitochondria. These observations clearly showed that mitochondrial function is principally differently regulated in these two types of muscles (Veksler *et al.*, 1995; Kuznetsov *et al.*, 1996). It means that in

oxidative muscles there must exist the mechanism that limits the diffusion of ADP into the mitochondria, probably at the porin channels (VDAC) in the outer membrane. As this type of control could be removed by limited proteolysis, it was concluded that some cytoskeletal proteins might participate in the control mechanism (Saks *et al.*, 1998). Later the dependence of respiration on the source of ADP was observed (Seppet *et al.*, 2001; Saks *et al.*, 2001). These studies demonstrated that mitochondria can be activated by endogenous ADP forming in the ATPase reactions much more effectively than by adding ADP exogenously. It was also shown that ADP produced by ATPases and stimulating mitochondria is not accessible for exogenous ADP trapping system consisting of the pyruvate kinase and phosphoenolpyruvate (PK+PEP). When the skinned fibers were treated with trypsin, the ADP became effectively trapped by PK+PEP system, and the effectiveness of endogenous ADP to activate respiration decreased.

These data could be taken to show that in oxidative red muscle cells mitochondria, consuming ADP and producing ATP, behave as if they were included into functional complexes with adjacent ADP producing systems — with the MgATPases of myofibrils and CaATPases of sarcoplasmic reticulum. ADP produced within these complexes (or intracellular energetic units — ICEUs) does not equilibrate easily with ADP in the bulk phase (cytosol). Moreover, within these functional units the energy is transferred mostly via enzyme networks composed of the CK and AK systems, and by direct channeling of the adenine nucleotides (Seppet *et al.*, 2002; Saks *et al.*, 2001). This new concept agrees with and supports the theoretical concepts of cell's architecture and metabolic channeling developed by others (Srere, 1985; Clegg, 1986; Ovadi, 1995). This concept of eukaryotic cell organization bears in mind that virtually all the cell architecture is interconnected by the micro-trabecular lattice containing cytoskeletal elements, with the intervening aqueous phase(s) being extremely dilute with respect to dissolved macromolecules (Clegg, 1986; Ovadi, 1995). At the same time, there is no sharp, rigid discontinuity between the architecture and the surrounding aqueous phase (Clegg, 1986). Klopfenstein *et al.* (1998) showed recently that direct interaction of endoplasmic reticulum with microtubules is mediated by an integral protein p63 and multiple connections between mitochondria and cytoskeleton elements have been described in the muscle and other types of cells (Kay *et al.*, 1997; Milner *et al.*, (2000), also reviewed by Penman (1995) and Rappaport *et al.*, (1998). Therefore, the structural organization of these functional complexes is probably also related to the organization of the cytoskeletal network of the cell. It is highly probable that the activities of ICEUs are synchronized in normal cells. The pathological conditions may impair the synchronized function. This is evident from recently described metabolic heterogenity of mitochondria in cardiomyocytes under conditions of substrate deprivaton, recently seen by confocal microscopy. It was shown that mitochondria in different parts of the cell may be oxidized to different extents,

and the redox potential of the respiratory chain can change spontaneously, by metabolic oscillations and waves (Romashko *et al.*, 1998). This may correspond to differences in the behaviour of different ICEUS when synchronization of their functions is disturbed. The cellular mechanism of the synchronization of the ICEUs remains unresolved (Saks *et al.*, 2001), and therefore needs further studies.

The concept of the ICEU explains why in ischemic heart the contraction stops despite high cellular ATP levels. It is likely that a ATP pool inside the ICEU is rapidly used up, resulting in the accumulation of ADP inside the ICEU and the inhibition of cross-bridge detachment and thus inhibition of contraction despite the presence of ATP in the bulk-water phase of the cell.

5. The biogenesis of mitochondria

Mitochondria have their own genetic system comprised of a circular DNA genome, the enzymes and cofactors required for its transcription and replication as well as the protein synthesis apparatus for the translation of mitochondrial mRNAs (Attardi and Schatz, 1988; Wallace, 1992; Shadel and Clayton, 1997). The biogenesis of mitochondria requires the expression of a large number of genes, most of which reside in the nuclear genome. There are only thirty seven mitochondrial genes in total, thirteen of them code for subunits of proteins of respiratory complex I, III, IV and complex V. Thus, the complexes involved in proton pumping and ATP synthesis are comprised of protein subunits encoded by both, mitochondrial and nuclear genes (Anderson *et al.*, 1981; Attardi and Schatz, 1988; Murdock *et al.*, 1999). All the hundreds of other mitochondrial proteins, including DNA polymerase, RNA polymerase, all ribosomal proteins *etc.* are coded by nuclear genes and imported from the cytosol.

The mitochondrial genetic information is very densely packed. There are no introns in mitochondrial genes and no gaps between them. It is established that there are below 1000 copies of the mitochondrial genome in somatic cells. Thus, despite of its small size mitochondrial DNA (mtDNA) comprises about 0.5% of the overall. DNA mass.

Mitochondrial DNA-depleted mutants provide a unique tool for a direct study of the role of mtDNA and encoded proteins (Attardi and Schatz, 1988; Miranda *et al.*, 1999; Tolkovsky *et al.*, 2002; Shen *et al.*, 2003). It has been found by using mtDNA-less cell line that mtDNA and encoded proteins are critical for mitochondrial function (Holmuhamedov *et al.*, 2003).

The data have been accumulated to demonstrate that the spatial distribution of mitochondria in the cell structures are controlled by the components of the cytoskeleton (Ball and Singer, 1982; Summerhayes *et al.*, 1983; Stromer and Bendayan, 1990; Drubin *et al.*, 1993). It is known, that microtubules (MTs)

serve as a pathway for intracellular organelle movements including mitochondria (Heggenes *et al.*, 1978; Nangaku *et al.*, 1994) and endoplasmatic reticulum (Bereiter-Hahn, 1990; Terasaki and Reese, 1994). When microtubules (MTs) are destabilized, mitochondria lose their normal spatial distribution in the cell and cluster around the nucleus (Wakabayashi and Spodnik, 2000). If the contact between mitochondria and endoplasmic reticulum is lost as a result of depolymerization of MTs, transport of materials from endoplasmic reticulum to mitochondria may be disturbed resulting in the disturbance of mitochondrial biogenesis (Spodnik *et al.*, 2002).

Genetic control of mitochondrial biogenesis requires the coordinated expression of many genes in both the nucleus and mitochondria, regulated by transcription factors (NRF-1, NRF-2) *etc.*

Two classes of nuclear transcriptional regulators implicated in mitochondrial biogenesis have emerged in recent years. The first includes DNA-binding transcription factors (NRF-1, NRF-2 *etc.*). The second, more recently defined group includes nuclear coactivators classified by PGC-1 and related family members (PRC and PGC-1β). PGC is coactivator of PPAR (peroxisome proliferator-activated receptor); PRC is PGC-1-related coactivator. These molecules do not bind DNA but can interact with DNA-bound transcription factors to activate or repress transcription and thereby regulate gene expression.

NRF-1 and NRF-2 recognition sites in nuclear genes are required for respiratory chain expression and function. It has been shown that NRF-1 is essential for rat and human cytochrome c expression and for human ATP synthase c subunit (Scarpulla, 1997) whereas NRF-2 is required for human ATP synthetase β-subunit (Virbasius and Scarpulla, 1991). Also, the regulatory role of NRF-1 to complex II of the respiratory chain has been proposed. One of the four subunits of complex II of the mitochondrial electron transport chain, the iron-sulfur subunit of succinate dehydrogenase is transcriptionally regulated by the nuclear respiratory factors NRF-1 and NRF-2 (Au and Scheffler, 1998).

An important feature of the second group of regulators, nuclear coactivators, is that they are responsive to physiological signals mediating thermogenesis, cell proliferation and gluconeogenesis. Mitochondrial proliferation occurs in muscle in response to chronic hypermetabolic conditions, such as endurance training, electrical stimulation and hyperthyroidism (Hood *et al.*, 1994; Wiesner, 1997).

Hypoxia exerts a reciprocal control on transcription of glycolytic (increase) and mitochondrial (decrease) enzymes (Webster *et al.*, 1990). This observation is considered to be mediated by the hypoxia inducible transcription factor (HIF) (Leary *et al.*, 1998).

There are differences between the proteins present in mitochondria from different tissues, reflecting the tissue specific patterns of nuclear gene expression. Protein turnover, however, seems to be rather slow and mitochondrial

protein composition does not respond very quickly to dietary or hormonal stimuli. The total number of mitochondria per cell can be changed (for example, through muscle activity) over the course of several weeks (Leary *et al.*, 1998). Turnover of mtDNA observed in adult female rats is 1.1–1.3% new mtDNA per day in rat cardiac and skeletal muscle (Collins *et al.*, 2003).

Massive amplification of mtDNA occurs during oogenesis (Michaels *et al.*, 1982). At birth, the production of mitochondria and respiratory enzymes is induced through both transcriptional and post-transcriptional mechanisms as the neonate adapts to extrauterine life, as shown in mouse (Piko and Taylor, 1987; Piko and Matsumuto, 1976).

Postnatal maturation of rat heart is associated with increase in tissue content of mitochondria related to increase of their size and number in the cell. Mitochondrial concentration of respiratory chain components also increase (Hallmann *et al.*, 1966; Schiebler and Wolf, 1966; Kinnula and Hassinen, 1977; Hirakow et al., 1980; Glatz and Veerkamp, 1982).

6. The cytoskeleton

The term of cytoskeleton is often considered synonymous with the three filaments most frequently imaged by fluorescence microscopy (microfilaments of actin, microtubules (MT) and intermediate filaments (IFs) (Cleveland and Mooseker, 1994; Penman, 1995). Thus, cytoskeleton is a complex network of filaments and tubules, which transmit mechanical and chemical stimuli within and between the cells (Choquet *et al.*, 1997; Wang, 1994). It contributes substantially to cell stability by anchoring subcellular structures, such as mitochondria, Golgi apparatus, nuclei, and myofibrils. The action of the cytoskeleton represents a stabilizing and mechanotransducing function that is supported by membrane-associated proteins, especially dystrophin that binds to both, intracellular actin and extracellular laminin (Klietsch *et al.*, 1993). A close integrin-cytoskeleton linkage system allows cells to respond to physical and biochemical influences exerted by the extracellular matrix. The extracellular matrix controls cytoskeletal mechanics and structure, particularly by binding of fibronectin to integrins (Choquet *et al.*, 1997; Wang, 1994). In case when the matrix resists movement, the linkage to the cytoskeleton is strengthened via an increased number of integrins (Choquet *et al.*, 1997). At the intercalated disk, the cytoskeleton is anchored to sites of cadherin-mediated adhesion between adjacent plasma membranes via catenins and desmoplakins (Koch and Franke, 1994).

6.1. Cytoskeletal proteins

The cytoskeleton of myocytes consists of actin, the IF desmin and of α- and β-tubulin that form the microtubules by polymerization (Hein *et al.*, 2000). Vinculin, talin, dystrophin, spectrin and ankyrin represent a separate group of membrane-associated proteins (Kost in *et al.*, 1998).

The proteins which contribute to cell shape, mechanical resistance, and morphological integrity can be subdivided on the basis of their structural and functional properties in four different groups (Kostin, 1998). The division of cytoskeletal proteins in cardiomyocytes is shown in Table 2.

The MTs are essentially composed of tubulin and their arrangement and function are regulated by microtubule-associated proteins (MAPS), the major classes of which are the microtubule associated motor-proteins (kinesin, dynein *etc.)* and the structurally associated proteins (tau, MAP 1, MAP 2), which modulate tubule stability and spatial arrangement (Rappaport *et al.*, 1998). The major component of microfilaments is actin. The third cytoskeletal structure consists of IFs. Main constituents of IFs are desmin and vimentin. Desmin is found to be essential for myofibrillar functional integrity and the maintenance of general cellular integrity, e.g. for the position of the nucleus (Capetanaki *et al.*, 1997). In mice with desmin null mutations, degeneration of cardiac muscle was observed, indicating the essential role of desmin for cell survival and sarcomerogenesis (Milner *et al.*, 1996; Thornell *et al.*, 1997).

Table 2. The division of cytoskeletal proteins in cardiac cell

True cytoskeletal proteins	Membrane-associated proteins	Sarcomeric skeleton	Proteins of intercalated disks	
			Desmosomes	Adherens and gap junctions
Tubulin	Dystrophin	Titin	Desmoplacin	N-cadherin
Desmin	Spectrin	Alpha-actinin	Desmocollin	Catenins
Actin	Talin	Myomesin	Desmoglein	Vinculin
	Vinculin	C-protein	Desmin	Connexin
	Plectin	M-protein	Plectin	Plectin
	Ankyrin			

Dystrophin is the protein that is encoded by the largest gene (6.3 kb) identified so far. The mutations in the dystrophin gene cause Duchenne muscular dystrophy (DMD) — a disorder of the skeletal muscle in human. The MDX mouse is a spontaneous mutant and an animal model for DMD. It has a point mutation in exon 23 of the dystrophin gene at position 21 on the long arm of X chromosome (Xp21) that eliminates the expression of dystrophin (Araki *et al.*, 1997).

6.2. The association of mitochondria with the cytoskeleton

In striated muscle, the desmin serves as a bridge between neighbouring Z-disks linking them together and integrating the contractile apparatus with the sarcolemma and the membrane of nuclei and mitochondria (Tokuyasu, 1983a, 1983b; Rappaport *et al.*, 1998). It is not completely clear whether desmin interacts directly with mitochondria via OM or is the interaction mediated by an IF-associated protein that interacts with mitochondria, as in the case of microtubules. Leterrier *et al.*, (1994) have shown that in case of brain mitochondria microtubule-associated proteins bind *in vitro* to specific sites of the OM and there exist cross bridging between mitochondria and the MT (Leterrier *et al.*, 1994). It has been found that MAP 2 interact with voltage-dependent anion channel (VDAC) in mitochondrial outer membrane (OM) (Linden *et al.*, 1989). Plectin, an IF linker protein, that in muscles colocalizes with desmin at Z-disks (Wiche *et al.*, 1983), localizes close to mitochondrial OM (Reipert *et al.*, 1999) and has been suggested to be the candidate for interaction between VDAC and IF-associated proteins (Capetanaki, 2002). It has been proposed that mitochondria-cytoskeleton interactions may modulate functional properties of mitochondria (Rappaport *et al.*, 1998). IF can influence mitochondrial function by changing mitochondrial shape by direct or indirect interactions with mitochondrial membrane proteins (Rappaport *et al.*, 1998). It has also been postulated that the cytoskeleton plays a role in the affinity of mitochondria for ADP *in vivo* (Saks *et al.*, 1998). Several studies have shown that the absence of desmin influences the coupling of CK and ANT as well as mitochondrial oxygen consumption and affinity for ADP in oxidative muscles, such as myocardium and *m. soleus*, Respiratory parameters for glycolytic *m. gastrocnemius* in case of desmin-defficiency were unaffected. Thus it appears that desmin cytoskeleton influences mitochondrial function *in situ* in mitochondria-rich muscle types (Kay *et al.*, 1997; Milner *et al.*, 2000).

It is concluded by Rappaport (1998) that cytoskeleton mediates mitochondrial movement and positioning and cytoskeletal network may participate in mitochondrial activity via outer membrane and cytoskeleton associated proteins (Rappaport *et al.*, 1998).

6.3. Developmental expression of cytoskeleton

All developing muscle cells, regardless of their type, simultaneously express IF desmin, vimentin, paranemin, and synemin. However, a difference is observed in the expression of paranemin in adult muscle. This protein is removed during differentiation of both fast-twitch and slow-twitch skeletal muscle but remains in mature myocardial cells (Price and Lazarides, 1983). On the other hand, synemin is expressed in all types of adult muscle cells mentioned above except for myocardial cells. Adult myocardial cells also lack vimentin, as its

presence is gradually reduced after hatching as shown in chicken development (Price and Lazarides, 1983). Since in adult striated muscle all expressed IF proteins are found predominantly in association with the peripheries of myofibrillar Z-disks, these results suggest that a change in the composition of skeletal and cardiac muscle Z-disks occurs during organism development and maturation (Price and Lazarides, 1983). The study of Oblinger and Kost (1994) documented the patterns of mRNA expression for five different tubulin genes and four of the MAP genes during development of hamster forebrain. The results revealed coordinated regulation of tubulin and MAP genes during development (Oblinger and Kost, 1994). It has been reported that five mouse tubulin genes, two (M alpha 1 and M alpha 2) that encode alpha-tubulin and three (M beta 2, M beta 4, and M beta 5) that encode beta-tubulin show regulated expression during development (Lewis *et al.*, 1985). The expression of M beta 4 increased dramatically during postnatal development (Lewis *et al.*, 1985). This finding coincides with the formation of mitochondrial arrays between the myofibrils in rat heart during postnatal life (Schiebler and Wolff, 1966; Tiivel *et al.*, 2001). Transcripts from all four other tubulin genes declined from maximum levels at or before birth (Lewis *et al.*, 1985). It has been concluded that each of the beta tubulin genes is functional, which is expressed according to the specific program during differentiation and are necessary for proper microtubule function in vertebrates (Lopata *et al.*, 1983).

7. Unsolved problems

At present it is not clear which proteins may participate in organization of the ICEUs and thereby exert control over ADP-activated respiration (oxidative phosphorylation). It is known that cytoskeletal proteins such as desmin colocalize with the cytolinker protein plectin, which, in turn, binds to mitochondria (Reipert *et al.*, 1999). The mitochondrial porin channel is known to form complexes with microtubule-associated proteins as well (Leterrier *et al.*, 1994). On the other hand, the gene deficient mice lacking desmin exhibit disturbed organization of the sarcomere structure together with the increased affinity of mitochondria to ADP (Kay *et al.*, 1997). Interestingly, measurements of desmin, α-actinin, and actin contents in *m. soleus* and EDL, of rat skeletal muscles and dystrophin-glycoprotein complex (DGC) components, indicate that the contents of most cytoskeletal proteins are higher in slow fibers than in fast-twitch ones (Chopard *et al.*, 2001). These results point to the possible role of the cytoskeletal proteins in regulation of mitochondrial function in oxidative muscles.

To identify the proteins participating in ICEUs several approaches can be utilized. Obviously, the first option would be isolation of the genes characteristic of the slow-twitch oxidative muscles, by the suppression subtractive

hybridization (SSH) for the cDNA libraries from oxidative and glycolytic muscles. The analogous studies with oligonucleotide arrays have recently revealed nine additional mRNAs representing transcription factors that were previously not known expressed in a fiber type manner. These newly identified factors/coregulators may be candidates for transcriptional regulation of the specificity of the metabolic and contractile characteristics of fiber types differentially expressed in *m. gastrocnemius* compared to *m. soleus* in mice (Campbell *et al.*, 2001).

Traditional subtractive hybridization technique *hitherto* applied is rather costly and time consuming. Therefore in this study methodically more optimal solution to the problem was used. It is hypothesized that if the ICEUs exist in oxidative muscle cells, the respective genes should be expressed in both myocardium and *m. soleus*, the typical representatives of oxidative muscles. Hence it would be reasonable to identify the genes commonly expressed in these muscles.

The second approach would base on the hypothesis that if the ICEUs do exist, the autoantibodies against this structure should be generated. Indeed, the autoantibodies have been useful tools for identification of novel proteins in cell biology (Tan, 1989; Machado, 1998). In PBC the antibodies are directed against (i) mitochondrial autoantigens, such as the E2 component (dihydrolipoamide acetyl transferase) of the pyruvate dehydrogenase (PDH), 2-oxoglutarate dehydrogenase, branched chain 2-oxo-acid dehydrogenase complex (Neuberger, 1997), ANT (Schultheiss, 1983), sulfit oxidase (Klein and Berg, 1991) trypsin sensitive M8-antigen associated with the mitochondrial outer membrane (Berg and Klein, 1989), (ii) the nuclear envelope proteins (Neuberger, 1997; Fritzler and Manns, 2002; Invernizzi *et al.*, 2001), and (iii) cytoskeletal proteins (actin, myosin, desmin, tropomyosin, α-actinin, filamin, and vimentin (Dighiero, *et al.*, 1990; Girard and Senécal, 1995). The same cytoskeletal proteins are autoantigenic targets also in chronic hepatitis (CH) patients, including autoimmune hepatitis (AIH) (Dighiero, *et al.*, 1990; Girard and Senécal, 1995; Toh, 1991). AIH is also characterized by antibodies to nuclear components and liver specific antigens (Wies, *et al.*, 2000). Thus, both of diseases, PBC and CH, share a common feature — presence of autoantibodies to different components of intracellular cytoskeletal system. These autoantibodies are considered to be non-species-specific and thus are applicable to search for their effects on mitochondrial function and localization in the permeabilized fibers of myocardium and skeletal muscles.

The third strategy is based on assumption that mechanism responsible for regulation of mitochondrial function must accompany the biogenesis of mitochondria both in time scale of the processes and in structural terms. Therefore, it would be important to assess mitochondrial function in a course of the development of different types of muscles.

AIMS OF THE STUDY

In the present study regulation of mitochondrial function in oxidative muscle fibers *in vivo* was addressed, in relation to glycolytic muscles, postnatal development, tissue-specific gene expression and disease.

In detail, the aims were:

1. To investigate the mechanisms of regulation of respiration in muscle fibers of different types in adult rat and mouse and in rat heart during the period of postnatal development.

2. To study the effect of immunoglobulins G from the sera of healthy persons and patients with liver diseases (primary biliary cirrhosis, chronic hepatitis) on ADP-activated respiration in skinned fibers from rat oxidative (myocardium, *m. soleus*) and glycolytic (*m. gastrocnemius*) muscles.

3. To construct the mouse cDNA library characteristic of the oxidative muscles (myocardium and *m. soleus*), by subtracting cDNAs expressed in glycolytic *m. extensor digitorum longus*.

MATERIALS AND METHODS

1. Animals

- *Rat model.* Outbred adult and 1–24 days old Wistar rats of either sex were used. The adult rats were anaesthetized by sodium pentobarbital (5 mg/100g BW, intraperitoneal) (Flecknell, P. A., 1987) or by ethyl ether. 1–24 days old rats were were anaesthetized by ethyl ether.
- *Mice model.* The 10–11month-old dystrophin and desmin knockout homozygous female mice were produced by Prof. H. Jockusch's group (University of Bielefeld) and control wild-type mice of same sex and age from the same source were used. The mice were anaesthetized by ethyl ether.

The animals were kept, fed and studied in accordance to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85–23, revised 1996).

2. Reagents

All the oligonucleotides and adapters used (Table 3) were ordered from Genset (Paris, France), except those produced by BD Biosciences Clontech (Palo Alto, CA, USA). ADP and ATP were obtained from Boehringer. All other reagents were purchased from Sigma.

3. Solutions

- Modified *Krebs-Henseleit solution* contained (mM) 118 NaCl, 4.7 KCl, 2.52 $CaCl_2$, 1.64 $MgSO_4$, 24.88 $NaHCO_3$, 1.18 KH_2PO_4, 5.55 glucose, 2 K-pyruvate, pH 7.4.
- *Solution A* contained (mM): 2.77 CaK_2EGTA, 7.23 K_2EGTA (concentration of free Ca^{2+} = 0.1 μmol/L), 49 potassium 2-(N-morpholino)-ethanesulfonate (MES), 20 imidazole, 20 taurine, 0.5 DTT, 5.25 ATP, 15 PCr, 9.5 $MgCl_2$ pH 7.0, adjusted by KOH.
- *Solution B* consisted (mM): 2.77 CaK_2EGTA, 7.23 K_2EGTA (concentration of free Ca^{2+} = 0.1 μmol/L), 20 imidazole, 3 KH_2PO_4, 5 K_2-glutamate, 2 K_2-malate, 0.5 DTT, 20 taurin, 4 $MgCl_2$, 100 MES, 5 mg/ml BSA, pH 7.0 titrated by KOH
- *1xTBE buffer* was used for nucleic acid electrophoresis, diluted from 5*xTBE* buffer containing 0.445 M Tris-Borate and 10 mM EDTA. Solid original for preparation of 5xTBE was obtained from NAXO Ltd.

- *10x PBS* consisted (M): 1.37 NaCl, 0.03 KCl, 0.09 $Na_2HPO_4 \cdot H_2O$

The solutions were prepared on the basis of deionized water (Milli-Q filters, Millipore Corp., USA.

Use of kits is specified in the text.

4. Methods

4.1. Preparation of skinned muscle fibers

Skinned fibers were prepared according to the method described earlier (Veksler *et al.*, 1987, 1995). The hearts and skeletal muscles (*m. soleus*, oxidative, slow-twitch) and white parts of *m. gastrocnemius* (glycolytic, fast-twitch) were quickly excised, rinsed in modified Krebs-Henseleit solution and put into solution A. Both of the muscles were kept ice-cold solution A, the hearts were cut into halves and muscle strips (3–4 mm long and 1–1.5 mm in diameter) were excised from endocardial side of left ventricles along fiber orientation to avoid mechanical damage to the cells. From the skeletal muscles the fiber bundles (4–5 mm long, about 1 mm in diameter) were taken. By using sharp-ended forceps or needles, the muscle fibers were separated from each other leaving only small areas of contact. After that the fibers were transferred into vessels with cooled (in ice) solution A containing 50 μg of saponin per ml and incubated at mild stirring for 30 min for complete solubilization of the sarcolemma. Permeabilized (skinned) fibers were then washed in solution B for 10 min; this washing procedure was repeated two more times to remove all metabolites, especially trace amounts of ADP completely. Removal of ADP can be seen easily from respiration recordings, which should show very reproducible basal State 2 rates (designated as V_o) not sensitive to inhibition by atractyloside (ATR) (see below).

4.2. Preparation of "ghost" fibers

Skinned fiber prepared as indicated above were incubated in solution containing 20 mM taurin, 0.5 mM dithithreitol, 10 mM $MgCl_2$, 10 mM ATP, 800 mM KCl, 50 mM Hepes, pH 7.1 adjusted with KOH for 30 min at 4 °C to extract myosin from the muscle cells. After that the fibers ("ghost" fibers) were washed 5 times in solution B.

4.3. Estimation of intactness of mitochondrial membranes

Cytocrome c test was used for estimation of the intactness of the outer membrane of mitochondria. The test was carried out in the oxygraphic medium in the presence of 125 mM KCl, which leads to dissociation of the endogenous cytochrome c from the membrane (Stoner and Sirak, 1969). To assess the status of the outer mitochondrial membrane the respiration of muscle fibers was maximally activated by 1 mM ADP followed by addition of 8 μM cytochrome c. In case of ruptured outer membrane, exogenous cytochrome c can pass into the intermembrane space and stimulate the respiration by compensating for the loss of that cytochrome through leaky membrane. This effect of cytochrome c is absent in conditions of intact outer membrane.

The intactness of inner mitochondrial membrane was assessed by addition of 35 μM ATR, inhibitor of ANT, into the oxygraphic medium. In intact mitochondria atractyloside reduces the ADP-stimulated respiration down to the basal levels (V_0), which indicates effective control of oxidative phosphorylation by ANT, whereas such a control is lost in case of impaired inner membrane.

4.4. Determination of the rates of mitochondrial respiration

The rates of oxygen uptake were recorded by means of the digital oxygen monitoring system (Rank Brothers, England, or Oroboros, Paar, Graz, Austria) equipped with Clark electrode. Determinations were carried out in a solution B at 25°C and the solubility of oxygen was taken as 430 ng-atoms per ml (Kuznetsov *et al.*, 1996). The amount of fibers used in experiments varied from 0.7–2.4 mg in 3 ml of reaction medium B. ADP for stimulation of respiration was added cumulatively and the maximum rates of ADP-activated respiration (V_{max}) and the Michaelis's constant (K_m) values for ADP were calculated on the basis of Michaelis-Menten equation. The respiratory control index (RCI) was calculated as a ratio of $(V_0 + V_{max})/V_0$ where V_0 and V_{max} are the respiration rate before addition of ADP and the maximal respiration rate at saturating ADP concentrations, respectively. After measurements, the skinned muscle preparations were removed from the chamber, blotted and dried overnight at 105°C for determination of dry tissue weight (DW). The rates of oxygen consumption were expressed in ng-atoms oxygen normalized per milligram of DW if not specified otherwise.

4.5. Analysis of IgG effects on mitochondrial respiration

The fibers from rat heart were skinned and incubated in solution containing 125 mM KCl, 20 mM Hepes, 4 mM glutamate, 2 mM malate, 3 mM Mg-acetate, 5

mM KH_2PO_4, 0.4 mM EGTA, 0.3 mM dithiothreitol and 2 mg/ml BSA, in the absence or presence of immunoglobulins G (IgG) (100 μg/ml) and 0.2 mM ADP at 25°C in a chamber (volume 3 ml) of oxygraph (Rank Brothers, UK). The rates of oxygen consumption by mitochondria in the skinned fibers were monitored by Clark electrode, assuming the solubility of oxygen in the medium to be 430 ng-atoms O/ml. The ratio between the rates of respiration with 1 mM ADP and before addition of ADP (basal respiration, V_0) was taken as the respiratory control index (RCI), to monitor coupling between oxidation and phosphorylation. The skinned fibers with RCI of 4–6 were used in the experiments to monitor the effects of IgG. After measurements the fibers were removed from the chamber and dried overnight at 105°C.

4.6. Imaging of mitochondria and IgG localization

To stain mitochondria, the muscle fibers were incubated in solution A (see above) containing 0.2 μM mitochondrion selective dye MitoTracker Red CMXRos (Molecular probes, Inc., Oregon, USA) with continuos stirring in the dark during 30 min. Thereafter the fibers were washed 3 times in solution A (without dye) to reduce the background fluorescence. Then the stained fibers were divided into three portions, and each of them was incubated in the standard oxygraphic medium, in conditions of oxidative phosphorylation registered by oxygraph (see above), in the presence of IgG isolated from either patient with PBC, CH or from a healthy control (HC) during 20 minutes with continuous stirring in the dark. After that the fibers were washed two times in solution A for 10 min in the dark, to remove the unbound immunoglobulins and incubated with FITC-conjugated rabbit immunoglobulins to human IgG (DAKO, Clostrup, Denmark) diluted 1:20 in solution A for 30 min. This was followed by washing the fibers twice in PBS for 10 min and the probes were fixed with 10% Histoprep (Fischer Scientific, Pittsburgh, PA, USA), placed on the specimen glass, attached with glycerol-PBS (1:1) drop and protected by coverslip. All these procedures were carried out at room temperature (20°C). The skinned fibers from the heart of a desmin knockout mouse were processed similarly, except that incubation with IgG was performed without parallel registration of the respiration rate. The specimens were imaged and scanned by MRC 1024 BioRad laser confocal microscope.

4.7. Determination of the tissue content of cytochrome aa_3

Tissue homogenates were prepared as described by Nishiki *et al.*, (1978). The tissue content of cytochrome aa_3 was assayed by registering the difference spectra of reduced and oxidized cytochrome in cardiac homogenates according to Fuller *et al.* (1985), using a Perkin-Elmer Lambda 900 spectrophotometer.

4.8. Assesment of creatine kinase isoenzyme profile and total activity

CK isoenzyme profile was assayed electrophoretically according to Vannier *et al.* (1996) and the total CK activity was estimated in tissue homogenates according to a method described elsewhere (Khuchua *et al.*, 1989).

4.9. Total RNA preparation

The total RNA was isolated from the ventricular myocardium, *m. soleus* and *m. extensor digitorum longus* of the mouse using total RNA isolating kit (Macherey-Nagel, Düren, Germany). All procedures were performed according to the manufacturer's protocol. The isolated RNA was ethanol-precipitated and resuspended in water in a final concentration of 10 ng/μl.

4.10. Total cDNA synthesis

The total RNA was reverse-transcribed to obtain the total cDNA using SMART cDNA synthesis protocol (Chenchik *et al.*, 1989). The resulting total cDNA was purified using NucleoSpin Extract Columns (Macherey-Nagel, Düren, Germany), ethanol precipitated and resuspended in water with concentration of 50 ng /μl.

4.11. Subtractive hybridization, PCR amplification and removal of the nested primers from the ends of cDNA fragments of HE population

Subtractive hybridization is used to isolate differentially expressed mRNAs and generate subtracted libraries (Diatchenko *et al.*, 1996). The DNA in which target sequences are to be found, is called "tester", and the reference DNA is called "driver". First, both DNA samples are digested with a restriction enzyme that generates blunt ends. The tester DNA is then divided into two portions, each of which is ligated to unique adapters that facilitate suppression polymerase chain reaction (PCR). The round of subtractive hybridization in the PCR-select technique includes two hybridization steps. In the first hybridization step, an excess of driver DNA is added to each population of adaptor-ligated tester. The samples are heat-denaturated and allowed to anneal. After several hours, due to the second order kinetics of hybridization, ss molecules corresponding to high and low abundance sequences become equalized. During the second hybridization step, the two primary hybridization samples are mixed together. Since the samples are not heat-

denatured, only the remaining subtracted ss tester DNA molecules are able to associate and form new hybrid. These new hybrid molecules are ds tester DNA molecules with different adaptor sequences at their ends.

a) Tester preparation. Here the first step was the restriction cleavage of total ds-cDNA obtained from the myocardium (H). Thus, 8 µl of respective total cDNA (50 ng/µl), 1 µl of Rsa I restriction enzyme (10 U/µl, New England Biolab, Beverly, MA, USA or Fermentas, Vilnius, Lithuania) and 1 µl of respective restriction buffer (from the enzyme supplier) were mixed and incubated at 37°C for 1 hour. Then the mixture was purified using NucleoSpin Extract Columns (Macherey-Nagel, Düren, Germany), cDNA fragments were ethanol-precipitated and resuspended in water with concentration of 50 ng/µl. As the second step, the adapters were added to two separate reaction mediums, both containing 2 µl of digested cDNA (50 ng/µl),), 1 µl T4 DNA ligase (New England Biolab, Beverly, MA, USA or Fermentas, Vilnius, Lithuania), 1 µl of T4 Ligase Buffer (respective supplier) and 4 µl water, but in the presence of 2 µl of adapter 1 (10 µM) in one and adapter 2R (10 µM) (Table 3) in another reaction medium. The reactions were incubated overnight at 16°C. The enzyme and excess of adapters were removed by using NucleoSpin Extract Columns (Macherey-Nagel, Düren, Germany), the tester cDNAs were ethanol-precipitated and resuspended in water with concentration of 10 ng/µl. Thus, two different tester preparations from the same cDNA fragment, one with adapter 1 and another with adapter 2R at the end of DNA, were obtained.

b) Driver preparation. The driver was prepared from the cDNA of *m. extensor digitorum longus* similarly to the tester's preparation (step (a) above), except that the resulting driver fragments were resuspended in water with concentration of 200 ng /µl and that the adapters were not ligated to the ends of cDNA.

c) Subtractive hybridization. 1.5 µl of driver dsDNA (300 ng) was added to a pair of Eppendorf tubes containing 1 µl of hybridization buffer (200 mM Hepes pH 8.3/2 M NaCl/0.1 mM EDTA, pH 8.0, 10%(wt/vol) PEG 8000) and adapter 1-ligated tester cDNA (15 ng) in one tube and adapter 2R-ligated tester cDNA (15 ng) in another, mixed, overlaid with mineral oil, denatured (5 min, 95°C) and hybridized for 10 hours at 68°C. Then these two samples were pooled and mixed, the freshly denatured (5 min, 95°C) driver dsDNA (200 ng) in 2 µl of hybridization buffer was added and the reaction (second hybridization) was allowed to proceed for 10 hours at 68°C. Thereafter the final hybridization mix was diluted for 10-fold in TE and stored at –20°C.

d) PCR amplification. The first step was accomplished in a 25 µl reaction mix containing 1 µl of diluted, subtracted cDNA, 1 µl of PCR Primer 1 (10 µM) and 23 µl of PCR master mixture prepared using Advantage 2 Polymerase Mix (BD Biosciences Clontech, Palo Alto, CA, USA), according to the instructions of the manufacturer. The PCR was performed with the following

parameters: initial incubation at 75°C for 5 min to fill the adapters, 27 cycles (at 94°C for 30 sec; 66°C for 30 sec and 72°C for 1.5 min) and the final extension at 68°C for 5 min. Then the amplified products were diluted 10-fold in the water and PCR-amplified in the next step, in 25 µl of the reaction mix containing 1 µl of diluted amplified cDNA, 1 µl of nested PCR primer 1 (10 µM), 1 µl of nested PCR primer 2R (10 µM) (Table 3) and 22 µl of PCR master mixture prepared using Advantage 2 Polymerase Mix (BD Biosciences Clontech, Palo Alto, CA,USA), according to the instructions of the manufacturer. The PCR amplification was performed within the 12 cycles (at 94°C for 30 sec; 68°C for 30 sec; 72°C for 1.5 min), with the final extension at 68°C for 5 min. The PCR products were analyzed on an agarose/SYBRGreen (Molecular Probes, Eugene, OR, USA) gel electrophoresis. Then the mixture was purified using NucleoSpin Extract Columns (Macherey-Nagel, Düren, Germany), cDNA fragments were ethanol-precipitated and resuspended in water with concentration of 50 ng /µl. The procedures characterized in (c) and (d) resulted in HE population of cDNA.

e) *Removal of the nested primer's sequencies from the ends of the DNA fragments of HE population.* Because the DNA fragments of HE population of cDNA generated by subtractive hybridization had different primers (nested primer 1 or nested primer 2R, Table 3) sequencies on its both ends, it was necessary to remove those sequences prior to addition of adapters in a course of the KDA procedure. The process of removal was accomplished as follows: 8 µl of HE cDNA (50 ng/µl), 1 µl of Rsa I restriction enzyme (10 U/µl, New England Biolab, Beverly, MA, USA, or Fermentas, Vilnius, Lithuania) and 1 µl of respective restriction buffer (from the enzyme supplier) were mixed and incubated at 37°C for 1 hour. Then the mixture was purified using NucleoSpin Extract Columns (Macherey-Nagel, Düren, Germany), the cDNA fragments were ethanol-precipitated and resuspended in water with concentration of 50 ng/µl. The efficiency of removal of nested primer's 1 and 2R sequences from the ends of HE fragments was monitored by PCR-amplification in 25 µl of the reaction mixture containing 1 µl of diluted (10x) RsaI cleaved HE, 1 µl of nested PCR primer 1 (10 µM), 1 µl of nested PCR primer 2R (10 µM) and 22 µl of PCR master mixture, the latter being prepared from Advantage 2 Polymerase Mix Kit (BD Biosciences Clontech, Palo Alto, CA, USA), according to the the manufacturer's instructions. The PCR amplification was performed as follows: at 94°C for 5 min, 30 cycles (at 94°C for 30 sec; 68°C for 30 sec; 72°C for 1.5 min), and the final extension at 68°C for 5 min. When the PCR products were tested with the agarose/SYBRGreen (Molecular Probes, Eugene, OR, USA) gel electrophoresis, no visible PCR products were recorded, which was taken to indicate that the nested primers sequences were totally removed from HE.

4.12. Kindred DNA amplification

Phase 1 (Fig. 1): cDNA preparation. The total cDNA of *m. soleus* was restriction cleaved exactly as in case of cDNA of myocardium (H) described above (paragraph (a) tester preparation). Then the adaptors were added, i.e. adapter 1 to cDNA of HE (nested sequences removed) and adapter 2R to cDNA of *m. soleus* cleaved with RsaI, according to the method described in paragraph (a) (tester preparation). The enzyme and adapters in excess were removed by using NucleoSpin Extract Columns (Macherey-Nagel, Düren, Germany), the DNAs were ethanol-precipitated and resuspended in water with concentration of 10 ng/µl. Thus, by completing this step, two different DNA preparations were obtained, one (*m. soleus*) with the adapter 1 and another (HE) with the adapter 2R at the end of its DNA fragments.

Phase 2: Hybridization of S with HE. The hybridization solution was generated by mixing 1.5 µl of adapter-ligated analyzable cDNA populations (S and HE, each 15 ng) with 1 µl of 4x hybridization buffer, prepared according to the PCR-Select cDNA Subtraction Kit instructions (BD Biosciences Clontech, Palo Aalto, CA, USA). The solution was overlaid with mineral oil, denatured (5 min, 95°C) and allowed to hybridize overnight (for 8 hours at 68°C). After that the final hybridization was diluted 10 times in TE and stored at –20°C.

Phase 3: The filling of the end of adapters and PCR amplification. The filling of adapters takes place in the process of initial incubation needed to prepare the template for PCR primers. For kindred DNA amplification (KDA) the two-step procedure of PCR-amplification was performed exactly according to that in PCR amplification in step (d) above. The PCR products obtained and considered to be the fraction SHE containing the cDNA fragments common to the myocardium (H) and *m. soleus* (S) were purified using the NucleoSpin Extract Columns (Macherey-Nagel, Düren, Germany), eluated in 50 µl of water and analyzed with the agarose/SYBRGreen (Molecular Probes, Eugene, OR, USA) gel electrophoresis.

Table 3. The oligonucleotides and adapters used in the study.

Primer name	Primer Sequence
SMART	5'-AAGCAGTGGTAACAACGCAGAGTACGCGGG-3'
SMART CDS Primer	5'-AAGCAGTGGTAACAACGCAGAGTACTTTTTTTT TTTTTTTTTTTTTTTTTTTT(A,C,G)(A,C,G,T)-3'
5′ PCR Primer	5'-AAGCAGTGGTAACAACGCAGAGT-3'
Adapter 1 Long Oligo	5'-CTAATACGACTCACTATAGGGCTCGAGCGG CCGCCCGGGCAGGT-3'
Adapter 1 Short Oligo	3'-GGCCCGTCCA-5'
Adapter 2R Long Oligo	5'-CTAATACGACTCACTATAGGGCAGCGTGGT CGCGGCCGAGGT-3'
Adapter 2R Short Oligo	3'-GCCGGCGCCA-5'
PCR Primer 1	5'-CTAATACGACTCACTATAGGGC-3'
Nested PCR primer 1	5'-TCGAGCGGCCGCCCGGGCAGGT-3'
Nested PCR primer 2R	5'-AGCGTGGTCGCGGCCGAGGT-3'

4.13. Cloning of the cDNAs produced by kindred DNA amplification

The KDA products were cloned, by using pTZ57R/T plasmid vector within the InsT/aclone PCR Product Cloning Kit, according to the manufacturer's protocol (Fermentas). The host strain used was JM109.

4.14. Preparation of DNA for Southern hybridization

The cloned DNA inserts were PCR amplified directly from the bacterial colonies, by transferring some bacterial cells of given colony into the 25 µl of the reaction mix containing 1 µl of nested PCR primer 1 (10 µM), 1 µl of nested PCR primer 2R (10 µM) (Table 2) and 23 µl of PCR master mixture prepared using Advantage 2 Polymerase Mix Kit (BD Biosciences Clontech, Palo Alto, CA, USA), as instructed by the manufacturer. The PCR amplification was performed within 30 cycles (at 94°C for 30 sec; 68°C for 30 sec; 72°C for 1.5 min) and the PCR products were purified using NucleoSpin Extract Columns (Macherey-Nagel, Düren, Germany) in eluation volume of

50 µl and analyzed on agarose/SYBRGreen (Molecular Probes, Eugene, OR, USA) gel electrophoresis.

4.15. Dot blot hybridization

5 µl of amplified cDNA fragments were directly dotted onto a nylon filter. All the processes — DNA labelling, hybridization and detection were performed using DIG-High Prime DNA Labelling and Detection Starter Kit I (Roche, Mannhein, Germany), according to the manufacturer's instructions.

4.16. Statistical analysis

Substrate-velocity curves and Lineweaver-Burk plots were created by GraphPad Prism version 3.0 (Graphpad Software Inc.). The data are expressed as means ± SEM if not specified otherwise. Statistical analysis was performed by Student's paired t-test. Differences at $P<0.05$ were considered significantly different.

RESULTS

1. Striking differences between the kinetics of regulation of respiration by ADP in oxidative and glycolytic muscles

The kinetics of *in vivo* regulation of mitochondrial respiration by ADP was studied in rat and mouse heart and *m. soleus* (both oxidative). Additionally, three glycolytic muscles of rat (*m. plantaris*, *m. quadriceps, m. gastrocnemius*) and two of mouse (*m. gastrocnemius*, EDL) were studied to determine kinetical characteristics of ADP-activated respiration (oxidative phosphorylation) in skinned fibers. Mitochondrial respiratory parameters were determined in the absence and the presence of creatine (20 mM), and the effect of proteolytic enzyme trypsin (5 μM) on the affinity to ADP was investigated. While mitochondrial respiration in skinned fibers from heart and *m. soleus* increased very slowly with elevation of ADP concentration, skinned fibers from *m. gastrocnemius* displayed very high sensitivity to ADP (Figure 1). Based on these data, mainly after linearization the dependences by double reciprocal plot (Figure 2B), V_{max} and the apparent K_m for ADP were calculated to characterize the affinity of mitochondria for ADP (Figures 1, 2). The data indicate striking difference in the values of apparent K_m for ADP determined in the skinned fibers from rat and mouse oxidative and glycolytic muscles (Table 4). Clearly, the K_m values were about an order higher in oxidative muscles than in glycolytic muscles (Table 4), whereas in latter ones the K_m was similar to that in isolated mitochondria. Differences in V_{max} correspond approximately to the known differences in the cell volume occupied by mitochondria in these muscles (Eisenberg, 1982; Schaper *et al.*, 1985).

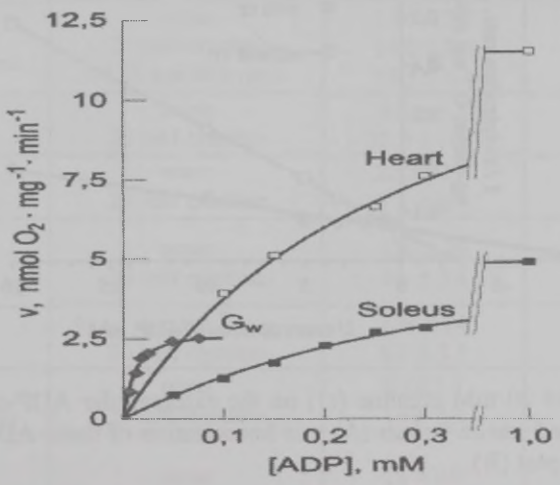

Figure 1. The dependence of the respiration rate in skinned muscle fibers from rat heart, *m. soleus* and *m. gastrocnemius* (G_w) on the extracellular ADP concentration.

In skinned fibers from rat (Figure 2A, Table 4) and mouse (Table 4) oxidative *m. soleus*, initiation of the mitochondria coupled CK reaction by addition of 20 mM creatine, which starts the production of ADP in the mitochondrial intermembrane space, significantly decreased the apparent K_m for externally added ADP (to about 70 μM, Figure 2). For cardiac fibers, the nature and effect of functional coupling between CK and ANT in the reaction of respiration has been described earlier (Veksler *et al.*, 1987; Saks *et al.*, 1993, 1994).

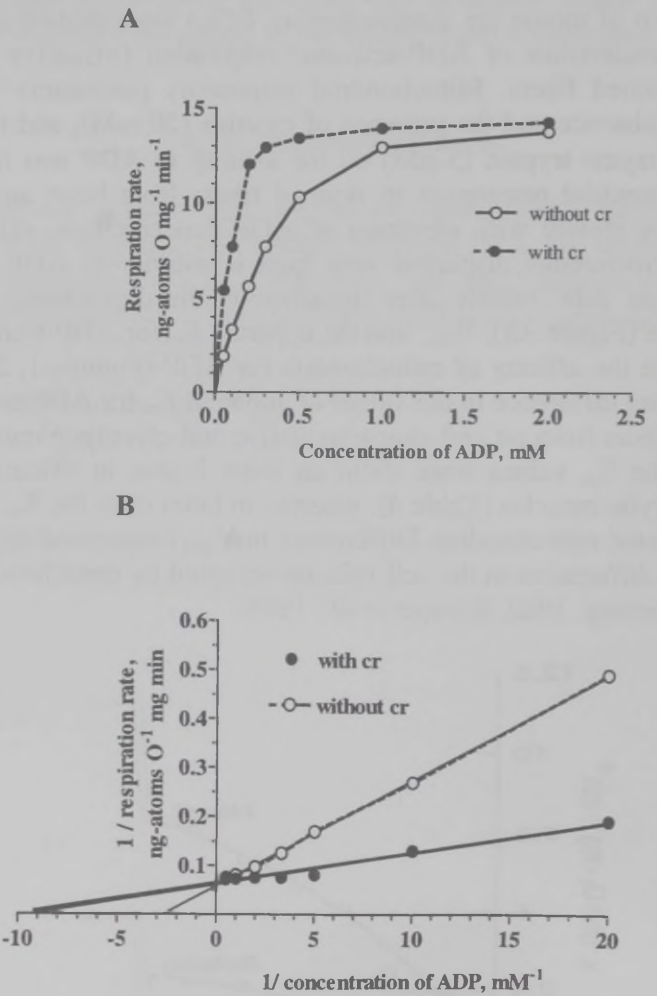

Figure 2. Effect of 20 mM creatine (cr) on the extracellular ADP-dependent respiration rates in skinned rat *m. soleus* (A) and linearization of these ADP-dependences in double-reciprocal plot (B).

The observation that in saponin-skinned muscle fibers from adult rat heart and *m. soleus* the apparent affinity of the mitochondrial oxidative phosphorylation system for ADP (K_m = 200–400 μM) (Table 4) is much lower than in isolated mitochondria (K_m = 10–20 μM) suggests a limited permeability of the OM to adenine nucleotides in oxidative muscle cells. It is possible, however, that high Km for ADP results from diffusion barrier due to large molecules such as myosin in the muscles cells. To control this, the myosin was removed from the muscle cells by KCl treatment. The "ghost" fibers obtained were analyzed. It was found that KCl treatment resulted in removal of 10–20% of cellular protein without any changes on the apparent K_m (Table 4). Thus, the diffusion through organized cell structures, such as myofibrils cannot be the reason for low affinity to ADP in oxidative muscle cells.

Table 4. K_m for ADP and V_{max} in different muscle fibers. The values of V_{max} about mouse are normalized per mg of wet weight of skinned tissue. Means ± SD are given for 5–12 experiments, n. d. — not determined.

Fibers	Treatment	K_m (ADP), μmoles	V_{max}, ng-at. O mg^{-1} min^{-1}
Rat ventricular myocardium	none	297 ± 35	28.7 ± 1.1
	20 mM creatine	85 ± 5.0	28.0 ± 4.0
	0.8 M KCl	351 ± 32	46.7 ± 1.5
	0.125 mg/ml trypsin	98 ± 8.0	26.0 ± 1.1
Rat *m. soleus*	none	354 ± 46	12.2 ± 0.5
	20 mM creatine	105 ± 15	16.0 ± 4.0
	0.8 M KCl	320 ± 22	17.0 ± 0.4
	0.125 mg/ml trypsin	59 ± 6.0	n. d.
Rat *m. gastrocnemius*	none	13.5 ± 2.7	6.2 ± 0.4
	20 mM creatine	14.0 ± 9.3	6.5 ± 2.6
	0.125 mg/ml trypsin	17.5 ± 3.4	n. d.
Rat *m. plantaris*	none	8.3 ± 5.4	7.8 ± 4.5
	20 mM creatine	20.0 ± 10.0	8.7 ± 2.3
Rat *m. quadriceps*	none	22.3 ± 1.4	8.2 ± 1.2
	20 mM creatine	3.5 ± 0.7	6.2 ± 0.5
Mouse ventricular myocardium	none	220 ± 26	12.1 ± 1.5
	20 mM creatine	44 ± 3.4	11.3 ± 3.3
Mouse *m. soleus*	none	324 ± 84	4.0 ± 0.9
	20 mM creatine	92 ± 3.1	2.7 ± 0.4
Mouse *m. gastrocnemius*	none	9.1± 1.7	1.5 ± 0.1
	20 mM creatine	14 ± 2.8	1.6 ± 0.2
Mouse *m. extensor digitorum longus*	none	16 ± 0.9	2.5 ± 0.2
	20 mM creatine	21 ± 3.1	3.2 ± 0.2

The kinetics of regulation of respiration by exogenous ADP in oxidative muscle fibers was changed significantly by the proteolytic treatment, as seen from the rapid decrease of apparent K_m (Table 4) for these fibers. In contrast, in skinned fibers from *m. gastrocnemius* trypsin treatment resulted in a transient increase of this constant (Table 4). Thus, the kinetics of regulation of respiration by ADP and the effect of the proteolytic enzyme are different in various types of muscles.

The cytochrome c test was used to investigate the state of the OM (Saks *et al.*, 1995) before and after trypsin treatment. The test indicated normal rates of respiration stimulated by a saturating concentration of ADP (not shown). Thus the OM was not destroyed by treatment of the skinned fibers with trypsin under these conditions. The result of this study and the dynamics of the proteolytic enzyme action described by Kuznetsov *et al.* (1996) indicate that there is probably a protein, or a protein complex, that is saturable with proteolytic enzymes and may control the permeability of porin pores in the OM of mitochondria.

2. Developmental changes in regulation of mitochondrial respiration by ADP and creatine in rat heart *in vivo*

The kinetics of *in vivo* regulation of mitochondrial oxygen consumption by ADP and creatine was also studied in rat heart during postnatal development.

Figure 3 shows that contrary to the developmental changes of mitochondrial affinity for ADP, the mi-CK activity assayed either directly in cardiac homogenates or by stimulation of respiration by 20 mM creatine in the presence of submaximal ADP concentrations (0.1 mM) (creatine index) shows delayed postnatal development. The activities are absent during the first week, rather low between the 2nd and the 3rd week of postnatal life and then rapidly increase and reach they high value characteristic of adult hearts within 40 days of development (Figure 3).

Parallel increase in mi-CK activity and creatine index shows that once expressed, mi-CK becomes functionally coupled to ANT. As a consequense, the ADP is produced in the intermembrane space of mitochondria by the activated mi-CK after the addition of creatine and this leads to the decrease of apparent K_m for ADP in the regulation of respiration (Seppet *et al.*, 1991; Saks *et al.*, 1995). Indeed, Figure 5 clearly shows corresponding dynamics in K_m values: due to the absence of mi-CK activities in the first days after birth (Figure 3), creatine has no effect on K_m for ADP (Figure 5). At three weeks creatine still exerts a very little control on the K_m for ADP (Figure 5). After that the affinity of oxidative phosphorylation system for ADP significantly increases (Figure 5) due to fast increase of mi-CK (Figure 3).

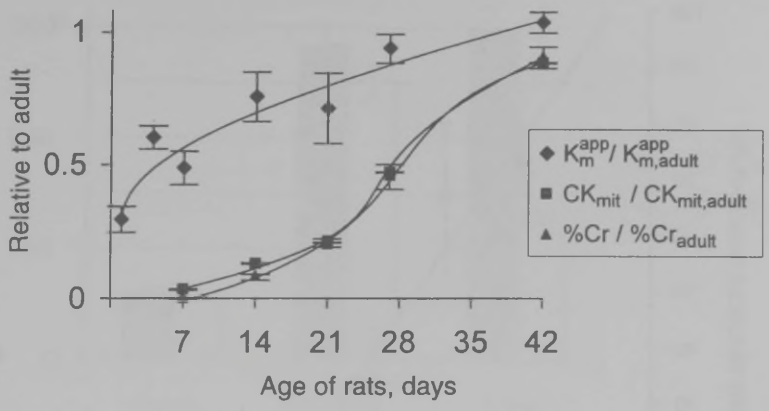

Figure 3. Developmental changes in affinity of mitochondrial respiration for ADP (Km), creatine index (% of activation of respiration by 20 mM creatine in the presence of 0.1 mM ADP), and activity of mi-CK, normalized to adult levels, in skinned cardiac fibers.

Analysis of respiration of skinned fibers revealed a rapid decrease in the apparent affinity of mitochondria to ADP during early postnatal development (within 1 week) that indicates the development of mechanism which increasingly limits the access of ADP to mitochondria (Figure 3). The postnatal maturation of cardiac muscle is associated with the developmental shift in metabolic control over oxidative phosphorylation from the mechanism controlling the diffusion of ADP through the mitochondrial OM to the mechanisms mediated by mi-CK. Interestingly, such a switch to another type of control exactly coincides with the period of rapid decrease in heart weight to body weight ratio (HW/BW) (Tiivel *et al.*, 2000), and a nice negative correlation between HW/BW ratio and creatine stimulated respiration (Figure 4). It can be seen that while postnatal development of the rat heart corresponds to decrease in HW/BW from 5.5 to approximately 3, a parallel increase in creatine-stimulated respiration does occur (Figure 4). Thus, postnatal increase in the effectiveness of heart function (estimated as decrease in HW/BW ratio) appears to be tightly related to the development of the mitochondrial mechanism of PCr synthesis in cardiac muscle.

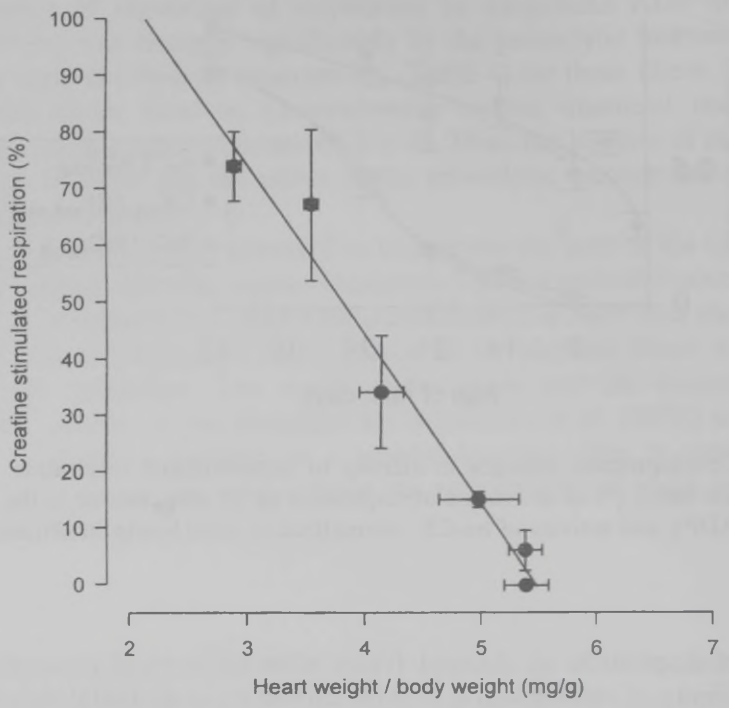

Figure 4. Negative correlation between the stimulation of respiration by creatine and HW/BW ratio.

Figure 6 demonstrates the tissue-specificity of these changes: in contrast to cardiac muscle the apparent K_m for ADP in the skinned fibers from glycolytic *m. gastrocnemius* is very low (10–15 μM) in newborn age, and does not change during the muscle development. Thus, age-dependent decrease of mitochondrial affinity to ADP is characteristic only of oxidative muscles.

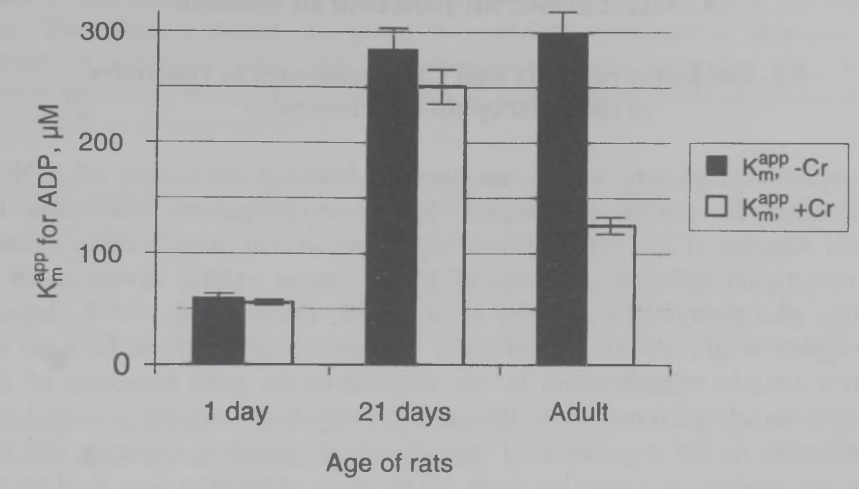

Figure 5. Developmental changes in creatine effect on the affinity for ADP in mitochondrial respiration.

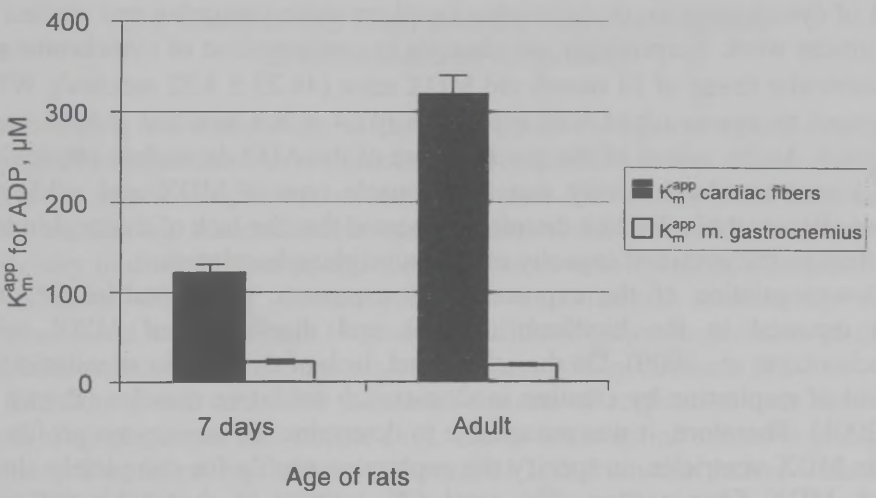

Figure 6. Comparison of the developmental changes in Km for ADP in skinned fibers from heart and *m. gastrocnemius.*

3. Mitochondrial function in disease

3.1. Oxidative capacity and CK isoenzymes in ventricles of the dystrophin-deficient mice

To complete the picture of the mechanisms limiting the access of ADP to mitochondria in oxidative muscles, further investigations addressed the diseased muscles of this type. Several reports show that mitochondria isolated from dystrophin-deficient muscles of MDX mouse exhibit disturbances of oxidative phosphorylation (Glesby *et al.*, 1988; Dunn *et al.*, 1993; Dupont-Versteegden et al., 1994). Therefore it was intresting to know how do the oxidative muscle mitochondria *in situ* respond to the gene knockout of this important muscle proteins. Thus, the current study dealt with the potential role of dystrophin in the regulation of mitochondrial oxidative capacity and CK isoenzyme profile in ventricles from MDX mice. Attention was paid to the content of cytochrome aa_3 as being characteristic of oxidative capacity.

It has been reported that cytochrome oxidase activity in diaphragms of three month old MDX mice decreased at the same range as downregulation of its mRNA expression (Gannoun-Zaki *et al.*, 1995). For the first time the tissue level of cytochrome aa_3 in dystrophin knockout mice ventricles was studied in the present work. Surprisingly, no changes in concentration of cytochrome aa_3 in ventricular tissue of 11 month old MDX mice (46.23 ± 4.32 nmoles/g WW) compared to age-matched wild-type ones (41.4 ± 8.4 nmoles/ g WW were observed. As the values of the maximal rate of the ADP-dependent respiration (V_{max}) were similar in every matching muscle type of MDX and wild-type mouse (Braun *et al.*, 2001) it therefore appeared that the lack of dystrophin had no effect on the maximal capacity of oxidative phosphorylation.

Downregulation of the expression of sarcomeric mitochondrial CK has been reported in the hindlimb muscles and diaphragm of MDX mice (Tkachenko *et al.*, 2000). On the other hand, lack of dystrophin attenuated the control of respiration by creatine in slow-twitch oxidative muscles (Brown *et al.*, 2001). Therefore, it was reasonable to determine the isoenzyme profile of CK in MDX ventricles, to specify the expression profile for completely slow-twitch MDX fiber portion. The total CK activity of dystrophin-deficient ventricles (206.8±22.6 μmoles min^{-1} g WW^{-1} was similar to that in control ones (235.3±14.3 μmoles min^{-1} g WW^{-1}) but the proportion of mi-CK was markedly increased at the expense of decreased BB- and MB-CK activities (Table 5). The results suggest that functional coupling between mitochondrial CK and ANT may be disturbed in skinned ventricular fibers of MDX mice due to imbalance in stoichiometry of these enzymes required for effective coupling (Kuznetsov and Saks, 1986; Saks *et al.*, 1987).

Table 5. CK isoenzyme profile (%) in skinned cardiac fibers of MDX and wild-type mice. The means ± S.E.M. are given. * — P<0.05 compared to wild-type. n — number of ventricles used, BB — homodimer of the brain-type CK, MB — heterodimer of the brain- and muscle-type CK, MM — homodimer of the muscle-type CK, mi-CK — mitochondrial CK.

Type of muscle	BB	MB	MM	Mi-CK
Wild-type n=4	6.1 ±1.1	21.8 ± 1..3	39.1 ± 1.5	33.0 ± 2.7
MDX n=5	2.6 ± 0.6*	16.3 ± 2.1*	36.5 ± 1.5	44.4 ± 3.6*

Although a number of reports have described impaired oxidative phosphorylation in dystrophin-deficient muscle cells (Glesby *et al.*, 1988; Dunn *et al.*, 1993; Even *et al.*, 1994; Dupont-Versteegden *et al.*, 1994) this study confirms the other data (Kemp *et al.*, 1993; Kuznetsov *et al.*, 1998 and Lodi *et al.*, 1999; Braun *et al.*, 2001) that imply the normal function of these processes.

3.2. Effect of IgG from patients with liver diseases on mitochondrial respiration

The research was undertaken to further address the role of cytoskeletal proteins in exerting control over mitochondrial respiration *in situ*. It was expected, that if the proteins capable to exert control over the mitochondrial function do exist, circulating autoantibodies against these proteins could be developed, especially in diseased states, and their effects on mitochondrial function could be revealed. Figure 7 demonstrates the experimental scheme by using permeabilized cardiac fibers to study the effect of immunoglobulins G (IgG) purified from the sera. of a patient with primary biliary cirrhosis (PBC).

Addition of 0.2 mM ADP increased the rate of respiration over the basal levels with glutamate and malate (V_0), due to initiation of oxidative phosphorylation. The following addition of purified IgG from the patient with PBC resulted in inhibition of ADP-stimulated respiration. The inhibition developed slowly; the steady state at maximally inhibited respiration was usually achieved not earlier than 10 min of incubation with IgG. (This explains why only submaximally activating concentrations of ADP were used in the experiments — to save oxygen in the reaction medium throughout the long-lasting experiment).

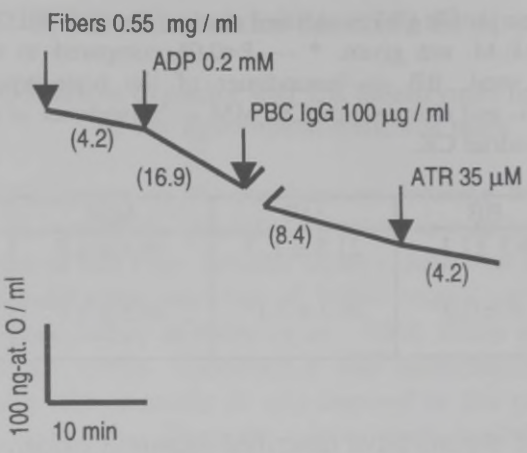

Figure 7. The scheme of oxygraphic measurements of mitochondrial oxygen consumption in skinned fibers from rat ventricular myocardium. The additions of fibers, ADP, immunoglobulin (PBC-IgG) and atractyloside (ATR) are indicated. Respiratory substrates (2 mM malate and 5 mM glutamate) were added into the medium before fibers. Respiration rates in parenthesis are expressed in ng-atoms O mg DW^{-1} min^{-1}. Note: The trace is interrupted after the addition of IgG for 10 min and only the last phase where steady state inhibition was reached is demonstrated.

In separate experiments it was shown that when IgG was added 20 min prior to the addition of ADP, the IgG exerted no significant effect on basal rate of respiration (V_0), whereas the ADP-dependent respiration became inhibited to the same extent as in case of being added after ADP. Thus, the effect of IgG was restricted to oxidative phosphorylation; the antibodies could control either the production of ADP by ATPases, or access of ADP to ANT at the level of mitochondrial outer membrane. The former aspect was addressed by decreasing the total ATPase activity in skinned fibers, by extracting myosin from the cells with KCl-treatment (ghost fibers, see Methods). However, the observed inhibitory effect (about 40%) of IgG on respiration corresponded to that in skinned fibers with normal myosin content (Results not shown). This suggests that inhibition of oxygen consumption in skinned fibers by IgG was not caused by its binding to the myosin ATPase.

To judge over the status of mitochondrial membranes in the presence of IgG, the effects of ATR and cytochrome c (Saks *et al.*, 1998) on ADP-dependent respiration were assessed. As shown in Figure 7, the addition of ATR, a specific inhibitor of ANT, abolished the ADP-activated respiration in skinned fibers exactly to the level seen before ADP addition. It means that inner mitochondrial membrane remained intact after permeabilization of the cell membrane by saponin (Saks *et al.*, 1998) before addition of IgG, enabling the respiration to be effectively controlled via ANT. On the other hand, in

separate experiments it was found that exogenous cytochrome c did not affect the ADP-activated respiration rates, both in the presence and absence of IgG (Recordings not shown). This observation, indicating intact outer mitochondrial membrane (Saks *et al.*, 1998) suggests that IgG exerted inhibitory effect on respiration without penetrating into intermembrane space of mitochondria in skinned fibers.

To determine whether the effects of IgG are related to reversible or irreversible binding, in several experiments the skinned fibers were washed during 30 min to remove the unbound IgG after registration of its inhibitory effect on respiration. It was found that this procedure had no effect, as the respiration rate remained essentially the same (5.36±0.51 and 5.40±0.68 ng-atoms O mg^{-1} min^{-1}, n=5) before and after PBC-IgG washout, respectively. Hence, irreversible binding of antibodies to intracellular sites in cardiac cells could be assumed.

3.2.1. Inhibition of mitochondrial respiration by IgG in cardiac fibers

Table 6 presents the mean values of the effects of IgG on respiration in different groups of IgG preparations. It can be seen that IgG from healthy donors significantly decreased the rate of oxidative phosphorylation in skinned cardiac fibers, relatively by 13% of the preincubation level. In contrast, IgG isolated from patients with PBC or CH equally suppressed this parameter to the markedly larger extent (by 42–44%). Notably, no quantitative difference between the two disease groups was observed.

Table 6. The effect of IgG from the sera of healthy donors, patients with PBC and CH on ADP-activated respiration in skinned fibers from rat ventricular myocardium.
*, *** — P<0.05 and 0.001 respectively compared to corresponding value without IgG. ¤¤¤ — P<0.001 compared to inhibition (in %) in healthy controls.

IgG donors	ADP-activated respiration, ng-atoms O mg^{-1} min^{-1}	ADP-activated respiration with IgG, ng-atoms O $mg^{-1}min^{-1}$	Inhibition by IgG, %
Healthy controls (7)	9.64 ± 0.72	8.27 ± 0.51*	13 ± 4
Patients with PBC (18)	11.36 ± 0.63	6.69 ± 0.46***	44 ± 3¤¤¤
Patients with CH (8)	9.15 ± 1.0	5.43 ± 0.57***	42 ± 3¤¤¤

3.2.2. Muscle type-specific inhibition of mitochondrial respiration by IgG

Figure 8 shows that IgG from the sera of patients with PBC and CH (including AIH) inhibited oxidative phosphorylation to the similar extent in skinned fibers of heart and skeletal muscle (*m. soleus*).

However, no inhibition occurred when the same immunoglobulin fractions were applied to glycolytic muscle (*m. gastrocnemius*). It appears therefore, that the glycolytic muscles either lack the autoantigens capable to react with IgG, or these autoantigens, though present, expose different epitopes compared to that in oxidative muscles. This result exhibits a special feature of oxidative muscles — to be a target for autoantibodies.

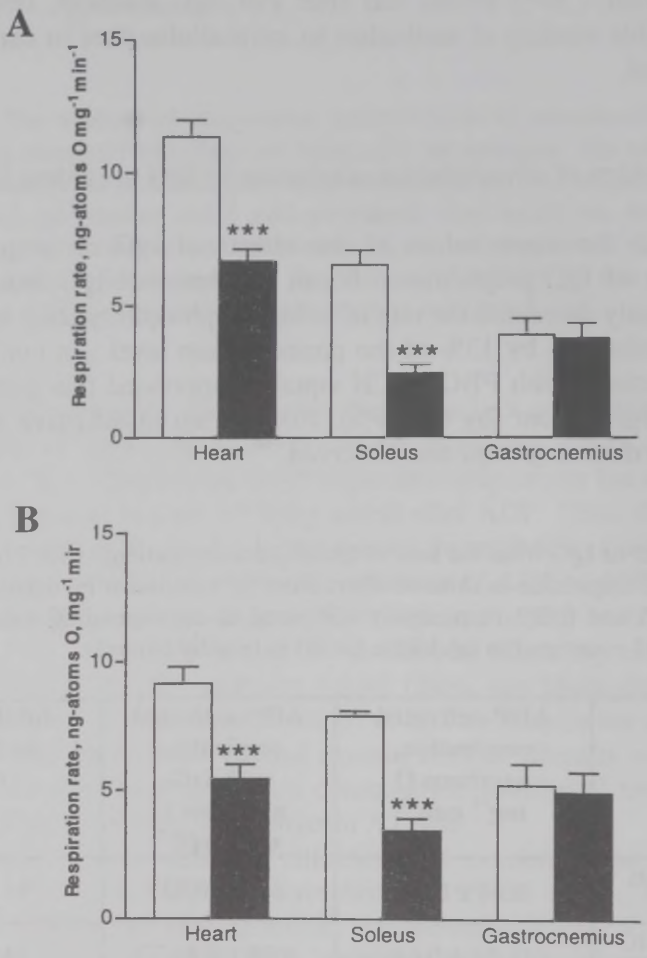

Figure 8. Effects of PBC-IgG (A) and CH-IgG (B) (black columns) on ADP-activated respiration in oxidative (heart, *m. soleus*) and glycolytic (*m. gastrocnemius*) muscles of rat.
*** — P<0.001 compared to respiration without IgG (white columns).

3.2.3. Structural evidence of binding of IgG to sarcomeric structures of skinned cardiac fibers as revealed by confocal microscopy

In general, Figure 9A,B,C,D demonstrates fairly preserved intracellular structures in skinned cardiac fibers from normal rat. This is evident from parallel rows of mitochondria separated by myofibrils (seen as dark lines between the mitochondrial rows). Intensive and uniform staining of mitochondrial membranes with Mitotracker Red indicates that all mitochondria were functionally normal, since maintenance of high membrane potential ($\Delta\psi$) is conditional for effective transport into and binding of this staining agent to mitochondria. When the skinned fibers were incubated with IgG from healthy subjects (Figure 9A), only a little amount of immunoglobulins was bound, compatibly with negligible inhibition of respiration (Table 6). One can also see that IgG was specifically localized between the mitochondria, thereby forming transversal fluorescent lines crossing the whole specimen. The positions of these parallel lines obviously corresponded to the Z-disks of the sarcomere. This is supported by other analyses of the localization of mitochondria with respect to sarcomere, both by laser confocal microscopy (Kay *et al.*, 1997) and electron microscopy (Nozaki *et al.*, 2001). Notably, the distance between neighbouring transversal green lines was about 2 μm, characteristic of relaxed state of the sarcomere due to low free Ca^{2+} content (0.1 μM) in the medium. In contrast to Z-disks, only negligible amounts of IgG were detected in the sarcomeric space between the Z-disks, and colocalization of IgG with mitochondrial membranes was not seen. Compared to the binding of normal IgG, the IgG from PBC patients appeared to accumulate in larger quantities, predominantly on Z-disks and less over sarcomeric area, that have seen there as green grains and fine lines on dark myofibrillal background. Like in experiments with IgG from healthy persons, IgG from PBC patients did not bind to the outer membrane of mitochondria (Figure 9B).

Figure 9C localizes IgG from AIH patient on the Z-disks and between them. In addition, and contrasting to PBC-IgG, these antibodies tended to form the transversal lines exactly between the Z-disks, which are likely corresponding to the M-line of sarcomere. Also, intensive accumulation of IgG between the cells and in the regions of intercalated disks where the proteins connecting the cardiomyocytes end-to-end localize could be observed (Figure 9D). This feature indicates once again effective removal of sarcolemma between the cardiomyocytes, this allowing IgG to penetrate easily to all intercellular spaces, and from there, into the intracellular cytoplasmic compartments of each individual cardiomyocytes. Localization of IgG between the mitochondria, within the Z-disk (Figure 9A,B,C,D) and between the Z-lines (Figure 9B,C,D) would occur if desmin serves as an autoantigen for IgG. The relevant control experiment was done by assessing the binding of IgG from AIH patient with subcellular structures in skinned cardiac fibers of desmin

knockout mouse (Figure 9 E,F). In accordance with earlier studies (Kay *et al.*, 1997), lack of desmin resulted in deterioration of myofibrils and mitochondria in the cardiomyocytes, with large mitochondrial accumulations between myofibrils separated from each other (Figure 9E,F). Notwithstanding the absence of desmin, the IgG still bound to Z-disks, sarcomeric space, M-lines and intercalated disks. These observations exclude the desmin of being a predominant antigen for binding of IgG to the sarcomeric structures.

4. Isolation the cDNA fragments in common between oxidative muscles

This issue arised from former results of the study suggesting that oxidative muscle cells may possess a similar mechanism of regulation of mitochondrial respiration, thus being responsible for low apparent affinity of mitochondria to ADP in these muscles, whereas the relevant mechanism seems to be lacking in the glycolytic muscles. Hence, isolation and identification of genes responsible for muscle-type specific control over oxidative phosphorylation, i.e. genes in common between myocardium and *m.soleus*, are required to study the nature of that mechanism. The aim of this part of the study was to reveal the cDNA fragments, that characterize oxidative muscle cells and also represent a pool of cDNA in common between in two oxidative muscles — murine myocardium and *m. soleus*. Figure 10 illustrates the principle of this strategy.

To evaluate differences between various muscles at the level of gene expression, total RNA was isolated from the tissues of interest (Figure 10) and thereafter the total cellular RNA pools were reverse-transcribed into the ds full-length cDNA by SMART technology. During second step subtracted heart-specific cDNA library (HE) was generated. Cardiac cDNA (H) was taken as tester and EDL cDNA (E) as driver. Then the novel cost-effective method of KDA was applied to isolate the SHE pool between the HE and the total cDNA of mouse *m. soleus* (Puurand *et al.*, 2003).

Unlike the SSH, which discards the common sequences of the distinct DNA population analyzed, the KDA isolates and amplifies these sequences within a single hybridization step. Here we demonstrate its usefulness in isolating the common genes in metabolically homologous muscles, as *m. soleus* and myocardium, sharing oxidative phosphorylation as a predominant mechanism for energy production.

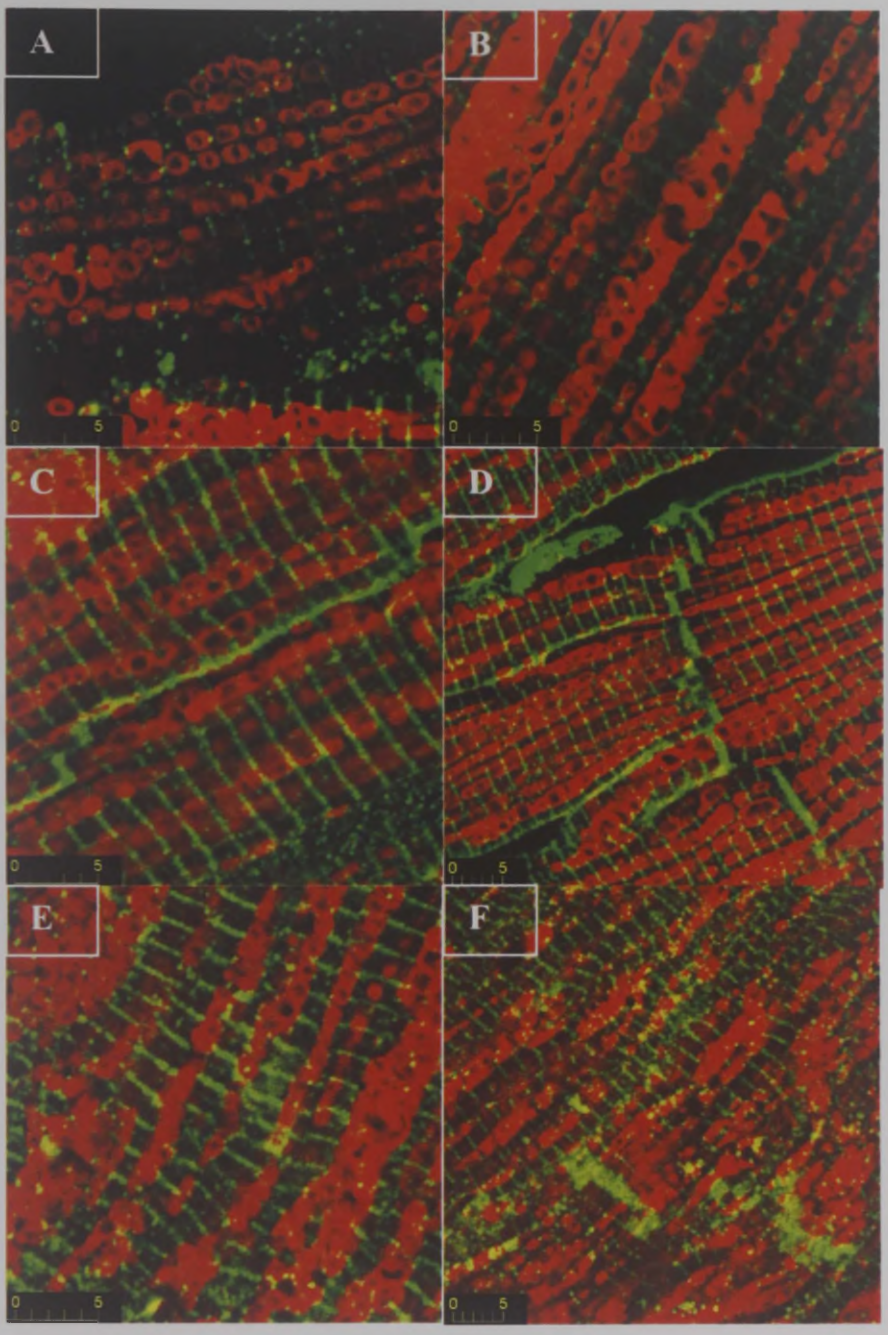

Figure 9. Double labelling immunofluorescence confocal microscopy of the skinned ventricular fibers from rat incubated with IgG from healthy control (A), with IgG from PBC patient (B), and with IgG from AIH patient (C and D). E,F – skinned fibers of the desmin knockout mice, incubated with IgG from patient with autoimmune hepatitis, as in panels C and D. The red color is Mitotracker Red associated with mitochondrial membranes. The green color corresponds to anti-human FITC-conjugated rabbit immunoglobulins. The bars are given in micrometers.

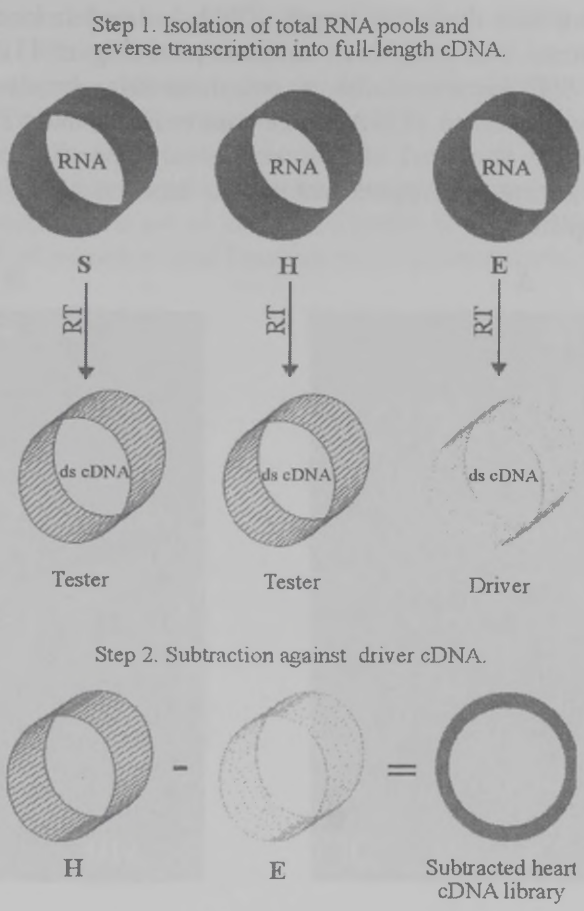

Figure 10. The principle scheme for isolation of genes common between mouse heart and *m. soleus*, but not expressed in EDL.

Figure 11A indicates three full-length cDNA isolated from different above-mentioned sources. The analysis of common pool (Figure 11B) indicated that the cDNAs in SHE became visible as two dominating bands of 200 and 250 bp. Commonly expressed cDNAs were inserted into the pTZ57R/T vector. Altogether several thousand clones, exclusively specific to red, oxidative muscles were generated. Approximately one hundred clones were randomly picked and amplified.

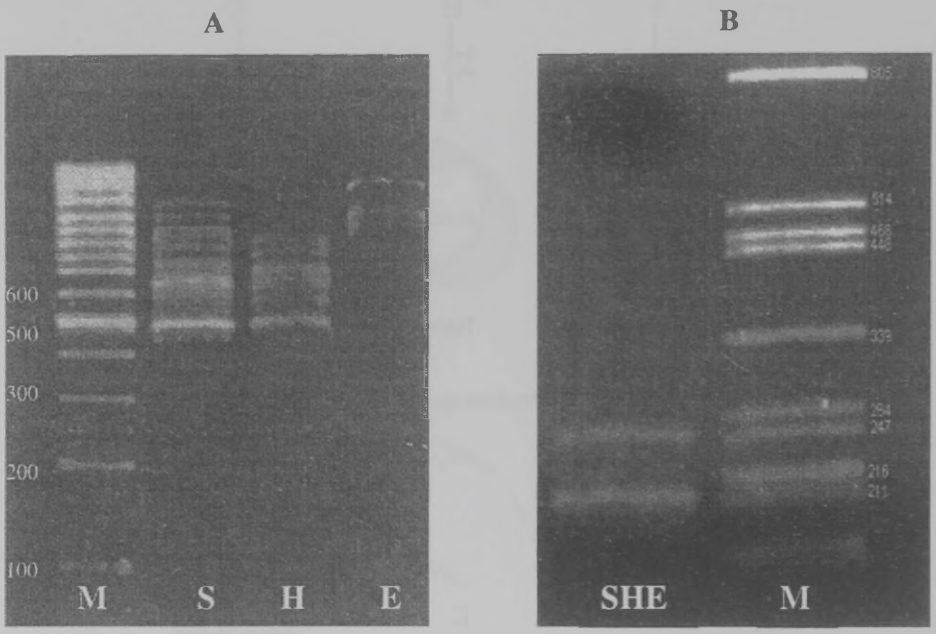

Figure 11. **A.** Agarose gel electrophoresis of total full-length cDNAs from *m. soleus* (S), heart (H), and *m. extensor digitorum longus* (E).
B. The dominating bands of common pool SHE. M — DNA size markers: Generuler 500 bp (A), λPSTI (B).

The efficiency of kindred DNA amplification was verified by Southern dot-blotting (Figure 12). In this analysis, 54 DNA fragments were randomly selected from the total set of cDNA and three blots were made keeping the same order of clones. One of the membranes was hybridized with the DIG-labeled *m. soleus* total cDNA (Figure 12A) and another with DIG-labeled mouse heart total cDNA (Figure 12B). The results of those two hybridizations were similar insofar as 54 clones hybridized with cDNA probes in both cases, and two negative controls (water and DNA of *E. coli*) did not hybridize. The third membrane (Figure 12C) depicts clones hybridized with DIG-labeled total cDNA of EDL and they were blotted in the same order as in Figure 12A and B. A very rare pattern of binding was observed, indicating that a large majority of genes, specific to EDL, were eliminated from the entire pool of

cDNA, in common between myocardium and *m. soleus* (SHE). The (+) sign marked in Figure 13C indicates the cDNA for G3PDH as a positive control. This glycolytic enzyme, which exists in all muscle tissue, was used to determine whether the hybridization was successful. This signal exceeded the five other spots on the filter. Thus, these results indicate that the common cDNA pool (SHE) did not contain the cDNA fragments characteristic of EDL and, as such, represented a set of candidate genes involved in muscle type-dependent control of mitochondrial function in oxidative muscle cells.

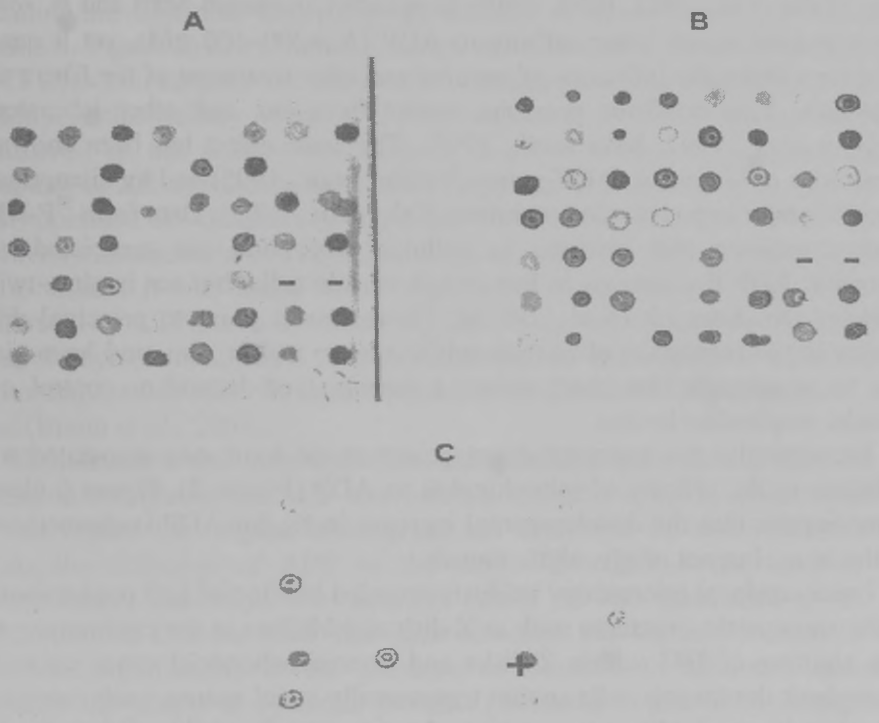

Figure 12. Evaluation of the common cDNA pool (SHE) by Southern hybridization. Three filters with the SHE clones, dot-blotted in the same order, were probed with total cDNA from *m. soleus* (A), heart (B) or EDL (C). The dots indicate binding of labeled cDNA of different sources to complementary cDNA sequencies of the SHE pool. (–) and (+) represent the negative and positive controls, respectively.

DISCUSSION

1. Regulation of oxidative phosphorylation — function of intracellular energetic units?

In this study the differences between the kinetics of regulation of respiration by ADP in oxidative and glycolytic muscles *in vivo* were observed. The glycolytic muscles (e.g. *m. gastrocnemius*) display high apparent affinity to ADP (K_m=10–20 μM) which is comparable to that in isolated mitochondria *in vitro* (Saks *et al.*, 1993, 1994, 1995). In contrast, oxidative heart and *m. soleus* fibers exhibit much lower affinity to ADP (K_m=200–400 μM), yet it can be increased under the influence of creatine and after treatment of the fibers with proteases. This confirms previous results from our and other laboratories (Seppet *et al.*, 1991; Saks *et al.*, 1995). The same effect has been shown in conditions of knockout of CK genes (Veksler *et al.*, 1995) and by disruption of the OM under hypoosmotic conditions (Saks *et al.*, 1995). Data from ^{31}P-NMR studies indicate that changes in cellular respiration are associated with cytosolic ADP fluctuations in fast-twitch muscle cells, but not in slow-twitch muscles (Kushmerick *et al.*, 1992b). These results point to principal differences in the regulation of mitochondria *in vitro* and *in vivo*, and have given rise to suggestion that there exists a muscle type-dependent control over cellular respiration *in vivo*.

Interestingly, the postnatal development in rat heart was associated with decrease in the affinity of mitochondria to ADP (Figure 3). Figure 6 clearly demonstrates that the developmental increase in K_m for ADP is characteristic of the heart, but not of glycolytic muscle.

Laser confocal microscopy analysis revealed binding of IgG predominantly to the sarcomeric structures such as Z-disk and M-lines in the cardiomyocytes. The staining of IgG within Z-disks and intermitochondrial space coincided throughout the muscle cells so that transversally serial spaces, each containing mitochondria and adjacent sarcomere, became clearly visible. When the IgG from a CH patient was incubated with the skinned myocardial fibers of the desmin knockout mice, its binding to Z-disks and the sarcomeric area was found to be similar to that in normal cardiac muscle. However, the transversal staining pattern was disintegrated, because of the slippage of the myofibrils in relation to each other and accumulation of mitochondria between them. Importantly, the sarcomere-specific binding of IgG was associated with the decreased respiratory capacity of mitochondria in skinned ventricularfibers. Therefore, it appears that the autoantibodies developed in human organism contain those that may recognize the proteins participating in the control over respiration.

Altogether these observations can be dicussed best in the light of the recent concept that in oxidative muscles the mitochondria and adjacent sarcomeres form complexes, termed as the intracellular energetic units, ICEUs. Inside the ICEUs the integration between the ATPases and mitochondria is ensured by creatine and adenylate kinases which, by functioning as the vectorial shuttles of the energy-rich phosphate moiety, are able to stimulate the respiration despite the constancy of cytoplasmic ADP concentration (Gellerich, 1992; Kushmerick and Meyer, 1992; Saks *et al.*, 1995; Kuznetsov *et al.*, 1996; Laterveer *et al.*, 1997; Dzeja *et al.*, 1998). Indeed, the fact that CK effectively operates, is supported by the observation that in the presence of 20 mM creatine the apparent mitochondrial affinity to exogenous ADP increases (Table 4, Figure 2) in oxidative muscles. In other words, coupling of mi-CK to ANT allows to increase the local ADP concentration inside the ICEU, or more exactly, in the vicinity of the ANT. The important role of adenylate kinases is confirmed in the studies by Dzeja *et al* (1998). They have demonstrated coupling of AK to mitochondria and energy consuming structures, such as the ATP-operated K^+-channels. In fact, there exists the third way of interaction between mitochondria and ATPases (Seppet *et al*, 2001; Braun *et al.*, 2001). In these studies it was found that a significant portion of the total ADP flux generated by ATPases is not freely equilibrating with the adenine nucleotide in the bulk cytoplasmic phase. Instead, it is directly transferred to mitochondria in normal cardiac cells. In mouse heart it corresponds to 35% of the total ADP flux (Braun *et al.*, 2001).

These studies rise very important and principal questions: Where are the diffusion boundaries for ADP and whether and how could they be regulated? In this regard the original assumption has been that the mitochondrial OM limits the diffusion of ADP to the ANT. This idea stems from several observations. Recently, the dynamic state of the permeability of the mitochondrial OM for ADP has been revealed; the barrier function of OM increases significantly in the presence of dextrans or albumins added to simulate the effects of cytosolic proteins (Gellerich *et al.*, 1993; Laterveer *et al.*, 1997). On the other hand, ultrastructural and biochemical studies demonstrate tight connections between mitochondria and cytoskeletal structures, particularly with annexin V and desmin (Sun *et al.*, 1993; Leterrier *et al.*, 1994 and Penman, 1995). Kay *et al.* (1997) have found that the absence of desmin results in the appearance of sarcomeres with disorganized structure and of a mitochondrial population displaying low affinity to ADP. Probably this population of mitochondria has lost control over the diffusion of ADP through the OM.

Another line of evidence shows that mitochondria isolated from dystrophin-deficient muscles exhibit disturbances of oxidative phosphorylation (Glesby *et al.*, 1988; Dunn *et al.*, 1993; Even and Decrouy, 1994 and Dupont Versteegden *et al.*, 1994) while, on the other hand, ^{31}P-NMR spectroscopy has revealed an increased concentration of free ADP in the skeletal muscles of Duchenne

muscular dystrophy (DMD) patients (Kemp *et al.*, 1993; Lodi *et al.*, 1999). These results can be interpreted as evidence that the cytoskeletal proteins such as desmin and dystrophin are important in governing the mitochondrial function via modulating the permeability of MOM and increasing the feedback signal by ADP to stimulate the mitochondria. Thus, the extramitochondrial proteins may exert control over outer membrane by limiting diffusion of ADP to mitochondria.

Experiments with IgG suggest, however, that diffusion restrictions for ADP may be localized not only at the level of the OM, but also within these structures that form and surround entire complexes of mitochondria and ATPases. This is evidenced by a finding that immunostaining localized predominantly in sites which may form the boundaries of hypothetical ICEUs, i. e. along the lines linking the neighbouring Z-disks and leaving mitochondria between them (Figure 9). It is not excluded that the Z-disks themselves also participate in forming the barriers described. These barriers not only tie mitochondria with adjacent ATPases, but also isolate some amount of adenine nucleotides from their cytoplasmic pool, so that adenine nucleotides cannot equilibrate easily between intra-ICEU- and extra-ICEU-spaces (Saks *et al.*, 2002). In these conditions, adhering of immunoglobulins to the full length of the barrier forming structures (green lines), would significally restrict diffusion of ADP from outside the cell and cytoplasm into ICEU. Consequently, less ADP can reach ANT to stimulate oxidative phosphorylation that explains the decreased rate of respiration (Table 6).

The specific subcellular localization of IgG binding (Figure 9) leads to seek the possible candidates for the target proteins within the sarcomere. In oxidative muscle the Z-disk comprises 5 proteins — α-actinin, γ-filamin, C-terminus of nebulette, N-terminus of titin and CapZ protein (Leibovitch *et al.*, 1995; Sorimachi *et al.*, 1997). The Z-disks of the same sarcomere are connected with intermediate filament protein desmin, which also cross-links individual myofibrils laterally at their Z-disks. It is known that desmin filaments colocalize with a cytolinker protein plectin, which, in turn, binds to mitochondria. Therefore the tandem of proteins — desmin and plectin is considered to play an important role in the positioning of mitochondria in the intermyofibrillar space exactly at the level between the two Z-disks (Reipert, *et al.*, 1999). In fact, Figure 9 fully supports this concept. Firstly, it clearly shows that mitochondria are fairly localized close to the neighbouring sarcomeres. Secondly, Figure. 9E,F indicates that in desmin-deficient mice heart, the positions of Z-disks in the neighbouring myofibrils became shifted and the intermitochondrial fibers were lost compared to normal myocardium (Figure 9 A,B,C,D) due to disintegration of the cellular structure. At the sametime, our results (Figure 13) suggest that desmin may not be the only candidate for binding IgG with sarcomeric structures. It would be more correct to assume that besides desmin, many other proteins such as actin, myosin, tubulin, troponin, α-actinin and tropomyosin found in patients with PBC and

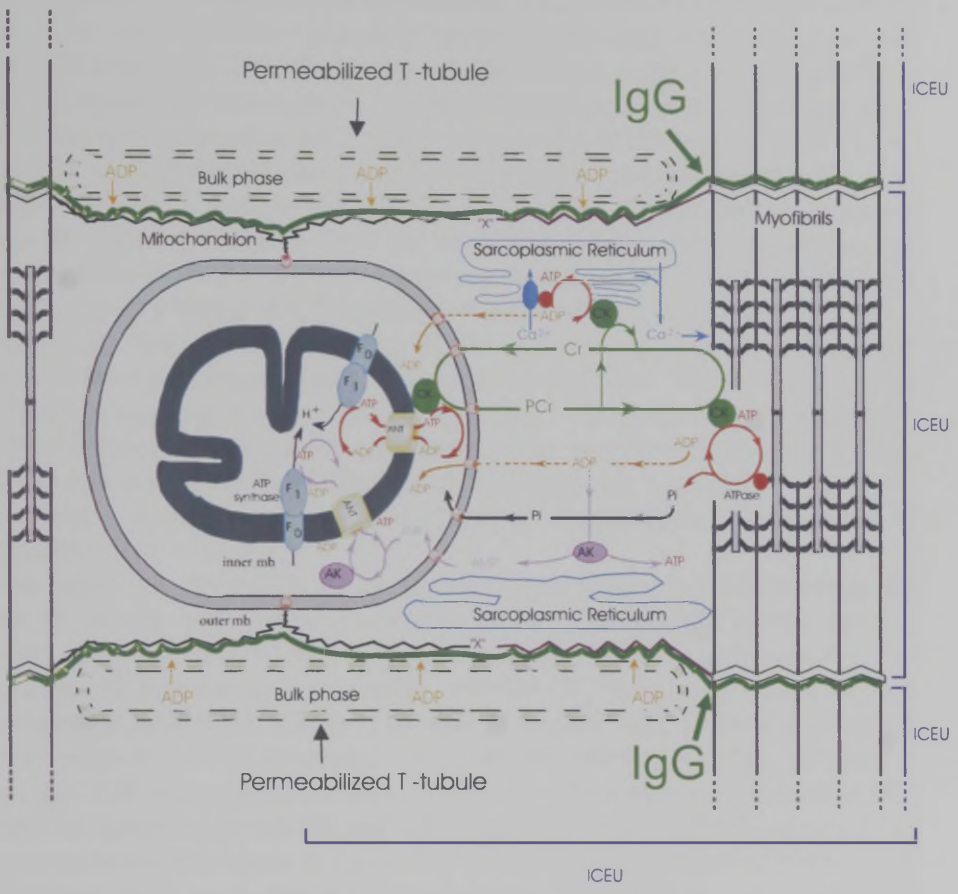

Figure 13. Scheme of ICEU in the cells of oxidative muscle. It is hypothetized that the protein(s) of unknown nature (X), most probably connected to cytoskeleton, fix the position of mitochondria adjacent to myofibrillar and SR ATPases, and compartmentalize these structures into the ICEUs isolated from the cytoplasmic bulk phase (Seppet *et al.*, 2001; Saks *et al.*, 2001) As a result, the exogenous ADP added to the skinned fibers cannot reach mitochondria easily and adenine nucleotides within ICEU do not equilibrate rapidly with their cytoplasmic counterparts. Binding of IgG (green lines, as in Figure 9) with protein X increases the restriction for diffusion of adenine nucleotides from the cytoplasm to ICEU, thus manifestating as inhibition of ADP- stimulated respiration.

CH (Dighiero *et al.*, 1990; Leibovitch *et al*, 1995 Girard and Senécal, 1995) play a role of potential antigens in this process. Consistently, the results of immunoblotting revealed serum IgG binding to a number of different proteins in skinned heart muscle, including those comigrating with α-actinin and desmin (Kadaja *et al.*, 2003). In general, there were more proteins capable to bind IgG in cardiac muscle than in *m. gastrocnemius*. Probably the immunoreactive bands that manifest themselves exclusively in the myocardium can be attributed to the proteins participating in control of mitochondrial respiration within the ICEU, and, therefore, conferring sensitivity of this unit to inhibitory action of IgG. At the same time the inhibition did not correlate with single band reactivity on immunoblots, suggesting that the effect on respiration could be mediated by binding of IgG to different molecules forming ICEU.

Whatever the proteins forming ICEU are, our results show that their expression is upregulated in early postnatal development. This is evidenced by the fact that apparent K_m for ADP in regulation of respiration increased within the first 3 weeks of postnatal development to the adult levels in rat heart. This process appears to be coinciding with mitogenesis, organization of mitochondria into eaxactly oriented rows (Tiivel *et al.*, 2000) and development of cytoskeleton.

We found that the mi-CK in rat heart becomes to be expressed between the first and second week of *post partum*, which confirms the earlier data by Dowell, (1987). The dynamics of biosynthesis of mi-CK exactly matched the activation of respiration by creatine (Figure 3), indicating that postnatal expression of mi-CK in muscle cells was associated with functional coupling of this enzyme to the processes of oxidative phosphorylation. In such a coupling, ATP produced in the mitochondria is effectively transformed to PCr in the cleft between the mi-CK and ANT, and the formed ADP is returned via ANT into the matrix, where it stimulates the respiration coupled to synthesis of ATP. Due to such a coupling the maximal rates of respiration can be achieved at much lower concentrations of cytosolic ADP than in the absence of coupling (Seppet *et al.*, 1991; Saks *et al.*, 1995). Therefore, the greater amount of mi-CK becomes attached to the inner mitochondrial membrane during the development, the larger is the decrease in the apparent Km for ADP, in the presence of creatine (Figure 3). It is known that postnatal expression of ANT does not limit this process, as synthesis of ANT precedes the induction of mi-CK (Schönfeld *et al.*, 1996).

The described results allow to delineate potnatal changes in the mechanisms of metabolic control as the function of compartmentalization of mitochondrial production of ATP and its use into ICEU. In newborn heart, the mitochondria are characterized by relatively low activity of ANT, due to which the ANT represents the main flux-limiting step (Glatz and Veerkamp, 1982). Since mitochondria are randomly localised in the cells and are not yet organized into ICEUs, there are no significant barriers to ADP diffusion. In these conditions the oxidative phosphorylation can be activated provided that

the cytosolic ADP concentrations increase in response to increased workload. In fact, the cytoslic ADP significantly increases as shown in neonatal sheep (Portman *et al.*, 1997). It satisfies requirements for activation of respiration. Maturation of cardiac muscle, associated with reorganization of mitochondrial localization into arrays between the myofibrils, i.e. with formation of ICEUs, results in increasing diffusion restrictions for ADP to the mitochondria. In this case the mitochondria cannot rely on the delivery of ADP from the cytoplasm outside the ICEU anymore. Instead they become dependent on production of ADP endogenously, in mi-CK and AK reactions, or on direct transfer of ADP, as evidenced from the dynamics of expression of mi-CK. These changes allow to increase the rate of ATP synthesis and provide ATPases with ATP without significant increases in the cytosolic ADP and ATP concentrations, i.e. to ensure the metabolic stability in cardiac cells and other oxidative muscle cells.

2. Identification of proteins participating in intracellular energetic units

In this study, the novel molecular method termed as the kindred DNA amplification (KDA) was applied to create tissue-specific cDNa library. It allows to cost-effectively isolate common cDNA fragments between two distinct cDNA populations. Unlike subtractive hybridization which discards common sequences, KDA isolates and amplifies these sequences within a single hybridization procedure. The utility of this method is demonstrated by cloning the genes in common between two different but metabolically homologous muscles, murine ventricular myocardium and *m. soleus*. It was hypothesized that both of the oxidative muscles, myocardium and *m. soleus* express few and probably the same protein(s) capable to exert intracellular control over mitochondrial respiration, whereas the glycolytic muscles lack these protein(s).

The results show that metabolically similar myocardium and *m. soleus* share a relatively little amount of expressed genes. Among them were identified tissue-specific mouse troponin T (TnT) (Huang and Jin, 1999), alpha-tropomyosin (Tpm) (Pieples and Wieczorek, 2000) and beta-tubulin, encoded by M-beta-4 genes (Lewis *et al.*, 1985). The studies on whether and how they are related to mechanisms of regulation of mitochondrial respiration are in progress.

CONCLUSIONS

1. The apparent affinity of mitochondria to exogenous ADP in regulation of respiration is much lower in skinned fibers of oxidative muscles than in glycolytic muscles. It increases in the presence of creatine and after trypsin treatment in oxidative muscles but not in glycolytic muscles. These observations point to different regulation of mitochondrial function due to muscle type-specific expression of extramitochondrial regulatory proteins in oxidative muscle cells.

2. In newborn rat heart mitochondrial respiration is stimulated by diffusion of ADP from the ATPases. Within 2–3 weeks of postnatal development the diffusion of ADP to mitochondria becomes progressively restricted and the control over respiration shifts towards mitochondrial CK, which by coupling to ANT allows to maximally activate the oxidative phosphorylation despite limited access of ADP.

3. Circulating autoantibodies of IgG type isolated from the sera of patients with autoimmune liver diseases inhibit mitochondrial ADP-regulated respiration in oxidative but not in glycolytic muscle cells. The inhibitory effect of IgGs is related to their specific interactions with sarcomeric structures, predominantly with the proteins projecting at the Z-disk and M-line areas.

4. The application of novel method enabled to isolate common cDNA fragments between oxidative muscles (myocardium and *m. soleus*). The clones from cDNA library created define tissue-specific expression pattern and provide the candidate genes involved in the control over cellular respiration in oxidative muscles.

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SUMMARY IN ESTONIAN

Mitokondrite funktsiooni regulatsiooni molekulaarsed aspektid oksüdatiivsetes lihasrakkudes

Käesolevas töös uuriti mitokondrite funktsiooni regulatsiooni oksüdatiivsetes lihaskiududes *in vivo* võrreldes glükolüütiliste lihastega ning seoses postnataalse arengu, koespetsiifilise geeniekspressiooni ja haigustega.

Tööülesanded olid järgmised:

1. Uurida mitokondrite hingamise regulatsioonimehhanisme täiskasvanud roti ja hiire eri tüüpi lihastes ning roti südames postnataalse arengu jooksul.
2. Uurida terve inimese ja maksahaigustega (primaarne biliaarne tsirroos, krooniline hepatiit) patsientide seerumitest eraldatud G-tüüpi immunoglobuliinide toimet mitokondrite ADP-aktiveeritud hingamisele oksüdatiivsete (müokard, *m. soleus*) ja glükolüütiliste (*m. gastrocnemius*) lihaste skinneeritud kiududes.
3. Konstrueerida hiire oksüdatiivsetele lihastele (müokard, *m. soleus*) iseloomulik cDNA kogu, subtrakteerides glükolüütilise lihase (*m. extensor digitorum longus*) cDNAd.

Töö tulemused võimaldavad teha järgmisi järeldusi:

1. Oksüdatiivsete lihaste skinneeritud kiududes on mitokondrite näiline afiinsus eksogeense ADP suhtes palju väiksem kui glükolüütilistes lihastes. Ta suureneb kreatiini juuresolekul ja trüpsiiniga töötlemise tagajärjel oksüdatiivsetes, kuid mitte glükolüütilistes lihaskiududes. Need andmed viitavad mitokondrite funktsiooni erinevale regulatsioonile, mis on tingitud ekstramitokondriaalsete regulatoorsete valkude lihastüübi-spetsiifilisest ekspressioonist oksüdatiivsete lihaste rakkudes.
2. Vastsündinud roti südames stimuleerib mitokondrite hingamist ATPaasidelt difundeeruv ADP. Postnataalse arengu 2–3 nädalal ADP difusioon progresseeruvalt pidurdub ning kontroll hingamise üle nihkub mitokondriaalse CK suunas, mis seotuna adeniinnukleotiidtranslokaasiga, võimaldab ADP piiratud kättesaadavusele vaatamata oksüdatiivset fosforüülimist maksimaalselt aktiveerida.
3. Autoimmuunsete maksahaigustega patsientide IgG-tüüpi antikehad inhibeerivad ADP-aktiveeritud hingamist oksüdatiivsetes, kuid mitte glükolüütilistes lihastes. IgG inhibeeriv toime oksüdatiivsele fosforüülimisele on seletatav nende spetsiifilise seostumisega sarkomeerides, peamiselt Z-ketta ja M-joone piirkonna valkudega.
4. Uudse uurimismetoodika rakendamine võimaldas isoleerida oksüdatiivsete lihaste (müokard ja *m. soleus*) ühised cDNA fragmendid. Saadud cDNA kogu kloonid määratlevad oksüdatiivsetele lihastele iseloomuliku geeniekspressiooni mustri ja võimaldavad identifitseerida oksüdatiivsete lihaste rakuhingamise kontrollis osalevad kandidaatgeenid.

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PUBLICATIONS

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Eur. J. Biochem. 241, 909–915 (1996)

Striking differences between the kinetics of regulation of respiration by ADP in slow-twitch and fast-twitch muscles *in vivo*

Andrei V. KUZNETSOV[1], Toomas TIIVEL[2,3], Peeter SIKK[2,3], Tuuli KAAMBRE[2,3], Laurence KAY[3], Zoya DANESHRAD[3], André ROSSI[2,3], Lumme KADAJA[4], Nadya PEET[4], Enn SEPPET[4] and Valdur A. SAKS[2,3]

[1] Neurobiochemisches Labor, Klinik für Neurologie, Universitätsklinikum Magdeburg, Germany
[2] Laboratory of Bioenergetics of Institute of Chemical and Biological Physics, Tallinn, Estonia
[3] Laboratory of Bioenergetics, Joseph Fourier University, Grenoble, France
[4] Laboratory of Pathophysiology, University of Tartu, Estonia

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The kinetics of *in vivo* regulation of mitochondrial respiration by ADP was studied in rat heart, slow-twitch skeletal muscle (soleus) and fast-twitch skeletal muscle (gastrocnemius, plantaris, quadriceps and tibialis anterior) by means of saponin-skinned fibres. Mitochondrial respiratory parameters were determined in the absence and presence of creatine (20 mM), and the effect of proteolytic enzymes (trypsin, chymotrypsin or elastase) on these parameters was investigated in detail. The results of these experiments confirm the observation of Veksler et al. [Veksler, V. I., Kuznetsov, A. V., Anflous, K., Mateo, P., van Deursen, J., Wieringa, B. & Ventura-Clapier, R. (1995) *J. Biol. Chem. 270*, 19921–19929], who studied muscle fibres from normal and transgenic mice, that the kinetics of respiration regulation in muscle cells is tissue specific. We found that in rat cardiac and soleus muscle fibres the apparent K_m for respiration regulation was 300–400 μM and decreased to 50–80 μM in the presence of creatine. In contrast, in skinned fibres from gastrocnemius, plantaris, tibialis anterior and quadriceps muscles, this value was initially very low, 10–20 μM, i.e. the same as that is in isolated muscle mitochondria, and the effect of creatine was not observable under these experimental conditions. Treatment of the fibres with trypsin, chymotrypsin or elastase (0.125 μg/ml) for 15 min decreased the apparent K_m for ADP in cardiac and soleus muscle fibres to 40–98 μM without significant alteration of V_{max} or the intactness of outer mitochondrial membrane, as assessed by the cytochrome *c* test. In fibres from gastrocnemius, trypsin increased the apparent K_m for ADP transiently. The effects of trypsin and chymotrypsin were studied in detail and found to be concentration dependent and time dependent. The effects were characterised by saturation phenomenon with respect to the proteolytic enzyme concentration, saturation being observed above 1 μM enzyme. These results are taken to show that in cardiac and slow-twitch skeletal muscle, the permeability of the outer mitochondrial membrane to adenine nucleotides is low and controlled by a cytoplasmic protein that is sensitive to trypsin and chymotrypsin. This protein may participate in feedback signal transduction by a mechanism of vectorial-ligand conduction. This protein factor is not expressed in fast-twitch skeletal muscle, in which cellular mechanism of regulation of respiration is probably very different from that of slow-twitch muscles.

Keywords: heart; skeletal muscle; respiration; regulation; adenine nucleotide.

The cellular mechanism of regulation of respiration *in vivo* is still unknown. The conventional explanation that the rate of respiration of mitochondria in intact cells is governed by the cytoplasmic ADP concentration according to a simple Michaelis-Menten type relationship is in disagreement with many experimental data [1–5]. First, it has been shown in numerous experiments on isolated hearts that the increases in workload and oxygen consumption are usually observed at stable, low and sometimes even decreasing steady-state ADP levels [1–5]. Second, Kushmerick et al. have found that in slow-twitch skeletal muscle, an increase of frequency of stimulation does not result in a proportional decrease of phosphocreatine content, which seems to be effectively and constantly resynthesised. Only in fast-twitch skeletal muscle seems there to be a reasonably simple Michaelis-Menten–type relationship between ADP content and oxygen consumption [6]. Third, experiments with skinned muscle fibres showed that in cardiac-muscle and red (slow-twitch)-skeletal-muscle cells the affinity of the mitochondrial oxidative-phosphorylation system to ADP is decreased by more than an order of magnitude compared with that of isolated mitochondria [1, 5, 7–9]. Recently, Veksler et al. have found that in the skinned fibres of fast-twitch skeletal muscle, in contrast to those of cardiac and slow-twitch skeletal muscle, the affinity of mitochondria for ADP is high and equal to that of isolated mitochondria [10]. Thus, it seems that the mechanism of regulation of respiration may be very different in different types of cells (i.e. tissue specific). The purpose of the present study was to investigate further this important question by use of skinned fibres from different types of muscles (rat heart, soleus, gastrocnemius, quadriceps, tibialis anterior red, plantaris and frog heart). The results verified that there are remarkable differences in the kinetics of regulation of mitochondrial respiration by ADP between these cells. Also, the effects of proteolytic en-

Correspondence to V. Saks, Laboratory of Bioenergetics Institute of Chemical and Biological Physics, Akadeemia tee 23, EE-0026 Tallinn, Estonia

zymes and creatine on respiration regulation were found to be remarkably different in the slow-twitch and fast-twitch muscle cells.

MATERIALS AND METHODS

Preparation of skinned muscle fibres. Wistar-line rats were used for these experiements. Skinned fibres were prepared according to the method described earlier [7–10]. The animals were anaesthetised with sodium pentobarbital (50 mg/kg body weight, intraperitoneal) and treated with heparin (1500 IU/kg body mass, intravenous). Their chests were opened, and the hearts, if still beating, were excised and put into cooled 2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 6.56 mM $MgCl_2$, 0.5 mM dithiothreitol, 50 mM Mes, 20 mM imidazole, 20 mM taurine, 5.3 mM ATP, 15 mM phosphocreatine, pH 7.1 (buffer A). Cooled hearts were cut in half and muscle strips (2–4 mm long, 1–1.5 mm diameter, 5–7 mg wet mass) cut from the endocardium of left ventricles along the fibre orientation to avoid mechanical damage of the cells. Muscle-fibre bundles (3–4 mm long, about 1 mm diameter) were taken also from soleus (oxidative, slow-twitch), gastrocnemius white (glycolytic, fast twitch), gastrocnemius red (oxidative/glycolytic, fast twitch), plantaris (oxidative/glycolytic, fast twitch, white), tibialis anterior (fast twitch, oxidative-glycolytic) and quadriceps (oxidative/glycolytic, mixed) [11–15]. By means of sharp-ended forceps or needles, the muscle fibres were separated from each other, leaving only small areas of contact. The fibres were transferred into vessels containing cooled (in ice) buffer A containing 50 μg/ml saponin and incubated with mild stirring for 30 min for complete solubilisation of the sarcolemma. Permeabilised (skinned) fibres were washed in 2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 1.38 mM $MgCl_2$, 0.5 mM dithiothreitol, 100 mM Mes, 20 mM imidazole, 20 mM taurine, 3 mM K_2HPO_4, pH 7.1 (buffer B), for 10 min; this washing procedure was performed three times to completely remove all metabolites, especially trace amounts of ADP. Complete removal of ADP can be demonstrated from respiration recordings, which should show very reproducible initial State-2 rates (designated as v_o) that are insensitive to inhibition by atractyloside.

The rates of oxygen uptake were recorded by means of the Yellow Spring Clark oxygen electrode in buffer B, containing respiratory substrates (5 mM pyruvate or 5 mM glutamate plus 2 mM malate) and 2 mg/ml BSA. Determinations were carried out at 25 °C and the solubility of oxygen was taken as 215 μM [7, 8].

Solutions. Composition of the solutions used was based on the ionic contents of muscle cell cytoplasm [16].

Electron microscopy. The ultrastructures of white and red skeletal muscles were observed by electron microscopy before and after saponin treatment. Tissue samples were fixed in glutaraldehyde (2.5% in cacodylate buffer) and treated as described [17, 18].

Treatment of fibres with proteolytic enzymes. Digestion of fibres was carried out in buffer B at 4 °C with 125 μg/ml trypsin (5 μM), chymotrypsin or elastase unless indicated otherwise.

Reagents. All reagents were purchased from Sigma, except ATP and ADP, which were obtained from Boehringer.

RESULTS

Electron microscopy. Fig. 1 shows the ultrastructure of the soleus (slow twitch, oxidative, red) and white gastrocnemius (fast twitch, glycolytic) skeletal muscles of the rat before and after permeabilisation by saponin. The differences between these two muscles are mostly of a quantitative nature and relate to differences in the cell volume occupied by mitochondria (2.2% and 5.9% in rat gastrocnemius and soleus muscles, respectively [11]), and in the levels of activities of glycolytic enzymes, creatine kinase (MM isoform) and myosin ATPase [12–15]. In both types of the cells, the localisation of mitochondria was similar: they were more or less homogeneously distributed in the cell and localised between myofibrils without significant clustering. This finding is in agreement with the detailed description given in [11]. Saponin treatment effectively removed the sarcolemma (Fig. 1C), and we can see some vesicularisation of the T-tubular system. From these data, we may conclude that average diffusion distances for extracellular ADP from outside the cells to the mitochondria should be very similar in these two muscles types. In cardiac cells, due to their smaller diameter, this distance should be shorter. In spite of these morphological similarities between fast-twitch and slow-twitch skeletal muscle, the kinetics of respiration regulation by ADP is different.

Kinetics of respiration regulation by ADP. Fig. 2 shows the results of the determination of the parameters of regulation of mitochondrial respiration by ADP in skinned fibres from rat heart, rat soleus and frog heart (A), and from white, red and mixed gastrocnemius (B). While mitochondrial respiration in skinned fibres from heart and soleus increased very slowly with elevation of the ADP concentration (almost millimolar concentrations of ADP were needed for maximal activation of respiration), skinned fibres from fast-twitch muscle (gastrocnemius) displayed very high sensitivity to ADP. The sensitivity of frog heart respiration was between these two types of muscles (Fig. 2; Table 1). From these data, the apparent K_m for ADP and V_{max} values may be calculated for characterisation of the affinity of mitochondria for ADP (Fig. 2C; Table 1). Fig. 2C demonstrates a striking difference in the values of apparent K_m for ADP between cardiac muscles and soleus, and fast-twitch gastrocnemius. There are about 30-fold differences between apparent K_m values for ADP for skinned fibres from slow-twitch and fast-twitch muscles (Table 1). Differences in V_{max} correspond very approximately to the known differences in the cell volume occupied by mitochondria in these muscles (30%, 5.9% and 2.2% in heart, soleus and gastrocnemius, respectively) [11, 19]. Skinned fibres from all other rat fast-twitch muscles studied, such as oxidative/glycolytic muscles (gastrocnemius red, quadriceps, tibialis anterior red and plantaris [11–15]) displayed very high affinity for ADP with respect to respiration regulation (Table 1).

In skinned fibres from slow-twitch soleus, activation of the mitochondria-coupled creatine kinase reaction by addition of creatine, which starts production of ADP in the mitochondrial intermembrane space, significantly decreased the apparent K_m for externally added ADP (to about 70 μM; Fig. 3). For cardiac fibres, this amplification effect of the coupled creatine kinase reaction on respiration regulation by ADP has been described in detail earlier [1, 8, 16]. For skinned fibres from gastrocnemius, this effect was not seen by the standard methods (oxygraphy) used in this work. The effect of creatine was practically absent in frog heart muscle.

As shown earlier for skinned cardiac muscles, hypoosmotic treatment of skinned fibres resulted in the appearance of a population of mitochondria with high affinity for ADP [9]. This effect is related to the damage of the outer mitochondrial membrane since it correlates with the effect of exogenous cytochrome *c* on the respiration [9]. Table 1 shows the same result for skinned fibres from soleus, which confirms that the low affinity of mitochondria for ADP *in vivo* is due to the decreased

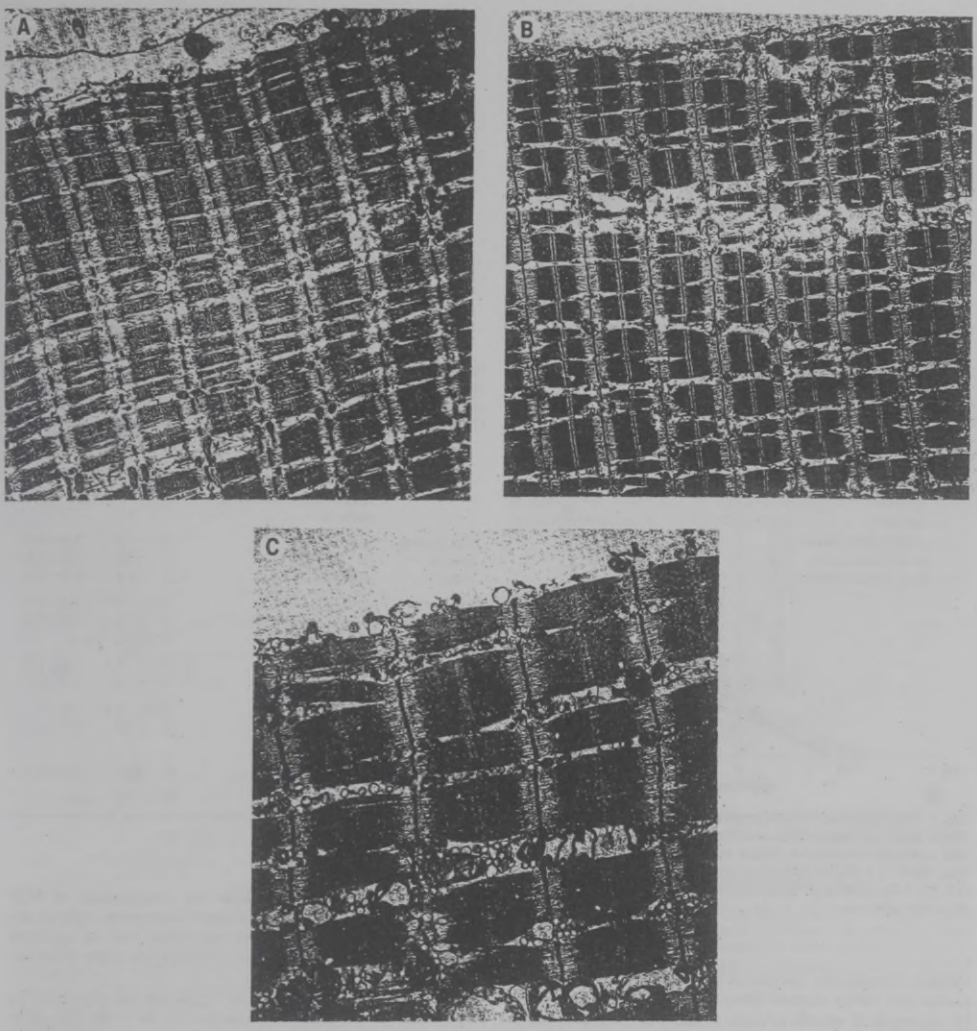

Fig. 1. Electron micrographs of fibres prepared from rat skeletal muscles. (A) Soleus before saponin treatment. The surface membrane of the cell is present but mechanically detached from myofibrils during fibres separation. Magnification, 3000×. (B) Gastrocnemius before treatment with saponin. The sarcolemma is present and mostly connected to subsarcolemmal myofibrils. Magnification, 3000×. (C) Fibres from gastrocnemius treated with saponin (50 μg/ml, 30 min). Complete dissolution of the sarcolemma and significant vesicularisation of the T-tubular system are seen. Magnification, 6000×.

permeability of the outer mitochondrial membrane for this substrate.

Effects of proteolytic enzymes (trypsin, chymotrypsin and elastase). The results shown in Fig. 4 confirm our earlier results that extraction of myosin by treatment of the cells with 800 mM KCl (to produce ghost fibres) does not change the apparent K_m for ADP. During this extraction, about 20% of cellular proteins are removed from the cells [8], which confirms that the surface membrane of the cells is almost completely destroyed and is not a barrier, even for protein molecules. Destruction of the sarcomers is responsible also for the elevation of V_{max} in these experiments. Thus, we cannot explain high apparent K_m values for ADP in skinned fibres by insufficient removal of the cellular surface membrane (sarcolemma). Nevertheless, treatment of the skinned fibres with the proteolytic enzymes chymotrypsin or

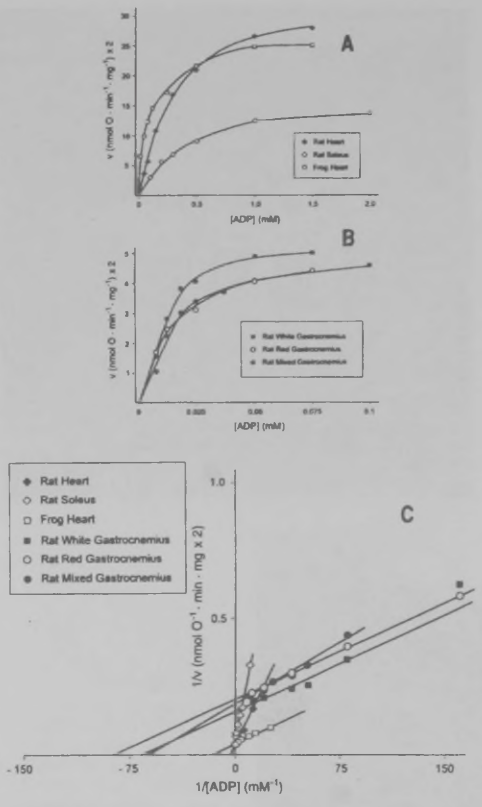

Fig. 2. The dependence of the respiration rate of the skinned muscle fibres from rat heart, soleus and frog heart (A) and from white, red and mixed gastrocnemius (B) on the extracellular ADP concentration. There is a 20-fold difference in the scale of the x-axes of (A) and (B). (C) Linearization of the dependences from (A) and (B) in double-reciprocal plots.

Table 1. Maximal oxygen-consumption rates and K_m^{app} (ADP) in different fibres. Means ± SD are given for 6–12 experiments. Trypsin, chymotrypsin and elastase were used in concentration of 125 μg/ml.

Fibre	Treatment	K_m^{app} (ADP)	V_{max}
		μM	nmol · min^{-1} · mg^{-1} × 2
Rat heart	none	297 ± 35	28.7 ± 1.1
	20 mM creatine	85 ± 5	28.0 ± 4
	ghost fibres	351 ± 32	46.7 ± 1.5
	trypsin	98 ± 8	26.0 ± 1
	chymotrypsin	143 ± 25	33.5 ± 2.0
	elastase	117 ± 24	27.0 ± 1.5
	creatine + chymotrypsin	120 ± 20	n.d.
Rat soleus	none	354 ± 46	12.2 ± 0.5
	20 mM creatine	105 ± 15	16.0 ± 4
	ghost fibres	320 ± 22	17.0 ± 0.4
	chymotrypsin	41 ± 8	11.8 ± 2
	elastase	42 ± 10	9.3 ± 1.0
	trypsin + trypsin inhibitor (Kunitz)	304 ± 25	10.7 ± 1
	trypsin	59 ± 6.0	n.d.
	creatine + trypsin	63 ± 3.0	n.d.
	hypoosmotic treatment, phase I	351 ± 48	n.d.
	hypoosmotic treatment, phase II	40 ± 8	n.d.
Rat gastrocnemius, white	none	14.4 ± 2.6	7.0 ± 0.5
	20 mM creatine	13 ± 10	5.4 ± 2.4
	trypsin	25 ± 4	8.9 ± 0.5
Rat gastrocnemius, red	none	12.5 ± 2.7	5.3 ± 0.3
	20 mM creatine	15 ± 8.5	7.6 ± 2.8
	trypsin	10 ± 2.8	n.d.
Rat quadriceps	none	22.3 ± 1.4	8.2 ± 1.2
	20 mM creatine	3.5 ± 0.7	6.2 ± 0.5
Rat plantaris	none	8.3 ± 5.4	7.8 ± 4.5
	20 mM creatine	20 ± 10	8.7 ± 2.3
Rat tibialis anterior red	none	15 ± 3	5.4 ± 0.3
Frog heart	none	94 ± 18	n.d.

elastase significantly decreased the apparent K_m for ADP in cardiac and soleus skinned fibres (Fig. 4B and Table 1). Probably, digestion of specific proteins sensitive to the proteolytic enzymes, rather than the destruction of cellular proteins in general (such as removal of myosin), changed the affinity of the mitochondrial systems to ADP in these cells. Therefore, the effects of these proteolytic enzymes were studied in more detail.

The cytochrome *c* test was used to investigate the state of the outer mitochondrial membrane before and after trypsin treatment. In KCl-containing medium, cytochrome *c* dissociates from the outer surface of the inner mitochondrial membrane and if the outer mitochondrial membrane is damaged it leaves the mitochondrial intermembrane space, which decreases the rate of respiration [9]. However, addition of exogenous cytochrome *c* completely restores respiration under these conditions [9]. The results of this test for investigation of the state of the outer mitochondrial membrane after treatment of the skinned fibres by trypsin are shown in Fig. 5. This figure shows high rates of respiration stimulated by a saturating concentration of ADP, which were not changed by addition of exogenous cytochrome *c*. Thus, the outer mitochondrial membrane was not destroyed by treatment of the skinned fibres with trypsin under these conditions.

Nevertheless, the kinetics of regulation of respiration by ADP were very significantly changed by the proteolytic treatment of cardiac and slow-twitch skeletal muscle, as seen from the rapid decrease of the apparent K_m for ADP in these fibres (Fig. 6 and Table 1). In contrast, in skinned fibres from gastrocnemius, trypsin treatment resulted in a transient increase of the apparent K_m for ADP. Thus, the kinetics of regulation of respiration by ADP and the effects of proteolytic enzymes are different in various types of muscles.

Two phases of trypsin and chymotrypsin action can be observed: a rapid decrease in the apparent K_m for ADP in skinned fibres from heart, which is followed by elevation of the apparent K_m. This second phase can probably be explained by inactivation of proteins in the mitochondrial membrane or damage to porin molecules, due to the prolonged action of trypsin or chymotrypsin. This second phase was not observed when elastase was used or soleus studied. Inactivation of proteins of the mitochondrial

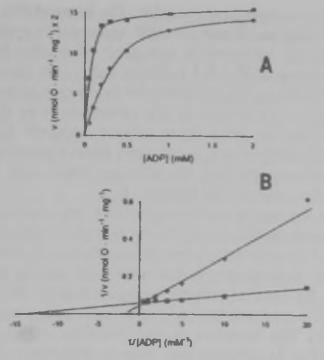

Fig. 3. Effect of creatine on the respiration rate. (A) The effect of creatine (20 mM) on the dependence of the respiration rate of the skinned fibres from rat soleus on the extracellular ADP concentration. (B) Linearization of the dependences shown in (A) in double-reciprocal plots. ◆, with creatine; ■, without creatine.

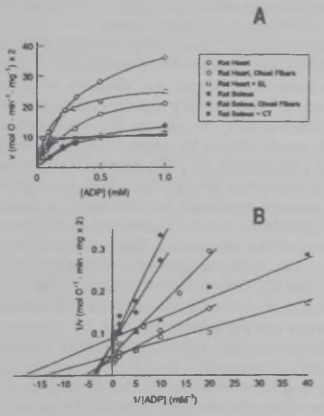

Fig. 4. Dependence of respiration rate on extracellular ATP concentration. (A) The dependences of the respiration rates of skinned cardiac and soleus fibres on the extracellular ADP concentration before and after extraction of myosin by 800 mM KCl (ghost fibres) or treatment of the fibres for 15 min with 125 μg/ml chymotrypsin (CT) or 125 μg/ml elastase (EL). (B) Linearisation of the dependences from (A) in double-reciprocal plots.

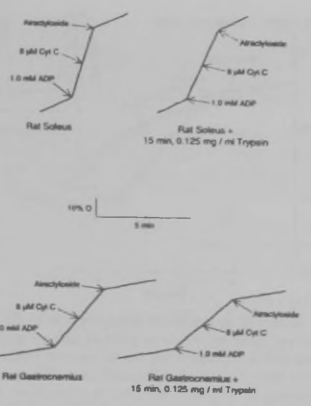

Fig. 5. Cytochrome *c* test for the intactness of the outer mitochondrial membrane in skinned fibres from rat soleus and gastrocnemius white muscles before and after treatment with 125 μg/ml trypsin for 15 min. Because of the different sizes of fibres, the respiration rates are different, but there is no change of respiration after addition of cytochrome *c* and thus no significant damage to the outer mitochondrial membrane under these conditions.

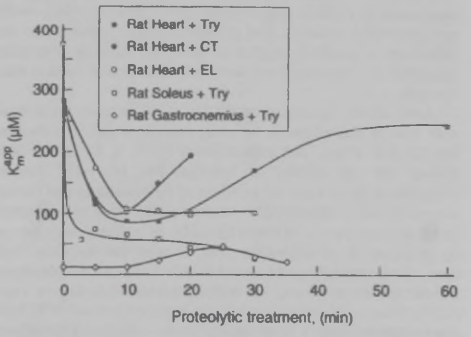

Fig. 6. Effects of proteases on apparent K_m for ADP. Changes in the apparent K_m for ADP in regulation of respiration of skinned fibres from rat heart, rat soleus and rat gastrocnemius white muscles with time in the presence of 125 μg/ml trypsin (Try), chymotrypsin (CT) or elastase (EL).

membrane might be the reason for the transitory increase in apparent K_m for ADP in skinned fibres from gastrocnemius. After long periods (more than 1 h) of trypsin action, the heart fibres became too digested to be correctly collected for dry mass (and V_{max}) determination, which was therefore artificially elevated (data not shown).

Fig. 7 shows a more-detailed analysis of the dynamics of the action of proteolytic enzymes (trypsin and chymotrypsin) on the respiration of skinned fibres from soleus and its dependence on the enzyme concentration. At a non-saturating ADP concentration (0.1 mM), trypsin or chymotrypsin exerted remarkable respiratory control independently of their concentration or time of action. The effect of chymotrypsin at low concentrations on the respiration of skinned fibres is shown in Fig. 7A. At a low concentration (0.5 μM) of chymotrypsin, it takes about 8 min before we observed a rather abrupt increase in the respiration rate. This time required to observe any change in respiration rate (respiratory control) after proteolytic enzyme addition is indicated in Fig. 7A as *t*, which we term the time of respiration activation. This time of activation decreased with elevation of the proteolytic enzyme concentration. Fig. 7B shows the quantitative relationship between this respiration-activation time and the proteolytic enzyme concentration. This relationship showed a saturation phenomenon: at enzyme concentrations higher than 1 μM a minimal activation time of 1−2 min was observed and it was not changed further with elevation of the enzyme concentration.

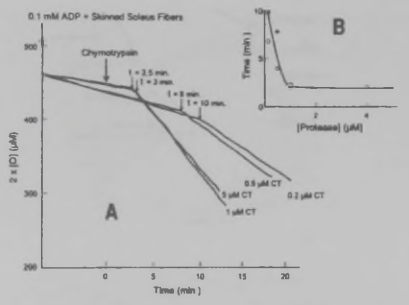

Fig. 7. Dynamics of the action of chymotrypsin on the respiration of skinned fibres from rat soleus muscle at non-saturating ADP concentrations (0.1 mM) depending on its concentration. (A) Oxygraph traces of the changes in oxygen concentration with time after addition of chymotrypsin (CT) to different final concentrations. Note the delay in the increase in respiration rate after addition of chymotrypsin. (B) time of respiration activation (respiratory control) as a function of chymotrypsin (CT, □) or trypsin (TR, ■) concentrations.

DISCUSSION

The results of this work confirm the earlier observation by Veksler et al. [10] that the kinetics of regulation of the rate of respiration by ADP *in vivo* is strikingly different in slow-twitch and fast-twitch muscles, and provide further evidence that this difference is probably related to the expression of extramitochondrial protein(s) in the slow-twitch skeletal and cardiac muscle cells.

Low affinity of mitochondria for ADP *in vivo*, in contrast with that of mitochondria *in vitro*, has been well documented for skinned cardiac and soleus fibres [1, 5, 7, 8]. It has been shown that the affinity of mitochondria for ADP can be increased in three ways: by swelling of mitochondria and disruption of the outer mitochondria membrane by hypoosmotic treatment; by treatment of skinned fibres by proteolytic enzymes; or by isolation of mitochondria. Similar observations have been made for permeabilised hepatocytes [9]. The most simple explanations for these effects, i.e. mitochondrial clustering or rapid ADP utilisation by adenylate kinase, in permeabilised cells have been excluded since a large change in the mitochondrial affinity for ADP is observed after trypsin action (Fig. 6) without any changes in mitochondrial localisation (or clustering) in the cells, and complete inhibition of adenylate kinase by diadenosine pentaphosphate has been shown not to change the affinity of mitochondria for ADP in skinned fibres [7, 9].

Another explanation, which is often proposed, is that the increased apparent K_m for ADP in cardiac and slow-twitch skeletal muscle fibres is a result of diffusion problems for extracellular ADP through damaged but not completely dissolved sarcolemma, and because of the long diffusion distance through organized cellular structures, such as myofibrils. Since these problems should be the same in slow-twitch and fast-twitch skeletal muscle, the results of this work, which show remarkable differences (more than 30-fold) between apparent K_m values for ADP in these cells, provide a rather strong argument against this explanation. Furthermore, in ghost fibres, the myosin is removed from the cells by KCl treatment, which results in removal of 10–20% of cellular proteins without any effect on the apparent K_m for ADP (Fig. 4), which indicates practically complete removal of sarcolemma as a diffusion barrier, even for big protein molecules. Data indicative of very significant adaptive changes also argue against this explanation. The permeability of the outer mitochondrial membrane for ADP (decrease in apparent K_m for this substrate) increased in transgenic mice after knock-out of creatine kinase [10] (MM isoform) and in the energy-deficient state of the heart [17], probably due to changes in the expression of some proteins but not in cell ultrastructure or diffusion distances. Since we have found similar apparent K_m values in skinned cardiomyocytes and skinned fibres consisting of numerous cardiomyocytes [20], the diffusion distance for ADP is not a factor that controls the apparent K_m.

Thus, the most plausible explanation seems to be that there is an extramitochondrial cytoplasmic protein, or group of proteins, expressed in cardiac and slow-twitch skeletal muscle but not in fast-twitch skeletal muscle that control the permeability of porin pores in the outer mitochondrial membrane for ADP. This protein may be connected to the cytoskeleton, as has been discussed earlier [1]. The dynamics of the proteolytic enzyme action described (Fig. 7) indicates that there is probably a protein, or a protein complex, that is saturable with proteolytic enzymes and may control the permeability of the outer-mitochondrial-membrane porin pores for ADP.

Precise identification of this protein or proteins, however, will take a significant amount of work.

The expression of this protein or protein complex is related to the cellular mechanism of regulation of respiration, which in muscle cells seems to be tissue specific.

Classical work by Chance and coworkers by means of phosphorus NMR have shown that skeletal-muscle exercise results in rapid metabolic changes, i.e. decrease of phosphocreatine content and a corresponding rise of cytoplasmic ADP. On the basis of these results, the concept of work/energy-cost-transfer functions was developed by means of modified Michaelis-Menten–type relationships [21, 22]. However, similar work carried out on isolated hearts showed that a large increase in the rate of oxygen consumption could be seen even at decreasing cytoplasmic ADP levels [1–5]. Kushmerick et al. have shown very different metabolic responses of fast-twitch and low-twitch skeletal muscles to increased frequency of stimulation and recovery after exercise [6]. Only in fast-twitch, glycolytic muscles, where mitochondria are activated only at the step of metabolic recovery after exercise and participate mostly in the recovery of high levels of phosphocreatine, could the results be explained by feedback control of respiration by ADP concentration according to a Michaelis-Menten relationship, and the authors concluded that the mechanisms of control of cellular respiration are quantitatively and qualitatively different in fast-twitch and slow-twitch skeletal muscle [6]. Our results are in excellent agreement with this conclusion, and probably clarify some of the important reasons for such a difference. In fast-twitch muscles, mitochondria display high affinity for ADP because of the high permeability of the outer mitochondrial porin pores for adenine nucleotides. In heart and slow-twitch skeletal muscle, outer-mitochondrial-membrane porin pores seem to be controlled by unknown proteins. In these cells, especially in the heart, mitochondrial oxidative phosphorylation is a basis for cellular energy metabolism and maintains constant levels of high-energy phosphate compounds (ATP and phosphocreatine) at any level of workload due to very effective feedback between contraction and respiration in these cells. The possibility that there is a structurally and functionally organised pathway for intracellular vectorial feedback signal conduction [23] based on cytoplasmic creatine-kinase and adenylate-kinase systems in these cells, as it has been recently proposed [1, 2, 24], is not excluded. It is possible that the protein factor(s) expressed in these cells that controls the affinity of mitochondria for extracellular ADP participates in the organisation of this vectorial signal-conduction

pathway, and that the affinity of mitochondria in these cells for endogenous and exogenous ADP might be different [1]. The coupled creatine-kinase system in mitochondria seems to be an important part of this signal-conduction pathway, serving as an amplifier of metabolic signals in mitochondria [1, 25] in which the apparent K_m for ADP *in vivo* is high, i.e. heart and slow-twitch soleus (Table 1). In frog heart, the specific form of creatine kinase [26] seems to be uncoupled from oxidative phosphorylation, since we observed no effect of creatine on respiration in frog heart. As a compensatory mechanism to increase the efficiency of regulation of respiration, mitochondrial affinity for ADP is probably increased in these cells (apparent K_m is decreased to 94 μM, compared with rat heart or soleus fibres in which the apparent K_m was 300–400 μM; Fig. 2). Similar adaptive changes in apparent K_m for ADP have been observed by Veksler et al. [10] in transgenic mice after deletion of MM creatine kinase.

The data available at present are not sufficient to answer the question of which protein structures of the cells are responsible for the striking difference between respiration regulation in fast-twitch and slow-twitch muscles observed in this study, and further experimental work is necessary to understand the reason for this difference.

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Developmental changes in regulation of mitochondrial respiration by ADP and creatine in rat heart *in vivo*

Toomas Tiivel,[1] Lumme Kadaya,[3] Andrei Kuznetsov,[2] Tuuli Käämbre,[1] Nadezhda Peet,[3] Peeter Sikk,[1] Urmo Braun,[3] Renée Ventura-Clapier,[4] Valdur Saks[1] and Enn K. Seppet[3]

[1]*Laboratories of Bioenergetics, Institute of Chemical and Biological Physics, Tallinn, Estonia and Joseph Fourier University, Grenoble, France;* [2]*Department of Transplantology, University Hospital, Innsbruck, Austria;* [3]*Department of Pathophysiology, University of Tartu, Estonia;* [4]*Laboratory of Cellular and Molecular Cardiology, Université de Paris-Sud, France*

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Abstract

In saponin-skinned muscle fibers from adult rat heart and m. soleus the apparent affinity of the mitochondrial oxidative phosphorylation system for ADP (K_m = 200–400 μM) is much lower than in isolated mitochondria (K_m = 10–20 μM). This suggests a limited permeability of the outer mitochondrial membrane (OMM) to adenine nucleotides in slow-twitch muscle cells. We have studied the postnatal changes in the affinity of mitochondrial respiration for ADP, in relation to morphological alterations and expression of mitochondrial creatine kinase (mi-CK) in rat heart *in vivo*. Analysis of respiration of skinned fibers revealed a gradual decrease in the apparent affinity of mitochondria to ADP throughout 6 weeks *post partum* that indicates the development of mechanism which increasingly limits the access of ADP to mitochondria. The expression of mi-CK started between the 1st and 2nd weeks and reached the adult levels after 6 weeks. This process was associated with increases in creatine-activated respiration and affinity of oxidative phosphorylation to ADP thus reflecting the progressive coupling of mi-CK to adenine nucleotide translocase. Laser confocal microscopy revealed significant changes in rearrangement of mitochondria in cardiac cells: while the mitochondria of variable shape and size appeared to be random-clustered in the cardiomyocytes of 1 day old rat, they formed a fine network between the myofibrils by the age of 3 weeks. These results allow to conclude that in early period of development, i.e. within 2–3 weeks, the diffusion of ADP to mitochondria becomes progressively restricted, that appears to be related to significant structural rearrangements such as formation of the mitochondrial network. Later (after 3 weeks) the control shifts to mi-CK, which by coupling to adenine nucleotide translocase, allows to maximally activate the processes of oxidative phosphorylation despite limited access of ADP through the OMM. (Mol Cell Biochem **208**: 119–128, 2000)

Key words: rat, development, skinned muscle fibers, oxidative phosphorylation, adenine nucleotide translocase, mitochondrial creatine kinase, outer mitochondrial membrane

Introduction

Postnatal maturation of the mammalian heart is associated with increased tissue content of the mitochondria related to increase of their size and number in the cell [1–3]. The specific activities of mitochondrial enzymes and the mitochondrial concentrations of the respiratory chain components also increase [4]. These changes result in a time-dependent augmentation in the overall respiration capacity of the cardiac

Address for offprints: E.K. Seppet, Department of Pathophysiology, Faculty of Medicine, University of Tartu, 19 Ravila Street, 50411 Tartu, Estonia

muscle, in order to meet increasing activity of the contractile function [4].

Much less is known about the metabolic regulation of the mitochondrial energy production during development. Studies on isolated mitochondria have revealed that the adenine nucleotide translocase (ANT) and the F_0F_1-ATP synthase exert the main control over respiration in the newborn animal [5, 6]. In later stages of development, the expression of ANT sharply increases, and the control over respiration shifts from ANT to another components, possibly being distributed between the substrate dehydrogenases, phosphate carrier and respiratory chain [7].

Recent studies have opened the new aspects of the metabolic regulation *in vivo*, i.e. under conditions of natural intracellular environment of mitochondria. It was shown by using saponin-skinned fibers of rabbit myocardium that early postnatal development is characterized by binding of mitochondrial creatine kinase (mi-CK) to the inner membrane of mitochondria due to which the respiration becomes stimulated by creatine [8, 9]. Deprivation of creatine kinase system function by feeding the developing rats with β-guanodinopropionic acid [10] has been shown to significantly impair the contractile function of developing rat heart. These experiments strongly suggest that compartmentation of different creatine kinase isoenzymes to intracellular organelles is important part of the postnatal cardiac maturation [8–10]. Nevertheless, this matter is still open for discussions, since recently no functional coupling between mi-CK and ANT was found in 1–2 week old rats [11]. Another set of important problems has emerged from the studies on the role of ADP as a signalling molecule in the muscle cells. It has been found recently that the skinned fibers prepared from the slow-twitch oxidative muscles (heart, m. soleus) exhibit greatly higher K_m for ADP (200–400 μM) than isolated mitochondria from all sources (10–20 μM) [13–15]. It was concluded that in slow-twitch muscles the permeability of outer mitochondrial membrane (OMM) for ADP is low, and probably controlled by unknown protein complex (protein x) [14, 15]. On the other hand, it is known that although the increased cardiac workload is linearly associated with the increased oxygen consumption, the cytosolic ADP concentration practically does not change in adult heart [6, 12]. In immature heart, however, significant elevations in cytosolic ADP have been observed in response to increased contractility [6]. On the basis of these results, profound alterations in the feedback mechanisms between the structures utilizing energy and mitochondria can be expected to occur during cardiac development. Therefore, the aim of the present study was to address the above mentioned problems in the developing rat heart. By using saponin-skinned cardiac fibers, we studied postnatal *in vivo* changes in capacity of oxidative phosphorylation, affinity of oxidative phosphorylation for ADP, and coupling of mi-CK to oxidative phosphorylation. Laser confocal microscopy was used to follow the changes in the position of mitochondria in the cells.

Materials and methods

Animals

The outbred adult Wistar rats and their puppies of mixed sex were used. This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

Preparation of skinned muscle fibers

After decapitation, the hearts were immediately excised, rinsed in modified Krebs-Henseleit solution (mM) 118 NaCl, 4.7 KCl, 2.52 $CaCl_2$, 1.64 $MgSO_4$, 24.88 $NaHCO_3$, 1.18 KH_2PO_4, 5.55 glucose, 2 K-pyruvate, pH 7.4. The muscle bundles (3–4 mm long, about 1 mm diameter) were excised from ventricular part of the hearts and/or from m. gastrocnemius, and transferred to the solution A containing (mM) 2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA (concentration of free Ca^{2+} = 0.1 μmol/L), 49 mM potassium 2-(N-morpholino)-ethanesulfonate (MES), 20 imidazole, 20 taurine, 0.5 DTT, 5.25 ATP, 15 PCr, 9.5 $MgCl_2$ pH 7.0, adjusted by KOH. In this solution, by means of sharp-ended forceps or needles, the muscle fibers were separated from each other. The fibers were then incubated with mild stirring for 30 min in solution A containing 50 μg/ml saponin for complete permeabilization of the sarcolemma. Permeabilised (skinned) fibers were washed by stirring for 10 min in a solution B consisting (mM): 2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA (concentration of free Ca^{2+} = 0.1 μmol/L), 20 imidazole, 3 KH_2PO_4, 5 K_2-glutamate, 2 K_2-malate, 0.5 DTT, 20 taurine, 4 $MgCl_2$, 100 MES, 5 mg/ml BSA, pH 7.0 titrated by KOH; this washing procedure was performed 3 times to completely remove all metabolites, including trace amounts of ADP. Complete removal of ADP can be demonstrated from respiration recordings, which should show very reproducible initial state-2 rates (designated as v_0) that are insensitive to inhibition by atractyloside. All procedures were carried out at 4°C.

Respiration measurements

The rates of oxygen uptake of muscle fibers were recorded by means of the oxygen monitoring systems (Rank Brothers, UK, or Yellow Spring System, USA) equipped with Clark electrode in a solution B at 25°C (the solubility of oxygen was taken as 430 ng-atoms/ml) [15]. ADP for stimulation of

oxidative phosphorylation was added cumulatively and the maximum rates of respiration (V_m) and the K_m values for ADP were calculated on the basis of Michaelis-Menten equation. In these assays, the v_0, i.e. the respiration rate in the absence of ADP, was subtracted from the ADP-stimulated respiration rates. The respiratory control index (RCI) was calculated as a ratio of $V_m + v_0/v_0$ where v_0 and $V_m + v_0$ are the respiration rates before addition of ADP and the maximal respiration rate at saturating ADP concentrations, respectively. After measurements, the skinned muscle preparations were removed from the chamber, blotted and dried overnight at 105°C for determination of dry weight. The rates of oxygen consumption were normalized per milligram of dry tissue weight.

Assay of functional coupling between mi-CK and ANT

The coupling of mi-CK to ANT was estimated using two approaches. (1) The K_m and V_m values were assayed from [ADP] vs. VO_2 rate relationships in the presence or in the absence of 20 mM creatine; the results were compared with corresponding kinetic parameters without creatine; (2) 20 mM of creatine was added to oxygraphic medium after recording of the respiration in the presence of submaximal concentration of ADP, 0.1 mM. The stimulation of respiration by creatine was expressed as the creatine stimulation index (%Cr) calculated according to the equation: $\%Cr = (V_{O2}^{Cr} - V_{O2}^{0.1\,ADP})/V_{O2}^{0.1\,ADP}$, where V_{O2}^{Cr} and $V_{O2}^{0.1\,ADP}$ denote the respiration rates with and without creatine, respectively.

Determination of the expression of mi-CK

Left ventricles were minced with scissors, placed into cold solution (1 g wet wt per 10 ml) containing (in mM): 100 K_2HPO_4, 1 EGTA, 15 N-acetylcysteine, pH 8.7 and homogenized with Ultra-Turrax homogenizer. The homogenates were incubated for 1 h at 4°C for complete extraction of CK, then centrifuged at 20,000 × g for 20 min, and the supernatant was used in the experiments. Total creatine kinase activity was measured at pH 7.1 and 22°C in a medium containing (mM): 0.5 ADP, 0.8 NADP, 20 glucose, 10 AMP (to inhibit myokinase) and 10 PCr in the presence of 2 IU/ml each of hexokinase and glucose-6P-dehydrogenase at pCa 9. Isoenzyme profile was determined using agarose electrophoresis (1%) performed at 200 V and 4°C for 1 h. Individual isoenzymes were observed by incubating the gel with staining solution soaked paper for 30 min. Staining solution contained (mM): 22 (morpholino)ethanesulfonate, 50 Mg-acetate, 70 glucose, 15 AMP, 120 N-acetylcysteine, 9 ADP, 9 NADP, 120 PCr, 18 IU/ml hexokinase and 6 IU/ml glucose-6P-dehydrogenase at pH 7.4 at 30°C. The isoenzyme bands were visualized by observing the fluorescence of NADPH.

Estimation of the intactness of the mitochondrial membranes

Cytochrome c test for estimation of the status of the OMM was carried out in the oxygraphic medium in the presence of 125 mM KCl as described earlier [16, 17]. To assess the intactness of the inner mitochondrial membrane, 35 μM atractyloside was added to the polarographic medium in the end of the experiment [18].

Treatment of fibers with trypsin

Digestion of fibers was carried out in solution B at 4°C with 125 μg/ml trypsin (5 μM) during 15 min followed by washing twice during 10 min in solution B without trypsin.

SDS-PAG electrophoresis

The tricine-SDS-PAGE system [19] with 13.3% acrylamide, 3% bis-acrylamide and 4% stack gel (82 × 50 × 0.75 mm) was used. The muscle fibers were mechanically disintegrated in liquid nitrogen and the muscle powder was solubilized in a 10-fold volume of the buffer solution containing (mM) 10 Tris-HCl, 1 $MgCl_2$, 1 EDTA, and 1 phenylmethylsulfonyl fluoride, pH 7.0. To prepare the samples for electrophoresis, the homogenates were diluted twice in a sample buffer with the final concentrations of 4% SDS, 12.5% glycerol, 50 mM Tris-HCl, 2% mercaptoethanol, 0.01% Serva Blue G, and incubated for 30 min at 40°C. Twenty μl of the sample (1 mg wet wt) was applied onto the gel in a Mini-Protean II (BioRad) chamber. The electrophoresis was run under current of 20 mA per gel for 3 h. Relative mobility of the proteins was determined using molecular weight markers (Sigma Co, USA). After electrophoresis, the gels were fixed in 50% methanol, 10% acetic acid for 30–60 min, stained in 0.025% Serva Blue G, 10% acetic acid, and destained in 10% acetic acid. The gels were scanned with LKB 2202 ULTRASCAN laser densitometer.

Imaging of mitochondria in skinned cardiac fibers

The cardiac fibers were gently stirred in solution B in the presence of 5 mM pyruvate and 2 mM malate (i.e. in conditions of basal respiration) and the mitochondrion-selective dye MitoTracker Red CMXRos (Molecular Probes Inc., OR, USA) (200 nM) in the dark during 30 min. Thereafter the fibers were washed 3 times in above-mentioned medium without dye during 15 min by stirring, to minimize background fluorescence. Finally, the stained fibers were placed on and attached to the specimen glass by a drop of glycerol-

PBS (phosphate-buffered saline) (mixed 1:1), and covered by the cover glass. All these procedures were performed at 25°C. The mitochondria were visualized and scanned by MRC 1024 BioRad laser confocal microscope with the PlanApo 60x/1.40 oil objective (Japan). The specimen was illuminated by krypton/argon laser (15 mW) light (568 nm) and the emitted light signal was filtered by 605DF32 filter and collected according to Kalman method using the BioRad aquisition system.

Results

Changes in heart to body weight weight ratio (HW/BW)

Figure 1 shows the changes in the HW/BW in rats during postnatal development. In newborn rats and during first 2 weeks of *post partum* life this ratio is very high, close to 6, and then decreases within 40 days to the normal value, characteristic of normal adult heart. These results are in concord with earlier observations [8, 11]. Postnatal decrease in HW/BW ratio indicates significant augmentation of the efficiency of cardiac muscle contraction in maintaining blood circulation in the body within the first 2 months of life. Therefore, it is interesting to correlate these changes with alterations in the intracellular bioenergetic parameters.

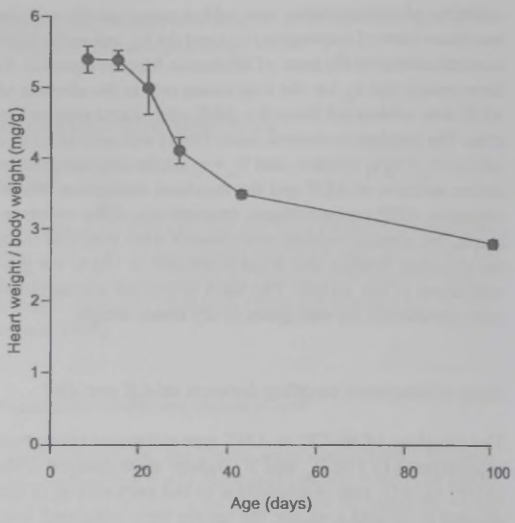

Fig. 1. Changes in heart weight (HW) to body weight (BW) ratio during postnatal development of the rat hearts (here and in following Figs (except Figs 7 and 8), the means ± S.E.M. of 7–24 experiments are given).

Changes in the basic characteristics of mitochondrial respiration

Figure 2 shows the developmental changes in several important parameters of mitochondrial respiration, measured in skinned cardiac fibers *in vivo*. The maximal ADP-activated respiration rate (V_m) increases most rapidly within the first week of the postnatal development, when approximately 50% of the adult levels is achieved. Further development is associated with slower increase in V_m, so that the adult levels are obtained not earlier than after 3 weeks. Such an retarded development in the maximal respiration rate after 1 week was observed in rat heart also earlier [8]. We did not find any significant alteration in the initial, State 2 respiration in the presence of substrates before addition of ADP (Fig. 2). Therefore, the respiratory control index, RCI, follows the course of development of the maximal rate of respiration (Fig. 2). Irrespective of the age, the cytochrome c test in skinned muscle fibers was negative suggesting intact OMM (results not shown).

Changes in the affinity of mitochondrial oxidative phosphorylation for ADP in vivo

Analysis of the respiratory properties of saponin-skinned muscle fibers, prepared from the different types of muscles have revealed that the apparent Km for ADP in regulation of ADP-dependent respiration in cardiac muscle and other slow-twitch oxidative muscles (i.e. m. soleus) is much higher (200–400 μM) than in isolated from all sources of mitochondria (10–20 μM), or in skinned fibers of glycolytic fast-twitch muscles [13–15]. These experiments suggested that in the cardiac cells (and other slow-twitch muscles) *in vivo*, the permeability of the OMM porin pores is controlled by some still unknown cytoplasmic proteins, probably associated with

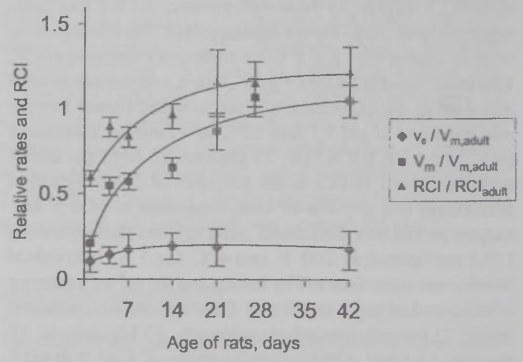

Fig. 2. Developmental changes in respiration rate without ADP (v_0), ADP-stimulated maximal rate of respiration (V_m, v_0 subtracted), and respiratory control index (RCI), normalised to adult (3 months-old) levels, in skinned cardiac fibers.

cytoskeleton [14, 15] (see also Discussion). Because of this, it was interesting to find out whether and how this type of control over mitochondrial activity changes during postnatal development. Accordingly, Fig. 3 shows that at birth (in the fibers taken from 1 day-old rat heart) the K_m for ADP is relatively low, as compared with the adult heart, and then steadily increases to the adult levels within 6 weeks. Thus, the *post partum* increase in the capacity of oxidative phosphorylation (V_m, Fig. 2), is associated with decreased affinity of mitochondria to ADP. Figure 4 shows the tissue-specificity of these changes: in contrast to heart muscle, the apparent K_m for ADP in the skinned fibers from fast skeletal muscle m. gastrocnemius is very low (10–15 μM) in newborn age, and does not change during the muscle development. Thus, age-dependent decrease of mitochondrial affinity to ADP is confined to oxidative slow-twitch muscles.

Changes in regulation of respiration by mi-CK

Figure 3 shows that contrary to the developmental changes of mitochondrial affinity for ADP, the mi-CK activity assayed either directly in cardiac homogenates or by stimulation of respiration by 20 mM creatine in the presence of submaximal ADP concentrations (0.1 mM) (creatine index, %Cr) shows delayed postnatal development. These activities are absent during first week, rather low between 2nd and 3rd weeks of postnatal life, but then rapidly increase and reach their high value characteristic of adult hearts within 40 days of development (Fig. 3).

Parallel increase in mi-CK activity and creatine index shows that once expressed, mi-CK becomes functionally coupled to ANT. Because then the ADP is produced in the intermembrane space of mitochondria by the mi-CK activated by addition of creatine, the apparent K_m for ADP in regulation of respiration decreases [13, 15]. Indeed, Fig. 5 clearly demonstrates corresponding dynamics in K_m values: due to the

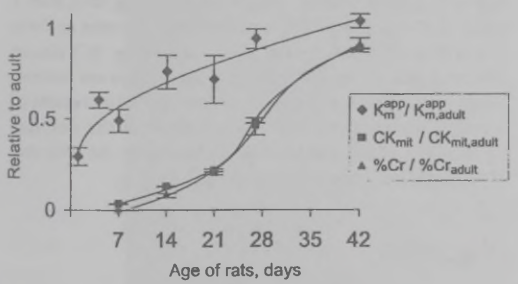

Fig. 3. Developmental changes in affinity of mitochondrial respiration for ADP (K_m), creatine index (% of activation of respiration by 20 mM creatine in the presence of 0.1 mM ADP), and activity of mi-CK kinase, normalised to adult (3 month-old) levels, in skinned cardiac fibers.

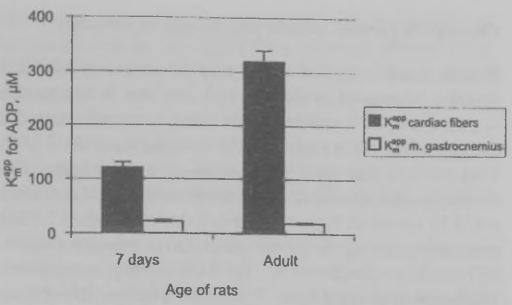

Fig. 4. Comparison of the developmental changes in K_m for ADP in skinned fibers from heart and m. gastrocnemius.

absence of mi-CK activity in first days after birth (Fig. 3), creatine has no effect on K_m for ADP. At three weeks it still exerts a very little effect on the K_m for ADP due to the low activity of mi-CK (Fig. 3), but after that the K_m for ADP in the presence of creatine significantly decreases, due to fast increase in expression of mi-CK. Thus, as it can be clearly seen in Fig. 3, the postnatal maturation of cardiac muscle is associated with the developmental shift in metabolic control over oxidative phosphorylation from the mechanism controlling the diffusion of ADP through the OMM to the mechanism mediated by the mi-CK. Interestingly, such a switch to another type of control exactly coincides with the period of rapid decrease in the HW/BW ratio (Fig. 1), and one can in fact find a nice negative linear correlation between these two parameters (Fig. 6), while development of the rat heart corresponds to decrease in HW/BW ratio from 5.5 to about 3, a parallel increase in creatine-stimulated respiration does occur. Thus, postnatal increase in the effectiveness of heart function (estimated as decrease in HW/BW ratio) appears to be tightly related to the development of the mitochondrial mechanism of PCr synthesis in cardiac muscle.

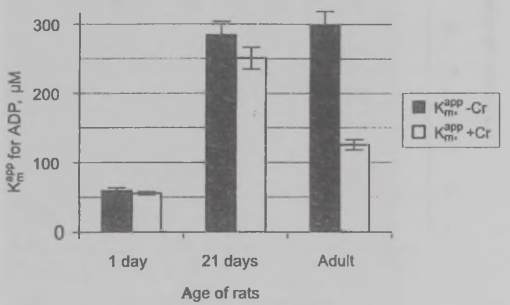

Fig. 5. Developmental changes in creatine effects on the affinity of mitochondrial respiration for ADP.

Changes in cardiac protein profile and morphology

Recent study revealed that a trypsin-sensitive 27.5 kD protein, expressed in slow-twitch, but not in fast-twitch muscles, can be a candidate for protein x controlling the activity of the porin pores (VDAC channels) in OMM [20]. Therefore, it was interesting to assay as to whether the developmental alterations in the tissue content of that protein could be revealed. Figure 7A demonstrates that the 27.5 kD protein was among the proteins the relative amount of which increased in parallel to K_m for ADP during maturation. Treatment of skinned fibers with trypsin before SDS-PAGE resulted in exclusion of this protein from the proteinogram (Fig. 7B). These results suggest that 27.5 kD protein is developmentally upregulated in rat heart. This makes it more important to identify the nature of this protein and its possible relevance to regulation of mitochondrial function *in vivo*.

Related to this issue are the changes in the fiber orientation and mitochondrial position relatively to fibers in cardiac cell during postnatal development. Indeed, recently we showed that in adult rat hearts the mitochondrial position is very precisely fixed between Z-lines, and alteration of this precise structural organisation in desmin-deficient cells leads to appearance of mitochondrial subpopulation with altered control characteristics [21]. Therefore, we used confocal microscopy and mitochondria-specific fluorescent probe to follow the changes in mitochondrial orientation in the skinned cardiac cells during development. Figure 8 shows that these changes are rather remarkable. At birth, the mitochondria

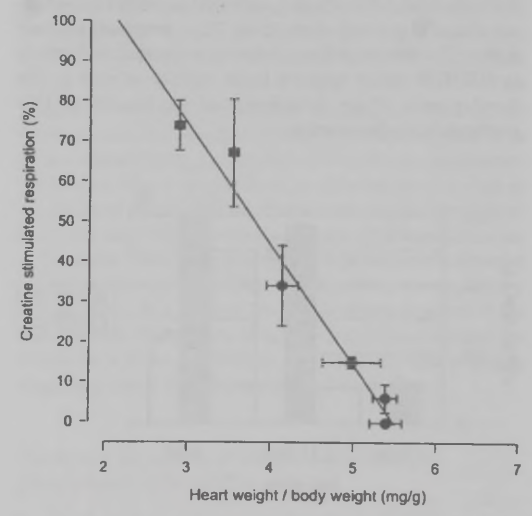

Fig. 6. Negative correlation between the stimulation of respiration by creatine and HW/BW ratio.

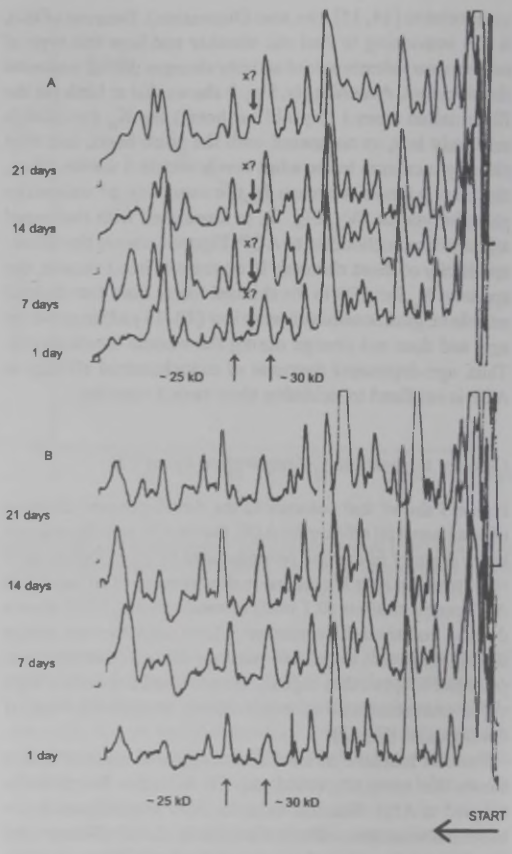

Fig. 7. Developmental changes in proteins from skinned heart fibers before (A) and after limited proteolysis with 125 μg/ml trypsin (B).

appeared to form random- shaped clusters (Fig. 8A); after 1 week of development the mitochondria become to form arrays (Fig. 8B), and by the third weeks (Fig. 8C) almost similar to adult (Fig. 8D) mitochondrial arrays are formed. These alterations are in concord with the earlier results of electron microscopy study, showing that the cellular organization of mitochondria is completed by the 24th day of the postnatal maturation of rat heart [3].

Discussion

The results of this study show that postnatal development of rat myocardium is associated with a gradual increase in the maximal capacity of oxidative phosphorylation (defined as V_m), reaching the adult levels by 4 weeks *in situ* (Fig. 1).

Similar dynamics has been reported for palmitate oxidation rate in rat heart homogenates during *post partum* maturation [4]. Two mechanisms underlie the rise in cardiac oxidative capacity: (i) increases in the specific activity of mitochondrial enzymes and cytochrome contents, and (ii) increase in the mitochondrial protein content per unit of the tissue weight [1, 2, 4, 5, 22–26]. Kinnula and Hassinen [1] have shown that the mitochondrial cytochrome content increases sharply during first two days after birth, reaching the adult levels already by the 7th day. Similarly, the ANT content and activity rise to 75% of adult levels during first 10 *post partum* days [5]. These changes are accompanied by the increase in the tissue content of mitochondria. However, the latter parameter continues to increase throughout the later period of development. Therefore, the tissue content of mitochondria becomes more than twice higher in adult rat heart than in the heart of 7 day-old rat [1]. This is in accord with the finding that the volume fraction of mitochondria increases after 10 days of rat cardiac development [27]. Thus, the initial rapid increase in V_m (Fig. 2) may be caused by increases in mitochondrial contents of adenine nucleotides, ANT and cytochromes, as well as by increased tissue content of mitochondria. The later increase in Vm could predominantly be due to further increase in the tissue content of mitochondria [2, 4].

In our experiments, no significant changes in basal respiration rate without ADP (V_0) was observed throughout the whole postnatal period (Fig. 2). Also, the respiration rates in the presence of saturating ADP returned to the levels equal to V_0 after addition of atractyloside to polarographic medium (not shown). This fact shows that the neonatal mitochondria possess the intact inner membrane. However, since in

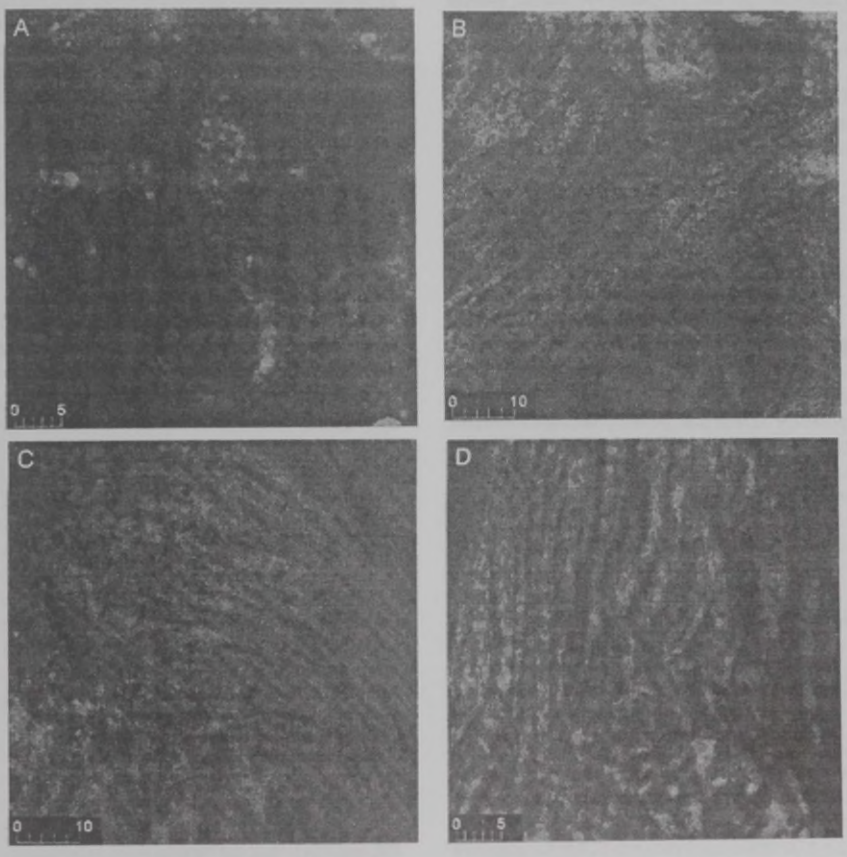

Fig. 8. Developmental changes in localization of mitochondria in rat skinned cardiac fibers. A – 1 day; B – 7 days; C – 21 days *post partum*; D – adult.

neonatal hearts mitochondrial content is lower than in adult hearts, the same respiration rate may be explained by increased proton permeability in neonatal mitochondria. This conforms to the earlier finding that the mitochondria from the neonatal liver exhibit higher passive proton permeability compared to adult liver mitochondria [28].

Due to unchanged V_0, the RCI increased in parallel to V_m (Fig. 2), demonstrating the developmental improvement of the coupling between the processes of substrate oxidation and phosphorylation of ADP.

The novel result of this study is that postnatal development in rat heart is associated with decrease in the affinity of mitochondria to ADP (Fig. 3.), as the K_m for ADP increased from the newborn value (K_m = 50 μM, Fig. 5) to adult levels (300 μM, Figs 4 and 5) during 1 month. The maximal value of K_m for ADP is well in the range (200–400 μM) observed earlier in the oxidative slow-twitch muscles like the heart and m. soleus of different species, including the human muscle [13–15]. This is by an order of magnitude higher than in mitochondria isolated from all possible muscle types (10–20 μM) [14]. Similarly to isolated mitochondria, the low value of K_m for ADP is also typical for the skinned fibers of the glycolytic fast-twitch skeletal muscles, like m. gastrocnemius [14, 15]. It was also demonstrated that in slow-twitch muscle fibers the K_m can be decreased by disruption of the OMM under hypoosmotic conditions, or by fast and rather selective treatment of muscle fibers with different proteases [14]. That the high K_m for ADP is not simply due to diffusion barrier for adenine nucleotides in the skinned muscle core, became evident from the findings that it remains high when either the myosin is extracted by high KCl treatment, or if the mitochondrial ATPases are partially inhibited by oligomycin [21]. Based on these results, it has been hypothesized that in the slow-twitch muscle cells *in vivo* the permeability of the OMM porin pores for ADP is low, possibly because some still unknown cytoplasmic protein (protein x) limits the diffusion of ADP through the membrane [14, 15]. At the same time, similar control mechanism appears to be absent in fast-twitch glycolytic muscle fibers [14–15]. Figure 4 clearly demonstrates that the developmental increase in K_m for ADP is characteristic of heart, but not of glycolytic muscle. Analysis of the cardiac protein profile before and after trypsin treatment showed that the expression of the putative candidate for protein x with molecular weight of 27.5 kD increases in parallel to that in K_m for ADP values. Therefore, we have most likely observed the developmental upregulation of the same specific mechanism which controls the access of ADP from the cytoplasmic compartment to the mitochondria, as earlier described in adult heart [14]. Comparison of the dynamics in K_m for ADP and of expression of 27.5 kD protein with laser confocal imaging (Fig. 8) shows that the maximum limitation of the ADP diffusion through the OMM is achieved by the same time when the formation of mitochondrial arrays between the myofibrils is completed, i.e. between the 3rd and 4th weeks of postnatal development. This finding coincides with earlier morphological studies showing that the cellular distribution of mitochondria becomes finished by the 24th day of rat's postnatal life [3]. The nature of the mechanism controlling the diffusion of adenine nucleotides through the OMM is not clear. Many facts suggest that it involves interaction of the mitochondria with other cytoskeletal proteins. According to Leterrier *et al.* [29], the OMM can be viewed as a receptor sites, to which several cytoskeletal proteins can bind. Among these proteins are the microtubule-associated proteins, the cytoplasmic motors kinesin and dynein, intermediate filaments, annexin V and desmin [29–31]. Sun *et al.* [31] have shown that annexin V, an intracellular protein, interacts with a 27 kDa mitochondrial protein that is possibly part of a larger complex. That desmin really plays a role in orientation of mitochondria was recently demonstrated by using the desmin gene-knockout mice: the loss of desmin was associated with the disorganisation of the mitochondrial arrays and the appearance of the random-positioned clusters of mitochondria between the disoriented myofibrils. In parallel, the kinetic analysis of the ADP-dependent respiration allowed to identify the population of mitochondria showing increased affinity to ADP, as compared to mitochondria in skinned fibers of wild type muscles [21]. This experiment also suggests that relatively low K_m for ADP in newborn rat heart fibers (Fig. 3) is associated with improper organisation of the intracellular structures.

Figure 3 demonstrates that the mi-CK in rat heart becomes to be expressed between the first and second week, and that significant increase in activity can be observed only after 3 weeks of postnatal development, which confirms the earlier data by Dowell [32]. Compared to other species, the mi-CK appears and develops postnatally significantly later in rat heart than in other species studied so far. In lamb and guinea-pig the activity can be detected before birth [33]. In rabbit, the activity of mi-CK creatine appears at 3 day after birth and increases to maximum/adult level at 18 days of age [8, 34]. Interestingly, the dynamics of biosynthesis of mi-CK is exactly matched by the activation of respiration by creatine in skinned cardiac fibers (Fig. 3), indicating the functional coupling between newly produced mi-CK and ANT. Similarly, concomitant progression in mi-CK activity and creatine-activated respiration of mitochondria has been observed in rabbit heart [8] and the rat skeletal muscles [35]. Thus, it seems that irrespective of the species, the postnatal expression of mi-CK in muscle cells is associated with functional coupling of this enzyme to the processes of oxidative phosphorylation.

In such a coupling, ATP, produced in the mitochondria is effectively transformed to PCr in the cleft between the mi-CK and ANT, and the formed ADP is returned via ANT into the matrix, where it stimulates the respiration coupled to

rephosphorylation of ADP. This mechanism has the following consequences: (i) it creates the intramitochondrial ADP-ATP cycle, which is isolated from the cytosolic ADP-ATP pools, and (ii) the maximal rates of respiration can be achieved at much lower concentrations of cytosolic adenine nucleotides than in the absence of coupling [13, 14]. Therefore, the greater amount of mi-CK becomes attached to the inner mitochondrial membrane during the development, the larger is the decrease in the apparent K_m for ADP, if respiration is stimulated by creatine (Fig. 5). Recently, the postnatal increase in expression of ANT was demonstrated [5, 6]. In rat, this process starts immediately after birth [5], hence preceding the change in mi-CK (Fig. 3). Obviously, early expression of ANT is an important prerequisite for establishment the interaction with mi-CK in the course of development.

The results of the present study, in conjunction with earlier information, allow to delineate the postnatal changes in the mechanisms of metabolic control as the function of the development of compartmentation of the cellular processes *in vivo*. In newborn heart, the mitochondria are characterized by relatively low activity of ANT together with a small pool of intramitochondrial adenine nucleotides [4, 5], due to which the ANT represents the main flux limiting step [4]. In the cells, they are not organized into arrays, but form separate random clusters (Fig. 8A). Besides, the mitochondria lack mi-CK, as well as they exhibit negligible barrier for ADP diffusion through the outer membrane (Fig. 3). In these conditions the oxidative phosphorylation can be activated provided that the cytosolic ADP concentration increases in response to increased workload. Indeed, very recently Portman *et al.* have observed a significant increase in the cytosolic ADP concentration in the neonatal sheep heart, in response to epinephrine stimulation [6]. Moreover, the Michaelis-Menten kinetics in respiration vs. ADP concentration was observed [6]. Thus, in neonatal heart, the cytosolic ADP can easily diffuse through the OMM, and via ANT stimulate the respiration, whereas the overall flux is limited at the level of ANT only. Maturation of cardiac muscle represents a shift to much higher level of compartmentation [36], and therefore, to another mechanisms of regulation. This is demonstrated morphologically as the formation of mitochondrial arrays between the myofibrils (Fig. 8, [37, 38]) so that the major part of mitochondria is positioned at the levels of each sarcomere between the Z lines by desmin filaments [21]. Ogata and Yamasaki have demonstrated that in adult muscle cells the mitochondria form three-dimensional organized networks, which are very different in glycolytic and oxidative muscles [38]. Our results show that kinetically mitochondria in these organized networks are characterized by decreased permeability of OMM to ADP (Fig. 3), and by coupling of Mi-CK to ANT (Fig. 5). On the other hand, the MM-CK is known to be functionally coupled to myofibrillar, sarcolemmal and sarcoplasmic reticulum ATPases [39, 40]. Thus, the isolated ATP-ADP cycles in different cellular compartments are united by different creatine kinases so that the random diffusion of metabolites between the mitochondria and ATPases is replaced by the vectorial ligand (i.e. Pi-group or creatine) transfer [39]. That this system operates very effectively is evident from the facts that increases in mitochondrial respiration linearly correspond to increments in cardiac workload, without significant alterations in the cytosolic ADP and ATP concentrations [6, 12], and from the negative linear correlation between the HW/BW ratio and creatine activated respiration in developing rat heart (Fig. 6).

In conclusion, the OMM and mi-CK exert their control over mitochondrial respiration in different periods of postnatal development. In early period, i.e. within 2 weeks, the diffusion of ADP to mitochondria becomes increasingly limited probably due to upregulation of protein x. Later (after 3 weeks) the control shifts to mi-CK, which, by coupling to ANT, allows to maximally activate the processes of oxidative phosphorylation despite limited access of ADP through the OMM. These developmental changes reflect significant alterations in compartmentation of cellular metabolic processes.

Acknowledgements

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Lack of dystrophin is associated with altered integration of the mitochondria and ATPases in slow-twitch muscle cells of MDX mice

Urmo Braun [a], Kalju Paju [a], Margus Eimre [a], Evelin Seppet [a], Ehte Orlova [a], Lumme Kadaja [a], Sonata Trumbeckaite [b], Frank N. Gellerich [b], Stephan Zierz [b], Harald Jockusch [c], Enn K. Seppet [a,*]

[a] *Department of Pathophysiology, Faculty of Medicine, University of Tartu, Ravila 19, 50411 Tartu, Estonia*
[b] *Muskellabor der Neurologischen Klinik, Martin Luther Universität Halle-Wittenberg, HallelSaale, Germany*
[c] *Lehrstuhl für Entwicklungsbiologie und Molekulare Pathologie, Fakultät für Biologie, Universität Bielefeld, Bielefeld, Germany*

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Abstract

The potential role of dystrophin-mediated control of systems integrating mitochondria with ATPases was assessed in muscle cells. Mitochondrial distribution and function in skinned cardiac and skeletal muscle fibers from dystrophin-deficient (MDX) and wild-type mice were compared. Laser confocal microscopy revealed disorganized mitochondrial arrays in m. gastrocnemius in MDX mice, whereas the other muscles appeared normal in this group. Irrespective of muscle type, the absence of dystrophin had no effect on the maximal capacity of oxidative phosphorylation, nor on coupling between oxidation and phosphorylation. However, in the myocardium and m. soleus, the coupling of mitochondrial creatine kinase to adenine nucleotide translocase was attenuated as evidenced by the decreased effect of creatine on the K_m for ADP in the reactions of oxidative phosphorylation. In m. soleus, a low K_m for ADP compared to the wild-type counterpart was found, which implies increased permeability for that nucleotide across the mitochondrial outer membrane. In normal cardiac fibers 35% of the ADP flux generated by ATPases was not accessible to the external pyruvate kinase-phosphoenolpyruvate system, which suggests the compartmentalized (direct) channeling of that fraction of ADP to mitochondria. Compared to control, the direct ADP transfer was increased in MDX ventricles. In conclusion, our data indicate that in slow-twitch muscle cells, the absence of dystrophin is associated with the rearrangement of the intracellular energy and feedback signal transfer systems between mitochondria and ATPases. As the mechanisms mediated by creatine kinases become ineffective, the role of diffusion of adenine nucleotides increases due to the higher permeability of the mitochondrial outer membrane for ADP and enhanced compartmentalization of ADP flux.

Keywords: Mitochondria; Adenosine 5′-triphosphatase; Muscle; Respiration; Regulation; Energy transfer

1. Introduction

The mechanisms exerting control over oxidative phosphorylation in muscle cells in vivo, i.e., the interaction of mitochondria with surrounding intracellular structures are unclear. According to classical concepts, the increased cytosolic [ADP] is a main signal for the stimulation of respiration [1,2]. Nevertheless, new data suggest that this theory cannot be universally applied. Recently, the dynamic state of

* Corresponding author. Fax: +372-7-374-372;
E-mail: enn@ut.ee

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the permeability of the mitochondrial outer membrane (MOM) for ADP has been revealed; the barrier function of MOM increases significantly in the presence of dextrans or albumins added to simulate the effects of cytosolic proteins [3,4]. Experiments using saponin-skinned fibers have demonstrated that fast-twitch muscles (e.g. m. gastrocnemius) display high apparent affinity to ADP (K_m = 10–20 μM) which is comparable to that in isolated mitochondria. In contrast, slow-twitch heart and m. soleus fibers exhibit much lower affinity to ADP (K_m = 200–400 μM), yet it can be increased (i) under the influence of creatine, (ii) after treatment of the fibers with proteases, and (iii) in conditions of knockout of the creatine kinase genes [5–9]. Data from ^{31}P-NMR studies indicate that changes in cellular respiration are associated with cytosolic ADP fluctuations in fast-twitch muscle cells, but not in slow-twitch muscles [10]. These results point to principal differences in the regulation of mitochondria in vitro and in vivo, and have given rise to speculation on muscle type-dependent control over cellular respiration in vivo. Accordingly, in glycolytic fast-twitch fibers the mitochondrial respiration can be effectively adjusted by cytoplasmic ADP as its concentration is changed largely in response to increasing workload and it may easily pass the porin pores between extra- and intramitochondrial compartments [6,8,10]. In oxidative slow-twitch myocytes the permeability of the porin pores to adenine nucleotides is restricted. Therefore, integration between the ATPases and mitochondria is ensured by creatine and adenylate kinases which, by functioning as the vectorial shuttles of the energy-rich phosphate moiety, are able to stimulate the respiration despite the constancy of cytoplasmic [ADP] [4,6,8,11,12].

The nature of the mechanisms limiting access of ADP to mitochondria in slow-twitch muscles is currently unclear. Ultrastructural and biochemical studies demonstrate tight connections between mitochondria and cytoskeletal structures, particularly with annexin V and desmin [13–15]. Kay et al. [16] have found that the absence of desmin results in the appearance of sarcomeres with disorganized structure and of a mitochondrial population displaying low affinity to ADP. Probably this population of mitochondria has lost control over the diffusion of ADP through the MOM. Another line of evidence shows that mitochondria isolated from dystrophin-deficient muscles exhibit disturbances of oxidative phosphorylation [17–20] while, on the other hand, ^{31}P-NMR spectroscopy has revealed an increased concentration of free ADP in the skeletal muscles of Duchenne muscular dystrophy (DMD) patients [21,22]. These results can be interpreted as evidence that the cytoskeletal proteins such as desmin and dystrophin are important in governing the mitochondrial function via modulating the permeability of MOM and increasing the feedback signal by ADP to stimulate the mitochondria.

The current study addresses further the potential role of dystrophin in the regulation of the mitochondrial function in vivo by using skinned muscle fibers of MDX mice. The advantage of the skinned fiber technique is that it allows the assay of the entire cellular mitochondrial population in its natural relationships with other intracellular structures [24]. Attention is paid to the controversy between the data showing either normal capacity of mitochondria to produce ATP [21,22], or muscle type-dependent alterations in oxidative phosphorylation [23]. For the first time the kinetics of the regulation of respiration by ADP and the status of the coupling between mitochondrial creatine kinase (mi-CK) and adenine nucleotide translocase (ANT) are characterized in muscles lacking dystrophin. We provide evidence that the deficit of dystrophin in MDX mouse slow-twitch muscle is associated with impaired coupling of mi-CK to oxidative phosphorylation, as well as with facilitated ADP transfer from ATPases to mitochondria.

2. Materials and methods

2.1. Animals

The 10–11-month-old dystrophin knockout homozygous female mice were produced by Prof. H. Jockusch's group (University of Bielefeld). The animals were kept, fed and studied in accordance to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Preparation of skinned muscle fibers

The mice received 5 units/g b.wt. of heparin intraperitoneally and were anesthetized with ethyl ether thereafter. The skin of the back limbs was quickly removed, the limbs and heart were excised and placed into ice-cold saline for fast cooling and removing the blood. Thereafter, thin muscle bundles (3–4 mm long, about 1 mm in diameter) from heart, m. soleus, m. extensor digitorum longus and m. gastrocnemius were excised in ice-cold solution A, and using sharp-ended needles the muscle fibers were separated from each other leaving only small areas of contact. Then the fibers were transferred to vessels with ice-cooled solution A containing 50 μg of saponin per ml and incubated at mild stirring for 30 min at 4°C for complete solubilization of the sarcolemma. Permeabilized (skinned) fibers were then washed three times in solution B for 10 min by stirring to remove completely all metabolites, especially the trace amounts of ADP.

2.3. Imaging of mitochondria in skinned cardiac fibers

The cardiac fibers were gently stirred in solution A in the presence of the mitochondrion-selective dye MitoTracker Red CMXRos (Molecular Probes, Eugene, OR, USA) (200 nM) in the dark for 30 min. Thereafter the fibers were washed three times in the above-mentioned medium without dye for 15 min by stirring, to minimize background fluorescence. The stained fibers were incubated in HistoPrep 10% prefilled buffered 10% formalin solution (Fisher Scientific, Pittsburgh, PA, USA), containing formaldehyde 4%, methyl alcohol 1%, phosphate buffer in water, for 15 min. Thereafter the fibers were placed on the specimen glass and attached to it with a drop of glycerol-PBS (phosphate-buffered saline) (mixed 1:1), and covered by the cover glass. All procedures were performed at 20°C. The mitochondria were visualized and scanned by MRC 1024 Bio-Rad laser confocal microscope with the PlanApo 60×/1.40 oil objective (Japan). The specimen was illuminated by krypton/argon laser (15 mW) light (568 nm) and the emitted light signal was filtered by 605DF32 filter and collected according to the Kalman method using the Bio-Rad acquisition system.

2.4. Determination of the tissue content of cytochrome aa_3

The tissue content of cytochrome aa_3 was assayed by registering the difference spectra of reduced and oxidized cytochromes in cardiac homogenates according to Fuller et al. [25], using a Perkin-Elmer Lambda 900 spectrophotometer.

2.5. Respirometric investigation of dependence of oxidative phosphorylation on ADP

The rates of oxygen uptake were recorded using a high resolution respirometer (Oroboros Oxygraph, Paar, Graz, Austria) equipped with a Clark oxygen electrode in solution B, complemented with 10 mM pyruvate and 2 mM malate at 25°C. The concentration of ADP in the incubation medium was cumulatively increased and the K_m and $V_{max(ADP)}$ values were calculated from the [ADP] versus respiration rate (the basal rate of respiration, v_0, subtracted) relationships on the basis of the Michaelis-Menten equation. The relative decrease in K_m values for ADP under the influence of 20 mM creatine, expressed as the ratio of K_m without creatine to that with creatine (creatine index), was used to measure the extent of functional coupling between mi-CK and ANT.

2.6. Determination of the rivalry between mitochondria and pyruvate kinase-phosphoenolpyruvate system for ADP produced in ATPase reactions

Approx. 3 mg of skinned muscle fibers were incubated in the spectrophotometric (Perkin-Elmer Lambda 900) cuvette containing medium B complemented with 5 mM phosphoenolpyruvate (PEP), 20 IU/ml pyruvate kinase (PK), 20 IU/ml lactate dehydrogenase (LDH) and 0.24 mM NADH at 25°C. The medium was continuously stirred with a magnetic stirrer operated by the Variomag Telemodul (H+P Labortechnic, Germany). The decrease in NADH in the incubation medium was registered at 340 nm before and after addition of 1 mM MgATP, as well as after subsequent additions of the substrates (10 mM glutamate and 2 mM malate) and 98 μM

atractyloside. Care was taken that neither of the coupled enzymes (PK nor LDH) was limiting the rate of the ATPase reaction, the latter being estimated from the stable and linear time-dependent portions of recordings. The total volume of the reaction mixture was 1.5 ml. The extent of mitochondrial rephosphorylation of the ADP produced in the ATPase reactions was quantified as the decrease in the flux through the PEP-PK system. In some experiments parallel changes in respiration were registered in order to demonstrate the coupling between changes in [NADH] and mitochondrial function (Fig. 3).

2.7. Determination of creatine kinase activity and isoenzyme profile

The cardiac tissue frozen at −70°C was thawed at 0°C and homogenized in medium containing (in mM): EGTA 1, dithiothreitol 1, $MgCl_2$, HEPES 5 and 1% Triton X-100 (1:20 w/v), pH 8.7 by an Ultra-Turrax homogenizer (Janke and Kunkel, Germany) (13 500 rpm) on ice during 30 s followed by a 1 min period of keeping the probe on ice. Thereafter the same cycles were repeated twice, and the homogenates were left on ice for 1 h to allow complete extraction of the creatine kinase. Then the CK activity was measured spectrophotometrically (Perkin-Elmer Lambda 900) in the stirred medium containing (in mM): glucose 20, adenosine monophosphate 20, dithiothreitol 0.3, magnesium acetate 3, NADP 1, ADP 1, Tris-HCl 50, pH 7.4, in the presence of 2 IU/ml hexokinase and 2 IU/ml glucose-6-phosphate dehydrogenase, at 25°C. The aliquot of muscle homogenate was added, and after stabilizing the optical density at 340 nm, the reaction was started by addition of 20 mM phosphocreatine and the rate of NADPH formation was registered. Care was taken that the creatine kinase reaction occurred linearly in time, and that the coupled enzyme system was not limiting the overall reaction rate. The CK isoenzyme profile was assayed electrophoretically according to Vannier et al. [26].

2.8. Reagents and solutions

All reagents were purchased from Sigma (St. Louis, MO, USA) except the enzymes which were obtained from Serva (Germany). Solution A contained, in mM: CaK_2EGTA 2.77, K_2EGTA 7.23, $MgCl_2$ 6.56, dithiothreitol (DTT) 0.5, potassium 2-(*N*-morpholino)ethanesulfonate (K-MES) 50, imidazole 20, taurine 20, Na_2ATP 5.3, phosphocreatine 15, pH 7.1 adjusted at 25°C. Solution B contained, in mM: CaK_2EGTA 2.77, K_2EGTA 7.23, $MgCl_2$ 1.38, DTT 0.5, K-MES 100, imidazole 20, taurine 20, K_2HPO_4 3, and 5 mg/ml bovine serum albumin (BSA), pH 7.1 at 25°C. The stock solutions of ATP or ADP used to add ATP or ADP into solution B contained $MgCl_2$ (0.8 mol/mol ATP or 0.6 mol/mol ADP).

2.9. Statistical analysis

One-way ANOVA with Newman-Keuls post hoc test was used for data analysis. The means ± S.E.M. are presented.

3. Results

Table 1 shows significantly increased heart weight of MDX mice compared to wild-type control mice. As the body weights of MDX mice also tended to be increased, the heart to body weight ratios became identical in both groups. Thus, the absence of dystrophin in MDX mice was not associated with the development of cardiac hypertrophy.

Fig. 1 demonstrates fairly organized mitochondrial arrays between the myofibrils in all muscle types of

Table 1
Anatomical data of the MDX and wild-type mice

Type of mouse	Body weight (g)	Heart weight (mg)	Heart/body weight ratio (mg/g)
Wild-type ($n=5$)	27 ± 0.6	132.8 ± 7.8	5 ± 0.3
MDX ($n=5$)	31.5 ± 2.1	158.7 ± 5.5*	5.1 ± 0.3

The means ± S.E.M. are given. n, number of animals. *$P < 0.05$ versus wild-type parameter.

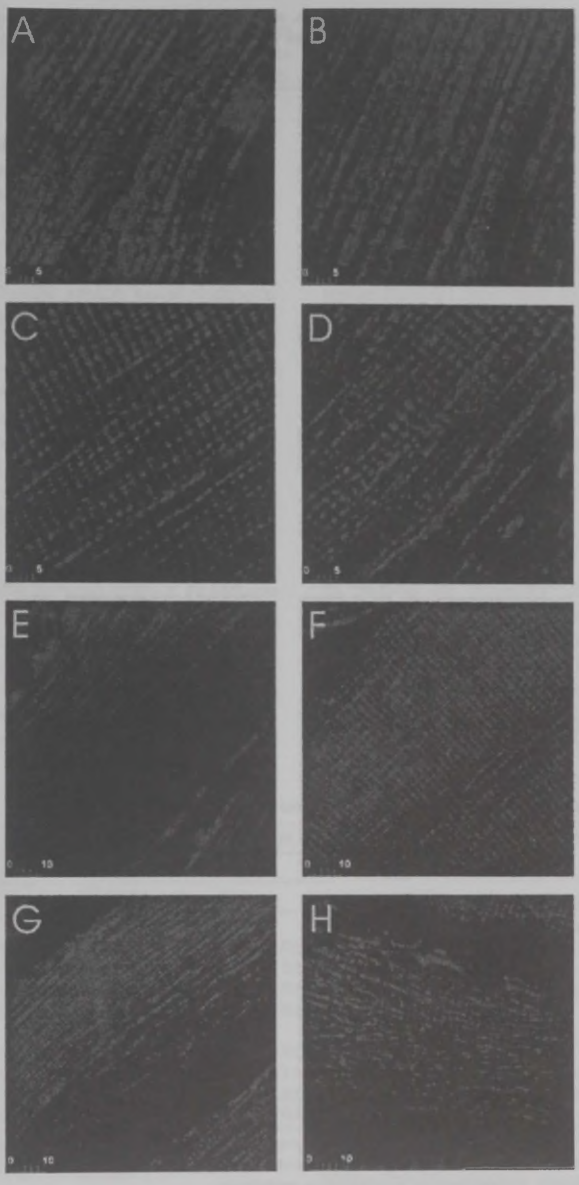
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Fig. 1. Laser confocal images of skinned fibers from heart (A,B), m. soleus (C,D), m. extensor digitorum longus (E,F) and m. gastrocnemius (G,H). The left panels show the muscles from wild-type and the right panels from MDX mice. The bars are given in micrometers.

the wild-type mice. The apparent density of mitochondria corresponded to the metabolic profile of the muscles, being the highest in ventricles (oxidative) and lowest in the white fibers of m. gastrocnemius (glycolytic). In accordance with earlier observations [27], the mitochondrial distribution appeared generally normal in MDX mice (Fig. 1B,D,F) except in m. gastrocnemius (Fig. 1H), where the mitochondria formed thinner and irregular lines compared to the wild-type counterpart.

Table 2 and Fig. 2 characterize the kinetics of ADP-stimulated respiration in the absence and presence of creatine. Irrespective of the presence of creatine, the values of the basal respiration rates (v_0, not shown), the maximal rate of the ADP-dependent respiration ($V_{max(ADP)}$) and the acceptor control index (V_{max}/v_0, calculated according to Saks et al. [24]), were similar in every matching muscle type of MDX and control mice. Likewise, the content of cytochromes aa_3 measured in ventricles of MDX mice was identical to that in controls (46.23 ± 4.32 and 41.4 ± 8.4 nmoles/g w.wt., respectively). It thus appeared that lack of dystrophin had no effect on the maximal capacity of oxidative phosphorylation, nor on coupling between oxidation and phosphorylation.

Consistently with previous studies, addition of 20 mM creatine to the medium resulted in a decreased K_m for ADP in wild-type ventricles and m. soleus (Fig. 2). Such an effect of creatine suggests the normal functional coupling between mi-CK and ANT, that enables the maximal stimulation of oxidative phosphorylation despite limited access of ADP via porin channels in these muscles [8]. In contrast, no influence of creatine on K_m for ADP was evident in glycolytic fibers such as m. extensor digitorum longus and m. gastrocnemius in normal mice. This is explained by the unrestricted diffusion of ADP through the MOM that allows to adjust the state 3 respiration independently of mi-CK in these muscles [8]. Fig. 2 demonstrates the absence of the creatine effect in glycolytic muscles of the MDX mice as well. However, in their ventricles and m. soleus creatine suppressed the K_m for ADP to a lesser extent, which resulted in a lower creatine index than in the corresponding muscles of the wild-type mice. Thus, lack of dystrophin attenuated the control of respiration by creatine in slow-twitch oxidative muscles. Decreased activity of mi-CK may be one of the possible causes for that phenomenon since downregulation of the expression of sarcomeric mi-CK has been re-

Table 2
Oxidative phosphorylation in skinned muscle fibers of MDX and wild-type mice

Type of muscle, parameter of oxidative phosphorylation	Type of mouse			
	Wild		MDX	
	−Cr	+Cr	−Cr	+Cr
$V_{max(ADP)}$ (nmoles O_2/min/mg w.wt.)				
Heart	6.07 ± 0.28 (7)	5.64 ± 0.63 (7)	5.87 ± 0.51 (6)	6.28 ± 0.40 (7)
M. soleus	2.00 ± 0.21 (5)	1.36 ± 0.09 (5)	1.52 ± 0.23 (5)	1.48 ± 0.19 (5)
M. extensor digitorum longus	1.27 ± 0.05 (5)	1.58 ± 0.06 (5)	1.29 ± 0.09 (5)	1.39 ± 0.08 (5)
M. gastrocnemius	0.79 ± 0.03 (4)	0.81 ± 0.11 (4)	0.67 ± 0.06 (5)	0.63 ± 0.06 (5)
V_{max}/v_0				
Heart	4.93 ± 0.21 (7)	5.51 ± 0.34 (7)	4.97 ± 0.45 (6)	5.58 ± 0.37 (7)
M. soleus	7.83 ± 1.04 (5)	7.91 ± 1.03 (5)	8.34 ± 1.54 (5)	6.73 ± 0.95 (5)
M. extensor digitorum longus	5.37 ± 0.71 (5)	6.38 ± 1.00 (5)	6.09 ± 1.20 (5)	5.99 ± 0.72 (5)
M. gastrocnemius	6.76 ± 0.83 (4)	7.41 ± 0.87 (4)	6.69 ± 1.49 (5)	6.24 ± 0.69 (5)

$V_{max(ADP)}$, maximal rate of ADP-dependent respiration, registered from the [ADP] versus respiration (basal rate before ADP addition, v_0, subtracted) relationship according to the Michaelis-Menten equation. V_{max}/v_0 represents an acceptor control ratio calculated as $[V_{max(ADP)}+v_0]/v_0$ [24]. The number of measurements is indicated in parentheses.

ported in MDX mice [28]. Therefore, the activities and isoenzyme profile of creatine kinase were determined in ventricles (the other tissues were not available in sufficient amounts for the analysis). Table 3 shows that the total creatine kinase activity of dystrophin-deficient ventricles was similar to that in control hearts, but the proportion of mi-CK was markedly increased at the expense of decreased BB- and MB-CK activities. Hence, the altered mitochondrial response to creatine (Fig. 2) could not be related to mi-CK activity, and therefore other factors should be considered (see Section 4).

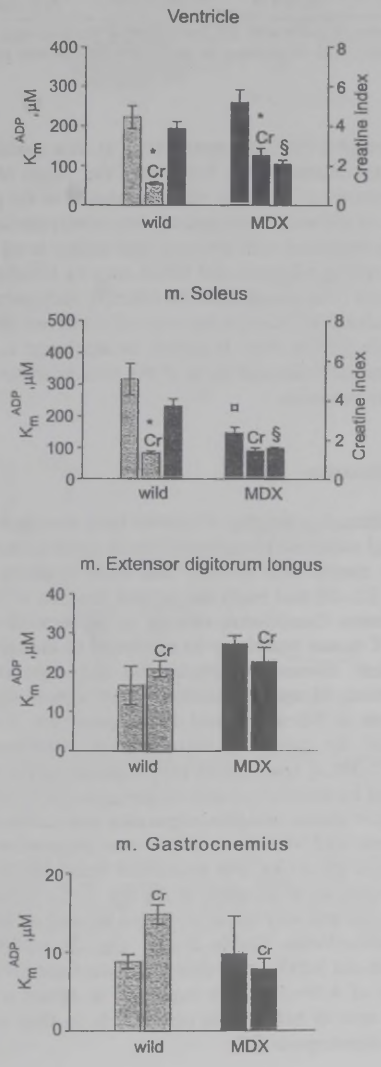

Fig. 2 also demonstrates another interesting observation: compared to normal m. soleus the K_m for ADP in the absence of creatine was decreased in the MDX counterpart. A similar change, suggesting an enhanced permeability of MOM for ADP, has been registered earlier in oxidative muscles in conditions of impaired or immature PCr energy transfer shuttle, as well as in the case of disintegrated sarcomeric structure due to lack of desmin [6,7,16,29]. It appears therefore that the absence of dystrophin in m. soleus promotes the development of the new muscle phenotype characterized by increased access of cytoplasmic ADP to mitochondria.

According to a recent hypothesis, mitochondria and ATPases form compartmentalized and functional complexes in which they interact not only via creatine and adenylate kinases, but also by a direct exchange of adenine nucleotides [30]. The question whether direct transfer of ADP to mitochondria is affected in MDX mice should be answered by analyzing the competition between mitochondria and the powerful PEP-PK system (with an activity exceeding that of total ATPase more than 100 times) for ADP produced in ATPase reactions (Fig. 3). It can be seen that addition of MgATP to the polarographic medium caused an activation of the total ATPase, which due to the presence of magnesium and calcium ions can be considered the sum of the myofibrillar MgATPase and sarcoplasmic reticular CaMgATPase (Fig. 3A,B). In this phase of the experiment, ADP was regenerated by the PEP-PK system as indicated by the high rate of NADH oxidation (Fig. 3B) and

← Fig. 2. Effect of 20 mM creatine on mitochondrial affinity to ADP (K_m^{ADP}). K_m^{ADP} without creatine/K_m^{ADP} with creatine = creatine index (gray columns). Cr, in the presence of creatine. $^*P<0.05$ compared to K_m^{ADP} without creatine; $^{\otimes}P<0.05$ compared to K_m^{ADP} corresponding parameter in wild-type muscle group; $^{\S}P<0.05$ compared to creatine index in wild-type muscle group.

Table 3
CK activities and isoenzyme profiles in the skinned cardiac fibers of MDX and wild-type mice

Type of muscle	Total CK activity (μmoles/min/g w.wt.)	Isoenzyme profile (%)			
		BB	MB	MM	MIT
Wild-type ($n=3$–4)	235.3±14.3	6.1±1.1	21.8±1.3	39.1±1.5	33.0±2.7
MDX ($n=5$)	206.8±22.6	2.6±0.6*	16.3±2.1*	36.5±1.3	44.4±3.6*

The means±S.E.M. are given. *$P<0.05$ versus wild-type parameter. n, number of hearts used. BB, homodimer of the brain-type creatine kinase; MB, heterodimer of the brain- and muscle-type creatine kinase; MM, homodimer of the muscle-type creatine kinase; MIT, mitochondrial isoenzyme of creatine kinase.

the low rate of respiration (Fig. 3C). The following addition of glutamate and malate caused a rapid augmentation of the respiration rate to its maximum level (Fig. 3C), which was associated with immediate suppression of the ADP flux through the PK-PEP system (Fig. 3B). Then the inhibition of respiration by blocking the ANT with atractyloside (Fig. 3C) restored the ADP flux through the PK-PEP exactly to the levels registered before addition of the substrates (Fig. 3B). This confirms that diminished ADP utilization by the PK-PEP system after addition of respiratory substrates resulted from the oxidative phosphorylation of ADP in mitochondria. Analysis of the full set of analogous experiments showed that after switching on the processes of oxidative phosphorylation the flux through the PK-PEP system became inhibited by 35% in wild-type cardiac fibers (Fig. 4A). This fraction of total ADP flux was compartmentalized in functional complexes and therefore was not accessible to the PK-PEP system. Based on the total ATPase activity (3.54±0.24 μmoles/min/g w.wt.), registered after addition of 1 mM ATP (Fig. 3B), the calculated absolute ADP flux taken up by mitochondria was about 1 μmole/min/g w.wt. in wild-type muscles (Fig. 4B). Compared to these data, the relative inhibition of ADP flux through the PEP-PK system by the mitochondrial function was stronger (Fig. 4A) and the total ATPase activity (7.48+0.55 μmoles/min/g w.wt.) higher in dystrophin-deficient ventricles. In combination, these two changes gave a 3-fold increase in absolute rates of the mitochondrial ADP phosphorylation as compared to the wild-type muscle group (Fig. 4B). It should be noted that in these experiments the mitochondria were incubated with glutamate and malate because the standard substrate pair, pyruvate-malate, could not be used in the presence of the coupled PK-LDH system (Fig. 3). In a special set of experiments it was found that the values of the parameters of oxidative phosphorylation in the presence of glutamate and malate were exactly similar to those registered with pyruvate and malate in all corresponding wild-type and MDX muscles (results not shown). This means that the observed differences in mitochondrial function between wild-type and MDX muscle groups (Fig. 4) cannot be attributed to the diverse substrate specificity of the mitochondrial oxidative processes.

4. Discussion

Although a number of reports have described impaired oxidative phosphorylation in dystrophin-deficient muscle cells [17–20], this study confirms the data [21–23] that imply the normal function of these processes. Controversy relating to the experimental MDX mouse model can be attributed to the age-dependent character of dystrophic and regenerative processes. Myopathic lesions progress to peak degeneration at 5–6 weeks, and at this point are characterized by extensive mitochondrial deterioration [20,31–35]. A regenerative period ensues and is controlled by the fetal myogenetic program [36,37]. During this phase, utrophin expression supersedes dystrophin and thereby prevents the progression of necrotic dystrophy and premature death [36,38,39]. Regeneration is complete at the age of 12–16 weeks [31], and one may therefore expect normal oxidative phosphorylation (Table 2 and Fig. 2) in 10–11-month-old MDX mice. Effective mitochondrial synthesis of ATP should be important to ensure a survival rate of MDX mice comparable to their wild-type counterparts [40].

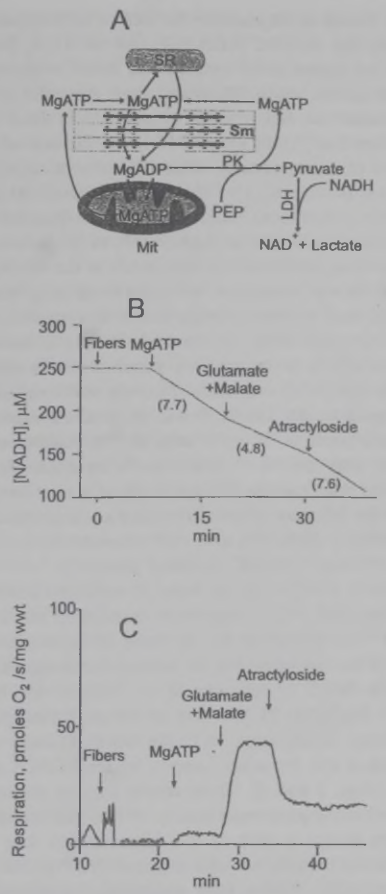

Fig. 3. Example of the analysis of rivalry between mitochondria and the external PK-PEP system for ADP generated in ATPase reactions (A) by measurements of the PK reaction (B) and respiration (C) in skinned cardiac fibers of wild-type mice. The experiments were performed at 25°C in solution B complemented with 5 mg/ml BSA, 5 mM PEP, 20 IU/ml PK, 20 IU/ml LDH, and 250 µM NADH. Further additions: fibers, 1 mM MgATP, 10 mM glutamate+2 mM malate, and 98 µM atractyloside. The numbers in parentheses indicate the rate of NADH oxidation (µM/min) in the presence of 2.6 mg/ml fibers (B).

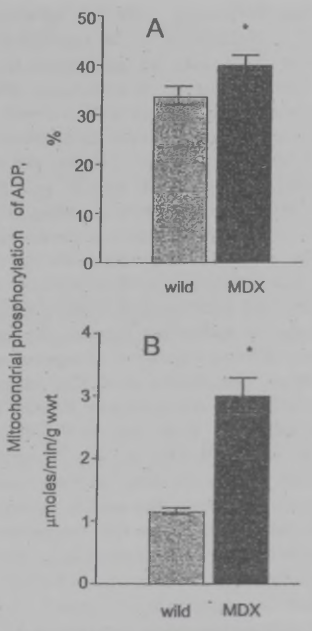

Fig. 4. Comparison of the mitochondrial phosphorylation of ADP in relative (A) and absolute (B) terms in cardiac fibers of MDX and wild-type mice. The data are obtained from the experiments shown in Fig. 3. The percentage of mitochondrial phosphorylation of ADP corresponds to the fraction of the total ADP flux that is inhibited after switching on the respiration by adding the substrates. The absolute flux is a difference between the rates of ADP phosphorylation by the PK-PEP system in the absence and presence of glutamate and malate. $^{*}P < 0.05$ compared to the corresponding parameter in wild-type myocardium.

We observed that the lack of dystrophin affects the intracellular energy transfer in slow-twitch muscles. First, the decrease in the creatine index (Fig. 2) suggests impaired coupling between the mi-CK and ANT. Mathematical modeling in this case predicts a reduction in cellular PCr contents together with accumulation of ADP [9]. This parallels the observations made by ^{31}P-NMR analysis in humans suffering from DMD or Becker muscular dystrophy (BMD) wherein PCr and ADP contents in muscles changed reciprocally, the changes enhanced after exercise and were more prevalent in patients with

DMD than in patients with less advanced BMD [21,22,41]. A disturbance in the interaction between mi-CK and ANT may be responsible for the decreased effect of creatine on respiration (Fig. 2). At present the molecular nature of that deviation is not clear; however, some putative mechanisms are apparent. It has been proposed that the precise molecular stoichiometry between mi-CK and ANT is a prerequisite for their effective coupling [42,43]. The expression of the mi-CK gene is decreased without alterations in ANT expression in the hindlimb muscles and diaphragm of the 3-month-old MDX mouse [28]. This finding points to the potential origin of the impaired interaction between the mi-CK and ANT, ergo different regulation of expression of these two proteins. Tkatchenko et al. [28] suggested that mitochondrial PCr synthesis may become limited in 3-month-old MDX mice, due to decreased mi-CK activity vs. unaltered ANT activity. Nevertheless Table 3 shows that in the elder animals other factors must play a role, since the mi-CK activity was actually increased. Although the mechanism causing increased mi-CK activity is unclear, it may serve as an adaptation to chronically insufficient PCr synthesis. If then the expression of creatine kinase is not matched with an equivalent increase in ANT expression, perhaps the coupling may be affected. On the other hand, as muscles of MDX mice exhibit abnormally high rates of oxyradical production [44] that entails peroxidation of cardiolipin [45], the generation of these species may be relevant. As fixation of mi-CK to the inner membrane critically depends on its binding to cardiolipin [46], its peroxidative modulation may attenuate the coupling of mi-CK to ANT.

Second, increased affinity of oxidative phosphorylation to ADP was registered in m. soleus of MDX mice (Fig. 2). A similar phenomenon, attributed to the decreased barrier function of MOM which allows the mitochondrial respiration to be controlled by cytosolic fluctuations of [ADP], has been observed in oxidative muscles after genetic or chemical modification of the creatine kinase system [7,47] and in cardiomyocytes of desmin-deficient mice [16]. These facts strongly suggest that the decreased K_m for ADP (Fig. 2), together with increased cellular [ADP] [21,22], indicate the increased role of the ADP diffusion in linking ATPases with mitochondria in m. soleus in response to the lack of dystrophin. To ensure the effective ADP diffusion to ANT, [ADP] must be higher in the cytosol and lower in the intermembranous space. However, high cytosolic [ADP] decreases the free energy of ATP hydrolysis [48], whereas low [ADP] near ANT limits the rate of oxidative phosphorylation. Within these constraints, increased permeability of the MOM is beneficial as it confers a decreased ADP concentration gradient [3]. Nevertheless, a shift of high [ADP] to the intermembrane compartment may then result in the following. First, direct activation of respiration may occur, which may explain creatine's inability to alter the K_m for ADP (Fig. 2). Second, it may destabilize the mi-CK forward reaction, which results in abnormally low [PCr] and reduced muscle work, especially at higher workloads [9]. It follows thus that excessive entry/deposition of ADP into the intermembranous space may represent either an independent factor able to compromise PCr synthesis or it may complicate the influence of other factors such as imbalanced expression of mi-CK and ANT, and/or the oxyradical defects in mi-CK described above.

Third, our data (Figs. 3 and 4) confirm the recent finding [30] that a significant portion of the total ADP flux generated by ATPases is directly transferred to mitochondria in normal cardiac cells. In mouse heart it corresponds to 35% of the total ADP flux (Fig. 3). The rest of the ADP flux (65%) is freely equilibrated with the whole cytosol, and therefore can be easily trapped by the PEP-PK system (Figs. 3 and 4). These results may be explained by microcompartmentalization of the cell structures which isolate a part of the mitochondria into the functional complexes with adjacent ADP producing systems [30]. Within such a complex, mitochondria and ATPases can interact via (i) simple diffusion of ADP/ATP and/or (ii) by vectorial transfer of the energy-rich phosphate group aided by adenylate and creatine kinases, the latter enzymes increasing the rate of energy channeling [30]. Besides MOM [6,11,16] the boundaries of these complexes may create additional diffusion constraints for exogenous ADP which may explain the high K_m for ADP in the heart and possibly in m. soleus (200–300 μM, Fig. 2) compared to that in isolated mitochondria [30]. A novel aspect of the current data is that compartmentalized ADP transfer to mitochondria in-

creases in dystrophin-deficient cardiac muscle (Figs. 3 and 4). Lack of dystrophin attenuates or stimulates the expression of a number of the proteins, such as titin, troponin I, α-tubulin and Rac1, which participate in the structural organization of muscle cells [28]. In addition, it is known that the cytoskeletal proteins can modulate the function of the contractile proteins [49], that about 40% of dystrophin is tightly bound to the contractile apparatus, and that the loss of that dystrophin fraction is critical for the development of cardiac insufficiency [50]. Thus increased mitochondrial uptake of ADP suggests altered cytoskeletal control over the function of the complexes between mitochondria and ATPases. It is possible that a mechanism develops to ensure an increase in ATPase activity and channeling of an ADP flux larger than in normal heart towards mitochondria, thereby stimulating respiration without decreasing the permeability of the MOM. This mechanism may be important in the compensation of the failed function of mi-CK (Fig. 2) in cardiac cells of MDX mice.

In conclusion, the dystrophin-deficient MDX mice exhibit a special myopathy which is characterized by normal oxidative phosphorylation with impaired coupling between the mi-CK and ANT and increased transfer of ADP from ATPases to mitochondria in slow-twitch oxidative muscles. As these properties are associated with defective energy transport they may constitute a basis for the development of muscle weakness in patients with DMD or BMD.

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IV

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Kindred DNA Amplification from Two Distinct Populations of cDNA Fragments

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ABSTRACT

Kindred DNA amplification is a novel and cost-effective method developed to isolate common cDNA fragments between two distinct cDNA populations. Unlike subtractive hybridization, which discards common sequences, kindred DNA amplification isolates and amplifies these sequences within a single hybridization procedure. The utility of this method is demonstrated by cloning the genes in common between two different but metabolically homologous muscles, murine ventricular myocardium and soleus. The reliability of kindred DNA amplification was confirmed by Southern hybridization.

INTRODUCTION

A vast number of biological processes, such as pre- and postnatal development differentiation, energy production, disease adaptation, and protection underscore the function of mammalian systems. Each of these processes is governed by specific gene expression that is regulated by two principal mechanisms. Differential expression operates via variations of sets of expressed genes. The second mechanism involves alterations in the expression level of an individual gene (epigenetic changes). At present, several methods based on cDNA subtraction are available for characterization of differential gene expression (1–3), whereas quantitative PCR together with micro-and macroarray techniques enables assessment of the epigenetic changes (4–6). In some cases, however, it is necessary to define the sequences in common between differentially expressed genes, for instance, in identifying genes specific to colon cancer (4). Then, time-intensive characterization of individual DNA populations followed by computer analysis to define the sequence differences has been used (4–6). We report on kindred DNA amplification, which is a novel and simple method to identify and select common DNA fragments between pools. Kindred DNA amplification utilizes DNA hybridization with subsequent suppression PCR (7,8), uisng modifications enabling one to selectively suppress or enhance the genes of interest. We have applied this technology to isolate the genes in common between two different muscles (myocardium and soleus), which are known to share mutual properties—predominantly dependence on the aerobic energy metabolism and regulation of mitochondrial function distinct from that of glycolytic muscles (9). This application stemmed from studies that demonstrated the low apparent affinity of mitochondrial respiration to exogenously added ADP in oxidative versus glycolytic muscles (extensor digitorum longus) (10,11). Selective proteolysis resulted in increased affinity of mitochondria to ADP in oxidative muscles but exerted no effect on that parameter in glycolytic muscles (10,11). It was hypothesized that both oxidative muscles, myocardium and soleus, likely express few and identical proteins capable of exerting intracellular control over mitochondrial respiration, whereas the glycolytic muscles lack these proteins (10,11). In oxidative muscle cells, these proteins may participate in the organization of the intracellular energetic units, representing the functional and structural complexes of mitochondria with adjacent ATPases. This type of organization likely confers specific regulatory properties of respiration to these muscles (12,13). To define the nature of these proteins, we isolated genes that are expressed in common between both types of oxidative muscles but not expressed in glycolytic muscle (extensor digitorum longus). Thus, muscle cDNA libraries for soleus (S), ventricular myocardium (H), and extensor digitorum longus (E) were generated, and H was subtracted against E, resulting in a cDNA population of heart-specific genes (HE). Finally, kindred DNA amplification was carried out to obtain common cDNA fragments from HE and S, and the shared genes (SHE) were confirmed by Southern analysis (14). This is a rapid method to isolate candidate genes for organization of intracellular energy production in oxidative muscle cells.

MATERIALS AND METHODS

Animals

The adult white laboratory male mice were used as the source of different muscle tissues. The animals were kept, fed, and studied in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Oligonucleotides and Adapters

All the oligonucleotides and adapters used (Table 1) were ordered from Genset (Paris, France), except those produced by BD Biosciences Clontech (Palo Alto, CA, USA).

Total RNA Preparation

The total RNA was isolated from the ventricular myocardium, soleus, and extensor digitorum longus of the mouse using total RNA isolating kit (Macherey-Nagel, Düren, Germany). All procedures were performed according to the manufacturer's protocol. The amount of total RNA isolated varied from 100 to 500 ng, depending on the type and mass of muscle obtained from one mouse. The isolated RNAs were ethanol-precipitated and resuspended in water in a final concentration of 10 ng/µL.

Total cDNA Synthesis

The total RNA was reverse-transcribed to obtain the total cDNA using the SMART™ cDNA synthesis protocol (15–17). The first step was the mixing of 4 µL respective total RNA sample (10 ng/µL), 2 µL 10 µM SMART oligonucleotide, and 2 µL 10 µM SMART CDS primer. The mixture was heated at 70°C for 2 min. Thereafter, the following additions were made: 4 µL 5× first-strand buffer (Invitrogen, Carlsbad, CA, USA), 2 µL 20 mM DTT, 2 µL 10 mM dNTP, 2 µL SuperScript™ II reverse transcriptase (Invit-

rogen), and 2 µL 20 U/µL ribonuclease inhibitor (MBI Fermentas, Vilnius, Lithuania). The resulting mixture was heated at 42°C for 2 h, followed by the addition of 80 µL TE buffer.

The second step was generation of total double-stranded cDNA from the total single-stranded cDNA obtained from the procedures in step one. It was started with mixing of 10 µL of the step-one mixture with 10 µL 10× Advantage 2™ PCR Buffer (BD Biosciences Clontech), 2 µL 10 mM dNTP, 2 µL 10 mM 5′-PCR Primer (Table 1), 2 µL Advantage 2 Polymerase Mix (BD Biosciences Clontech), and 74 µL water. PCR was performed within the following parameters: 30 cycles of 95°C for 30 s, 65°C for 30 s, and 68°C for 6 min, and a final extension at 68°C for 9 min. Starting from cycle no. 18, 10 µL of the reaction mixture were removed after every third cycle and analyzed on a 2% agarose/SYBR Green® gel (Molecular Probes, Eugene, OR, USA) to find out the optimal number of PCR cycles. After that, this PCR regime was applied to all muscle samples studied. The resulting total cDNA was purified using NucleoSpin® Extract Columns (Macherey-Nagel), ethanol-precipitated, and resuspended in water at a concentration of 50 ng/µL.

Subtractive Hybridization, PCR Amplification, and Removal of the Nested Primer's Sequences from the Ends of cDNA Fragments of HE Population

Tester preparation. Here, the first step was the restriction cleavage of total double-stranded cDNA obtained from the myocardium (H). Thus, 8 µL of respective total cDNA (50 ng/µL), 1 µL *Rsa*I restriction enzyme (10 U/µL; New England Biolabs, Beverly, MA, USA, or MBI Fermentas), and 1 µL respective restriction buffer (from the enzyme supplier) were mixed and incubated at 37°C for 1 h. Then, the mixture was purified using NucleoSpin Extract Columns, and cDNA fragments were ethanol-precipitated and resuspended in water at a concentration of 50 ng/µL. In a second step, the adapters were added to two separate reaction mediums, both containing 2 µL 50 ng/µL digested cDNA, 1 µL T4 DNA ligase (New England Biolabs or MBI Fermentas), 1 µL T4 Ligase Buffer (respective supplier), and 4 µL water, but in the presence of 2 µL 10 µM adapter 1 in one and adapter 2R (10 µM) (Table 1) in the other. The reactions were incubated overnight at 16°C. The enzyme and excess adapters were removed by using NucleoSpin Extract Columns, and the tester cDNAs were ethanol-precipitated and resuspended in water at concentrations of 10 ng/µL. Thus, two different tester preparations from the same cDNA fragment, one with adapter 1 and another with adapter 2R, were obtained.

Driver preparation. The driver was prepared from the cDNA of extensor digitorum longus similar to the tester preparation, except the resulting driver fragments were resuspended in water at concentrations of 200 ng/µL and the adapters were not ligated to the ends of cDNA.

Subtractive hybridization. 1.5 µL driver dsDNA (300 ng) was added to a pair of Eppendorf® tubes containing 1 µL hybridization buffer [200 mM HEPES, pH 8.3, 2 M NaCl, 0.1 mM EDTA, 10% (w/v) PEG 8000] and 15 ng adapter 1-ligated tester cDNA in one tube and 15 ng adapter 2R-ligated tester cDNA in the other, mixed, overlaid with mineral oil, denatured for 5 min at 95°C, and hybridized for 10 h at 68°C. These two samples were then pooled and mixed, freshly denatured (5 min at 95°C) driver dsDNA (200 ng) in 2 µL hybridization buffer was added, and the reaction (second hybridization) was incubated for 10 h at 68°C. Thereafter, the final hybridization mixture was diluted 10-fold in TE and stored at -20°C.

PCR amplification. The first step was accomplished in a 25-µL reaction mixture containing 1 µL diluted, subtracted cDNA, 1 µL 10 µM PCR primer 1, and 23 µL PCR master mixture prepared using Advantage 2 Polymerase Mix, according to the manufacturer's instructions. PCR was performed using the following parameters: initial incubation at 75°C for 5 min, then 27 cycles at 94°C for 30 s, 66°C for 30 s, and 72°C for 1.5 min, and a final extension at 68°C for 5 min. The amplified products were then diluted 10-fold in water and amplified in 25 µL of the reaction mixture containing 1 µL diluted amplified cDNA, 1 µL 10 µM nested PCR primer 1, 1 µL 10 µM nested PCR primer 2R (Table 1), and 22 µL PCR master mixture. PCR amplification was performed for 12 cycles at 94°C for 30 s, 68°C for 30 s, and 72°C for 1.5 min, with a final extension step at 68°C for 5 min. The PCR products were analyzed on an agarose/SYBR Green gel. Then, the mixture was purified using NucleoSpin Extract Columns, and cDNA fragments were ethanol-precipitated and resuspended in water at a concentration of 50 ng/µL, resulting in an HE population of cDNA.

Removal of the nested primer's sequences from the ends of the DNA fragments of HE population. Because the DNA fragments of HE population of cDNA generated by subtractive hybridization had different primer (nested primer 1 or nested primer 2R; Table 1) sequences on both ends, it was necessary to remove those sequences before the addition of adapters during the kindred DNA amplification procedure (see below). The sequences were removed as follows: 8 µL 50 ng/µL HE cDNA, 1 µL 10 U/µL *Rsa*I restriction enzyme, and 1 µL respective restriction buffer (from the enzyme supplier) were mixed and incubated at 37°C for 1 h. Then, the mixture was purified using NucleoSpin Extract Columns, and the cDNA fragments were ethanol-precipitated and resuspended in water at a concentration of 50 ng/µL. The efficiency of removal of primers 1 and 2R from the ends of the HE fragments was monitored by PCR amplification in a 25-µL reaction mixture containing 1 µL diluted (10×) *Rsa*I-cleaved HE, 1 µL 10 µM primer 1, 1 µL 10 µM primer 2R, and 22 µL PCR master mixture. The PCR amplification was performed as follows: 94°C for 5 min, 30 cycles at 94°C for 30 s, 68°C for 30 s, and 72°C for 1.5 min, and the final extension at 68°C for 5 min. When the PCR products were analyzed by the agarose/SYBR Green gel electrophoresis, no visible PCR products were observed, indicating that the nested primer sequences were totally removed from HE.

Kindred DNA Amplification

Phase 1 (Figure 1): cDNA preparation. The total cDNA of soleus was

restriction-digested exactly as for the cDNA of myocardium (H) described above. The adapters were then added to cDNA of soleus cleaved with *RsaI*, as described above. The excess enzyme and adapters were removed by using NucleoSpin Extract Columns, and the DNA was ethanol-precipitated and resuspended in water at a concentration of 10 ng/µL. Thus, two different DNA preparations were obtained, one (soleus) with the adapter 1 and another (HE) with the adapter 2R at the end of the DNA fragments.

Phase 2: hybridization of S with HE. The hybridization solution was generated by mixing 1.5 µL adapter-ligated cDNA (S and HE, each 15 ng) with 1 µL 4× hybridization buffer, prepared according to the PCR-Select cDNA Subtraction Kit instructions (BD Biosciences Clontech). The solution was overlaid with mineral oil, denatured for 5 min at 95°C, and allowed to hybridize overnight for 8 h at 68°C. The final hybridization was then diluted 10-fold in TE and stored at -20°C.

Phase 3: Filling in the end of adapters and PCR amplification. The filling in of adapters takes place in the initial incubation needed to prepare the PCR template. For kindred DNA amplification, the two-step procedure of PCR amplification was performed exactly as described for the PCR amplification step above. The PCR products obtained were considered to be the SHE fraction containing the cDNA fragments in common between the myocardium (H) and soleus (S) were purified using the NucleoSpin Extract Columns, eluted in 50 µL water, and analyzed by agarose/SYBR Green gel electrophoresis.

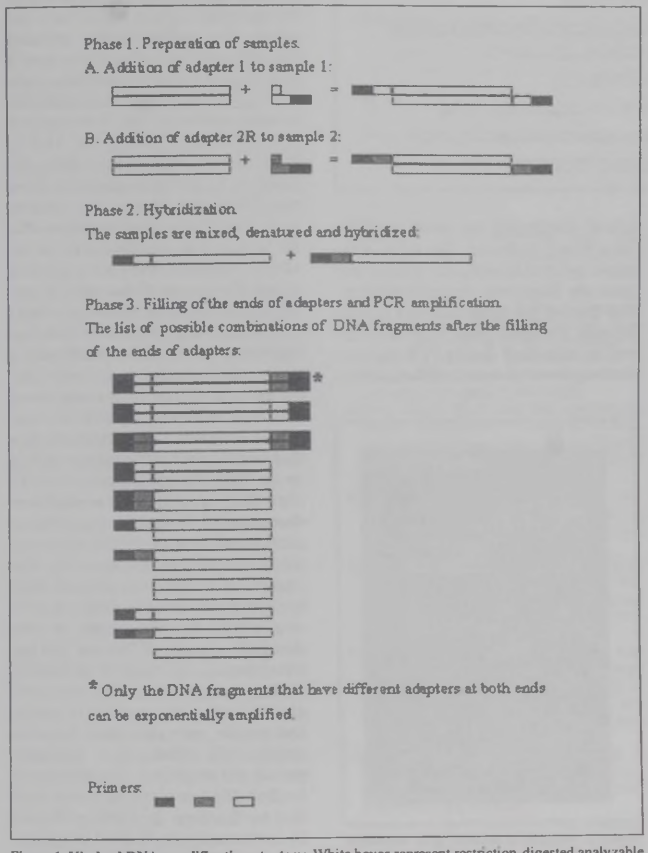

Figure 1. Kindred DNA amplification strategy. White boxes represent restriction-digested analyzable DNA fragments. The double-labeled boxes, black combined with white and gray combined with black, represent adapter 1 and adapter 2R long strands, respectively. Black boxes also correspond to the PCR primer 1 sequence, whereas the small white and gray boxes represent the PCR primer 1 and primer 2R sequencies, respectively. The smallest white and gray boxes represent adapter 1 and adapter 2R short strands, respectively (see also Table 1).

Cloning of the Kindred DNA Amplification cDNAs

The kindred DNA amplification products were cloned by using pTZ57R/T plasmid vector within the InsT/aclone PCR Product Cloning Kit according to the manufacturer's protocol (MBI Fermentas). The host strain used was JM109.

Preparation of DNA for Southern Hybridization

The cloned DNA inserts were amplified by PCR directly from the bacterial colonies. Bacterial cells of a given colony were transferred into 25 µL of the reaction mixture containing 1 µL 10 µM nested PCR primer 1, 1 µL 10 µM nested PCR primer 2R (Table 1), and 23 µL PCR master mixture. PCR amplification was performed by 30 cycles at 94°C for 30 s, 68°C for 30 s, and 72°C for 1.5 min, and the PCR products were purified using NucleoSpin Extract Columns in an elution volume of 50 µL and analyzed by agarose/SYBR Green gel electrophoresis.

Dot-Blot Hybridization

Five microliters of amplified cDNA fragments were directly blotted onto a nylon filter. All the processes—DNA labeling, hybridization, and detection—were performed using DIG-High Prime DNA Labeling and Detection Starter Kit

Table 1. Oligonucleotides and Adapters Used in the Study

Primer Name	Primer Sequence (5′→3′)
SMART	AAGCAGTGGTAACAACGCAGAGTACG CGGG
SMART CDS primer	AAGCAGTGGTAACAACGCAGAGTACTT TTTTTTTTTTTTTTTTTTTTTTTTTTTT (A,C,G)(A,C,G,T)
5′ PCR primer	AAGCAGTGGTAACAACGCAGAGT
Adapter 1 long oligonucleotide	CTAATACGACTCACTATAGGGCTCGAG CGGCCGCCCGGGCAGGT
Adapter 1 short oligonucleotide	ACCTGCCCGG
Adapter 2R long oligonucleotide	CTAATACGACTCACTATAGGGCAGCG TGGTCGCGGCCGAGGT
Adapter 2R short oligonucleotide	ACCGCGGCCG
PCR primer 1	CTAATACGACTCACTATAGGGC
Nested PCR primer 1	TCGAGCGGCCGCCCGGGCAGGT
Nested PCR primer 2R	AGCGTGGTCGCGGCCGAGGT

I (Roche Applied Science, Mannhein, Germany), according to the manufacturer's instructions.

RESULTS AND DISCUSSION

The Principles of Kindred DNA Amplification

Figure 1 shows the principles of kindred DNA amplification. In phase 1, the adapters are added to the ends of restriction-digested tester DNA fragments: adapter 1 to one population of DNA fragments (Figure 1, phase 1A) and adapter 2R to the other (Figure 1, phase 1B). In phase 2, the samples (DNA fragments with adapters) are mixed, denatured, and hybridized. As the ligation of adapters does not occur with 100% fidelity, the reaction mixture contained several probable combinations of DNA fragments, both with and without adapters (Figure 1, phase 3). The method is based on a concept that only those DNA fragments that have different adapters on its both ends can be amplified exponentially (Figure 1, phase 3). These particular fragments can be generated only if they were present in both DNA sample populations analyzed. The sense strand of the fragment with one adapter's long strand at its end hybridizes with the antisense strand, possessing the other adapter's long strand at its end. The DNA fragments present in only one sample can generate fragments during hybridization having the same adapters at both its ends. However, these fragments cannot be amplified during PCR because the long inverted repeats of the adapters form stable "panhandle-like" structures after each denaturing-annealing PCR cycle (1–3).

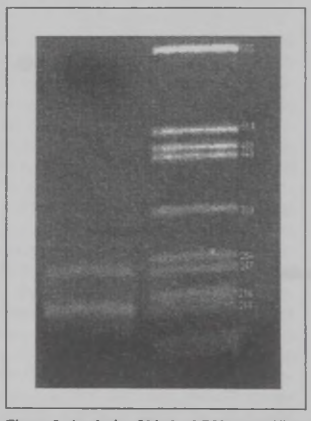

Figure 2. Analysis of kindred DNA amplification by 2% agarose gel electrophoresis. The dominating bands of cDNAs in common (SHE) between heart and soleus are visible at 200 and 250 bp (left lane). Lambda DNA/*Pst*I size marker's fragments (MBI Fermentas) are visualized in the right lane.

Application of Kindred DNA Amplification to Identify cDNA Fragments in Common between Different Types of Oxidative Muscle

The aim of these experiments was to reveal the cDNA fragments that characterize oxidative muscle cells and also represent a pool of DNA in common between two different types of oxidative muscles—murine myocardium and soleus. Our earlier studies indicated that different types of oxidative muscle may possess a similar regulatory mechanism of mitochondrial respiration that is responsible for the low apparent affinity of mitochondria for ADP in these muscles, whereas this mechanism seems to be absent in glycolytic muscles (10,12,13). Hence, the isolation and identification of genes responsible for muscle type-specific control of oxidative phosphorylation are required to reveal the nature of that mechanism. We determined that obtaining a large cDNA pool is preferable for isolating regulatory genes, and consequently, a new method was designed. Accordingly, the total cDNA from mouse heart was first subtracted against the total cDNA of extensor digitorum longus, a fast-twitch glycolytic muscle, resulting in the HE cDNA population. The kindred DNA amplification method was then applied to isolate the common cDNA pool between the HE and the total cDNA of soleus (Figure 1). The analysis of the products obtained (Figure 2) indicates that the cDNAs in common (SHE) became visible as two dominating bands of 200 and 250 bp, which were inserted into the pTZ57R/T vector. Altogether several thousand clones, exclusively specific to oxidative muscle, were generated. Approximately 100 clones were randomly picked and amplified. The efficacy of kindred DNA amplification was verified by Southern dot-blotting (Figure 3). In this analysis, 54 DNA fragments were randomly selected from the total set of cDNA, and three identical blots were performed. One of the membranes was hybridized with the DIG-labeled soleus total cDNA (Figure 3A)

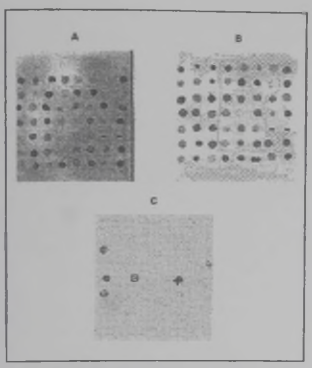

Figure 3. Evaluation of the common cDNA pool (SHE) by Southern hybridization. Three identical filters blotted with the SHE clones were probed with total cDNA from soleus (A), heart (B), or extensor digitorum longus (C). The dots indicate binding of labeled cDNA of different sources to complementary cDNA sequences of the SHE pool. (-) and (+) represent the negative and positive controls, respectively.

and another with DIG-labeled mouse heart total cDNA (Figure 3B). The results of those two hybridizations were similar insofar as 54 clones hybridized with cDNA probes in both cases, and two negative controls (water and *E. coli* DNA) did not hybridize. The third membrane (Figure 3C) depicts clones hybridized with DIG-labeled total cDNA of extensor digitorum longus, and they were blotted in the same order as in Figure 3, A and B. A very rare pattern of binding was observed, indicating that a large majority of genes, specific to extensor digitorum longus, were eliminated from the entire pool of cDNA in common between myocardium and soleus (SHE). The (+) sign marked in Figure 3C indicates the cDNA for G3PDH used as a positive control. This glycolytic enzyme, which exists in all muscle tissue, was used to determine whether the hybridization was successful. This signal superseded the five other spots seen on the filter. Thus, these results indicate that the common cDNA pool (SHE) obtained by kindred DNA amplification did not contain the cDNA fragments characteristic of extensor digitorum longus and, as such, represented a set of genes specific for slow-twitch oxidative muscle.

In conclusion, the method described here serves as a reliable and practical tool for cloning genes in common from different tissues. This method permitted us to rapidly isolate candidate genes involved in muscle type-dependent control of mitochondrial respiration in oxidative muscle. The function of these genes remains to be defined in future studies.

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Ulo Puurand, Lumme Kadaja, and Enn K. Seppet
University of Tartu
Tartu, Estonia

V

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IgG from patients with liver diseases inhibit mitochondrial respiration in permeabilized oxidative muscle cells: Impaired function of intracellular energetic units?

Lumme Kadaja,[1] Kai E. Kisand,[2] Nadezhda Peet,[1] Urmo Braun,[1] Kaja Metsküla,[2] Kaupo Teesalu,[2] Riina Vibo,[1] Kalle V. Kisand,[1] Raivo Uibo,[2] Harald Jockusch[1] and Enn K. Seppet

Departments of [1]Pathophysiology and [2]Immunology, Faculty of Medicine, University of Tartu, Tartu, Estonia; [3]Lehrstuhl für Entwicklungsbiologie und Molekulare Pathologie, Fakultät für Biologie, Universität Bielefeld, Bielefeld, Germany

Abstract

The effect of IgG purified from the sera of healthy persons and patients with primary biliary cirrhosis (PBC) and chronic hepatitis (CH) on ADP dependent respiration (oxidative phosphorylation) in skinned muscle fibers from rat oxidative muscles (heart and *M. soleus*) and glycolytic skeletal muscle (*M. gastrocnemius*) was studied. The results show that IgG from three different sources inhibited the rate of respiration by 13, 44 and 42%, respectively, these effects being equally expressed in both types of oxidative muscles, whereas no inhibition was observed in glycolytic muscle. The following washout of unbound IgG did not abolish the inhibition of respiration suggesting that the specific interaction of IgG with antigens had taken place. Laser confocal analysis revealed binding of IgG predominantly to the sarcomeric structures such as Z-disk and M-lines in the cardiomyocytes. The staining of IgG within Z-disks and intermitochondrial space coincided throughout the muscle cells so that transversally serial spaces, each containing mitochondria and adjacent sarcomere, became clearly visible. When the IgG from a CH patient was incubated with the skinned myocardial fibers of the desmin knockout mice, its binding to Z-disks and the sarcomeric area was found to be similar to that in normal cardiac muscle. However, the transversal staining pattern was disintegrated, because of the slippage of the myofibrils in relation to each other and accumulation of mitochondria between them. These observations support the recent hypothesis that in oxidative muscles the mitochondria and adjacent sarcomeres form complexes, termed as the intracellular energetic units, ICEUs. Moreover, they indicate that human autoantibodies can be useful tools for localizing the proteins responsible for formation of ICEUs and modulation of their function. Thus, it appears that the proteins associated with the Z-disks and M-lines may participate in formation of ICEUs and that binding of IgG to these proteins decreases the access of exogenous adenine nucleotides to mitochondria, which manifests as decreased rate of ADP-dependent respiration. (Mol Cell Biochem **000**: 000–000, 2003

Key words: primary biliary cirrhosis, chronic hepatitis, antimitochondrial antibodies, oxidative phosphorylation, saponin-skinned muscle fibers, intracellular energetic units

Introduction

Respiration of permeabilized cells of oxidative muscle exhibit strikingly low affinity to exogenous ADP compared to that in glycolytic muscle or isolated mitochondria [1]. This phenomenon suggests the principal differences in the mechanisms of regulation of oxidative phosphorylation by ADP *in vivo* and *in vitro*, and between different types of muscle cells.

Address for offprints: E.K. Seppet, Department of Pathophysiology, Faculty of Medicine, University of Tartu, 19 Ravila Street, 50411 Tartu, Estonia (E-mail: enn@ut.ee)

Our recent studies [2, 3] have revealed that in skinned fibers of oxidative muscle ADP generated locally by ATPases does not equilibrate rapidly with the ADP in the medium surrounding the fibers, neither are mitochondria easily accessible for exogenous ADP. It was also shown that in conditions of oxidative phosphorylation the mitochondria effectively compete for ADP in the presence of exogenously added phosphoenolpyruvate and pyruvate kinase (PEP + PK) system, whereas pretreatment of skinned fibers with trypsin increases the efficiency of utilization of endogenous ADP by the PEP + PK. However, even in trypsinized fibers addition of 20 mM creatine decreases the proportion of ADP reaching the PEP + PK system. Based on these results, it was hypothesized that in oxidative muscle cells the mitochondria and ATPases are compartmentalized into the complexes of organized metabolic pathways, termed as the intracellular energetic units (ICEUs). Within these units the mitochondria are integrated with ATPases with creatine and adenylate kinase systems and via direct channeling of ATP and ADP. Compartmentation of adenine nucleotides and energy transfer systems into ICEU increases the efficiency of regulation of respiration [2, 3].

Structurally the existence of ICEU is supported by the observations that in cardiomyocytes the mitochondria are positioned exactly regularly so that each individual mitochondrion forms a unit with adjacent sarcomere [3, 4]. Several observations suggest that the cytoskeletal proteins are responsible for such an precise organization. Accordingly, desmin-deficient mice exhibit disintegrated sarcomeres together with lost contacts between them and mitochondria, these changes being associated with increased affinity of mitochondria to exogenous ADP in the slow-twitch fibers [5]. On the other hand, lack of dystrophin results in impaired integration of the mitochondria and ATPases in slow-twitch muscle cells of dystrophin knockout mice, a model of Duchenne disease, as evidenced by increased access of ADP for mitochondria together with disturbed coupling between mitochondrial creatine kinase and adenine nucleotide translocase (ANT) [6].

The present study was undertaken to further address the role of cytoskeletal proteins in exerting control over mitochondrial respiration *in situ*. We investigated the effect of IgG (obtained from patients with chronic liver diseases of different etiology, but frequently associated with circulating autoantibodies to cytoskeletal proteins) on mitochondrial function. This approach was chosen because the autoantibodies have been useful probes for identification of novel proteins in cell biology [7, 8]. In primary biliary cirrhosis (PBC), the antibodies are directed against (i) mitochondrial autoantigens, such as the E2 component (dihydrolipoamide acetyl transferase) of the pyruvate dehydrogenase (PDH), 2-oxoglutarate dehydrogenase, branched chain 2-oxo-acid dehydrogenase complex [9, 10], ANT [11], sulfit oxidase [12], trypsin sensitive M8-antigen associated with the mitochondrial outer membrane [13], (ii) the nuclear envelope proteins [9, 10, 14], and (iii) cytoskeletal proteins (actin, myosin, desmin, tropomyosin, α-actinin, filamin, and vimentin) [15, 16]. The same cytoskeletal proteins are autoantigenic targets also in chronic hepatitis (CH) patients, including autoimmune hepatitis (AIH) [15–17]. AIHis also characterized by antibodies to nuclear components and liver specific antigens [18]. Thus, both diseases, PBC and CH, share a common feature – presence of autoantibodies to different components of intracellular cytoskeletal system. Because these autoantibodies are considered to be non-species-specific, they were applied to search for their effects on mitochondrial function and localization in the permeabilized fibers of myocardium and skeletal muscles of rat and mice.

The results show that the human diseased sera studied possess autoantibodies that inhibit mitochondrial respiration selectively in oxidative muscle cells (heart and *M. soleus*), but not in glycolytic muscles. In cardiac muscle this effect was associated with binding of the autoantibodies to the intermitochondrial areas, Z-disks and M-lines. Therefore, it may be expected that these structures contain proteins capable to exert control over the function of ICEU [2, 3].

Materials and methods

Patients

The sera from 16 patients with PBC, 8 patients with CH from which 6 were diagnosed as autoimmune hepatitis (AIH) and 2 as alcoholic liver disease (ALD), and 6 healthy control persons were used in the study [19]. Indirect immunofluorescence test revealed antibodies to mitochondria in all PBC patients, to nuclear components in 12 sera and to smooth muscle antigens in 3 sera. From AIH patients 3 possessed antibodies to nuclear components, 3 patients to smooth muscle, one to reticulin and one had thyroid microsomal antibodies. One patient with ALD had antibodies to smooth muscle. Thirteen patients with PBC had antibodies to PDH detected by ELISA [19]. In patients with CH the active hepatitis was histopathologically confirmed.

Animals

Adult outbred Wistar rats of either sex weighing 300-350 g and desmin knockout homozygous female mice produced by Professor Harald Jockusch's group (University of Bielefeld, Germany) were used in the experiments. The animals were kept, fed and studied in accordance to the Guide for the care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication no. 85-23, revised 1996).

Separation and concentration of IgG on protein A Sepharose

IgG was separated from the human serum samples by fast protein liquid chromatography (FPLC) using 1 ml column filled with protein A Sepharose 4 Fast Flow (Pharmacia Biotech AB, Uppsala, Sweden) in 0.1 M Tris/HCl buffer, pH 8.3. IgG was eluted using 0.2 M glycine/HCl pH 2.7. The samples were immediately neutralised with 1 M Tris/HCl buffer pH 9.0, concentrated on polyethylene glycol 20000 (Fluka, Buchs, Switzerland) and dialysed overnight against 0.15 M phosphate-buffered saline (PBS) pH 7.3. The protein content in concentrated IgG solution was determined by Bradford [20].

Preparation of skinned muscle fibers

Skinned fibers were prepared according to the method described earlier [2, 3]. The animals were anaesthetized with sodium pentobarbital (50 mg/kg body wt, i.p.). The hearts and skeletal muscles (*M. soleus*, oxidative, slow-twitch) and *m. gastrocnemius* white (glycolytic, fast-twitch) were quickly excised, rinsed in ice-cold solution C and put into cooled solution A. In the same solution, the hearts were cut into halves and muscle strips (3–5 mm long and 1–1.5 mm in diameter, 5–10 mg of wet wt) were excised from endocardial side of left ventricles along fiber orientation to avoid mechanical damage of the cells. From the skeletal muscles the fiber bundles (3–4 mm long, about 1 mm in diameter) were taken. By using sharp-ended forceps or needles, the muscle fibers were separated from each other leaving only small areas of contact. After that the fibers were transferred into vessels with cooled (in ice) solution A containing 50 µg of saponin per ml and incubated at mild stirring for 30 min for complete solubilization of the sarcolemma. Permeabilized (skinned) fibers were then washed in solution B for 10 min; this washing procedure was repeated two more times to remove completely all metabolites, especially trace amounts of ADP. Complete removal of ADP can be easily seen from respiration recordings which should show very reproducible initial State 2 rates (designated as v_o) not sensitive to inhibition by atractyloside (see below).

Preparation of 'ghost' fibers

Skinned fibers prepared as indicated above were incubated in solution containing 20 mM taurin, 0.5 mM dithithreitol, 10 mM $MgCl_2$, 10 mM ATP, 800 mM KCl, 50 mM Hepes, pH 7.1 adjusted with KOH for 30 min at 4°C to extract myosin from the muscle cells. After that the fibers ('ghost' fibers) were washed 5 times in solution B.

Analysis of the effects of IgG on mitochondrial respiration in skinned muscle fibers

The skinned fibers (0.7–2.4 mg dry wt) were incubated in solution containing 125 mM KCl, 20 mM Hepes, 4 mM glutamate, 2 mM malate, 3 mM Mg-acetate, 5 mM KH_2PO_4, 0.4 mM EGTA, 0.3 mM dithiothreitol and 2 mg/ml BSA, in the absence or presence of IgG (100 µg/ml) and 0.2 mM ADPat 25°C in a chamber (volume 3 ml) of oxygraph (Rank Brothers, UK). The rates of oxygen consumption rates by mitochondria in the skinned fibers were monitored by Clark electrode, assuming the solubility of oxygen in the medium to be 430 ng-atoms O/ml [1]. The ratio between the rate of respiration with 1 mM ADP and that before addition of ADP (basal respiration, v_o) was taken as the acceptor control index (ACI), to monitor coupling between oxidation and phosphorylation. The skinned fibers with ACI of 4–6 were used in the experiments to monitor the effects of IgG. After measurements the fibers were removed from the chamber and dried overnight at 105°C. The rates of oxygen consumption were normalised per mg of dry muscle weight.

Estimation of intactness of the outer and inner mitochondrial membranes in skinned fibers

To assess the status of the outer mitochondrial membrane the respiration of muscle fibers was maximally activated by 1 mM ADP followed by addition of 8 µM cytochrome c. In case of ruptured outer membrane, exogenous cytochrome c can pass into the intermembrane space and stimulate the respiration by compensating loss of that cytochrome through leaky membrane. This effect of cytochrome c is absent in conditions of intact outer membrane. The intactness of inner mitochondrial membrane was assessed by addition of 35 µM atractyloside, inhibitor of ANT, into the oxygraphic medium. In intact mitochondria atractyloside reduces the ADP-stimulated respiration down to the basal levels (v_o), which indicates effective control of oxidative phosphorylation by ANT, whereas such a control is lost in case of impaired inner membrane [21].

SDS-PAGE and immunoblotting of skinned muscle fibers

To prepare protein samples for electrophoresis, skinned fibers from rat heart muscle and *M. gastrocnemius* were solubilised in 5 volume of buffer containing 9 M urea, 40 mM Tris/HCl pH 9.5, 2% CHAPS, 70 mM DTT, 1 mM EDTA, 2 mM Pefabloc SC, 2 µM pepstatin and 2.1 µM leupeptin. First, muscle fibers were ultrasonicated in ice-cold solubilisation buffer and then extracted for 1 h at 4°C, followed by centrifugation at 20,000 g for 20 min. Protein concentrations in su-

pernatants were measured by Bradford [20]. Protein samples were diluted in SDS-sample buffer (0.0625 mM Tris-HCl, pH 6.8, 3% SDS, 10% glycerol, 0.1 M DTT, 0.02% bromophenol blue) and subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with protein load 10 mg per cm of gel [22]. The separated proteins were transferred from the gel onto nitrocellulose membrane by semi-dry electrotransfer at 0.8 mA/cm² for 80 min in the buffer containing 25 mM Tris, 192 mM glycine, 0.05% SDS and 10% (vol/vol) methanol. The membranes were blocked with 3% skimmed milk and 0.05% Tween 20 in Tris buffered saline (25 mM Tris pH 7.4, 150 mM NaCl) (TBS) for 1 h. The strips cut from membrane were incubated overnight at 4°C with human sera diluted 1:100 in blocking buffer or with control antibodies, rabbit anti-desmin diluted 1:400 and anti-α-actinin diluted 1:200. The strips were washed four times during 30 min with TBS, 0.05% Tween 20 and then incubated for 1 h with alkaline phosphatase conjugated rabbit antibodies to human IgG or swine antibodies to rabbit Ig (DAKO, Glostrup, Denmark), both diluted 1:1000 in blocking buffer. After final washing the bound antibodies were detected by substrate reaction with nitro blue tetrazolium/5-bromo-4-chloro-indolyl phosphate.

Imaging of mitochondria and IgG localization by laser confocal microscopy

To stain mitochondria, the muscle fibers were incubated in solution A (see above) containing 0.2 μM mitochondrion selective dye MitoTracker Red CMXRos (Molecular probes, Inc., OR, USA) with continuous stirring in the dark during 30 min. Thereafter the fibers were washed 3 times in solution A (without stain) to reduce the background fluorescence. Then the stained fibers were divided into three portions, and each of them was incubated in the standard oxygraphic medium, in conditions of oxidative phosphorylation registered by oxygraph (see above), in the presence of IgG isolated from either patient with PBC, CH or from a healthy control (HC) during 20 min with continuous stirring in the dark. After that the fibers were washed two times in solution A for 10 min in dark, to remove the unbound immunoglobulins and incubated with FITC-conjugated rabbit immunoglobulins to human IgG (DAKO, Clostrup, Denmark) diluted 1:20 in solution A for 30 min. This was followed by washing the fibers twice in PBS for 10 min and the probes were fixed with 10% Histoprep (Fischer Scientific, Pittsburgh, PA, USA), placed on the specimen glass, attached with glycerol-PBS (1:1) drop and protected by coverslip. All these procedures were carried out at room temperature (20°C). The skinned fibers from the heart of a desmin knockout mouse were processed similarly, except that incubation with IgG was performed without parallel registration of the respiration rate. The specimens were imaged and scanned by MRC 1024 BioRad laser confocal microscope.

Solutions

Solution A contained, in mM: CaK_2EGTA 2.77, K_2EGTA 7.23, $MgCl_2$ 9.5, dithiothreitol (DTT) 0.5, potassium 2-(N-morpholino)ethansulfonate (K-Mes) 49, imidazole 20, taurine 20, Na_2ATP 5.25, phosphocreatine 15, pH 7.0 adjusted at 25°C. The concentration of free Ca^{2+} = 0.1 μM.

Solution B contained, in mM: CaK_2EGTA 2.77, K_2EGTA 7.23, $MgCl_2$ 4, DTT 0.5, K-Mes 100, imidazole 20, taurine 20, K_2HPO_4 3, glutamate 5, and malate 2, 5 mg/ml BSA pH 7.0 adjusted at 25°C. The concentration of free Ca^{2+} = 0.1 μM.

Solution C contained, in mM: 118 NaCl, 4.7 KCl, 2.52 $CaCl_2$, 1.64 $MgSO_4$, 24.88 $NaHCO_3$, 1.18 KH_2PO_4, 5.55 glucose, 2 K-pyruvate, pH 7.4.

10x Phosphate Buffered Saline (PBS) (g per liter) 80 NaCl, 2 KCl, 14 $Na_2HPO_4 \times H_2O$ or 11.5 Na_2HPO_4 2 KH_2PO_4 or 2.79 $KH_2PO_4 \times 3H_2O$.

Reagents

All reagents and anti-desmin and anti-a-actinin antibodies were purchased from Sigma (St. Louis, MO, USA) if not stated otherwise. The solutions were prepared on the basis of deionized water (Milli-Q filters, Millipore Corp., USA).

Statistical analysis

Statistical analysis was performed by Student's paired *t*-test or by ANOVA. The results are given as mean ± S.E.M. if not specified otherwise.

Results

Muscle type-specific inhibition of mitochondrial respiration by IgG

Figure 1 shows the scheme of the typical experiment with normal skinned cardiac fibers. Addition of 0.2 mM ADP increased the rate of respiration over the basal levels with glutamate and malate (v_0), due to initiation of oxidative phosphorylation. This rate corresponds to state 3 respiration in isolated mitochondria [23]. Typically to skinned fibers, there was no transition from state 3 to state 2, because of continuous regeneration of ADP by ATPases consuming mitochondrially produced ATP [21]. The following addition of purified IgG from the patient with PBC resulted in inhibition of ADP-stimulated respiration. The inhibition developed slowly; the steady state at maximally inhibited respiration was usually achieved not earlier than 10 min of incubation with IgG. (This explains why only submaximally activating con-

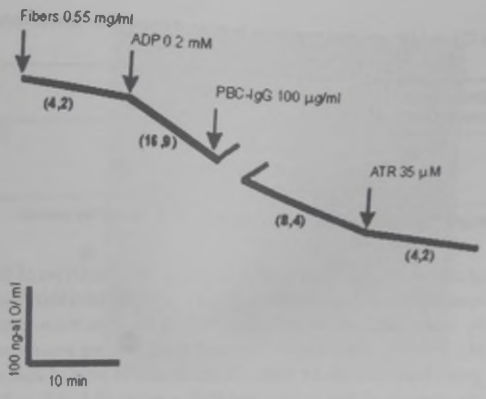

Fig. 1. The scheme of oxygraphic measurements of mitochondrial oxygen consumption in skinned fibers from rat ventricular myocardium. The additions of fibers, ADP, immunoglobulin (PBC-IgG) and atractyloside (ATR) are indicated. Respiratory substrates were added into the medium before fibers. Respiration rates in parenthesis are expressed in ng-atoms O mg dry weight^{-1} min^{-1}. Note: The trace is interrupted after the addition of IgG for 10 min and only the last section of recording where steady state inhibition was reached is demonstrated.

centrations of ADP were used in the experiments – to save oxygen in the medium throughout the long-time experiment). In separate experiments it was shown that when IgG was added 20 min prior to addition of ADP, the IgG exerted no significant effect on basal rate of respiration (v_0), whereas the ADP-dependent respiration became inhibited to the same extent as when being added after ADP. Thus, the effect of IgG was restricted to oxidative phosphorylation; the antibodies could control either the production of ADP by ATPases, or access of ADP to ANT at the level of mitochondrial outer membrane. The former aspect was addressed by decreasing the total ATPase activity in skinned fibers, by extracting myosin from the cells with KCl-treatment (ghost fibers, see Materials and methods). However, the observed inhibitory effect (about 40%) of IgG on respiration corresponded to that in skinned fibers with normal myosin content (results not shown). This suggests that inhibition of oxygen consumption in skinned fibers by IgG was not caused by its binding to the myosin ATPase. To judge over the status of mitochondrial membranes in the presence of IgG, the effects of atractyloside (ATR) and cytochrome c [21] on ADP-dependent respiration were assessed. As shown in Fig. 1, addition of ATR, a specific inhibitor of ANT, abolished the ADP-activated respiration in skinned fibers exactly to the level seen before ADP addition. It means that inner mitochondrial membrane remained intact after permeabilization of the cell membrane by saponin [21] and addition of IgG, enabling the respiration to be effectively controlled via ANT. On the other hand, in separate experiments it was found that exogenous cytochrome c did not affect the ADP-activated respiration rates, both in the presence and absence of IgG (Recordings not shown). This observation, indicating intact outer mitochondrial membrane [21], suggests that IgG exerted inhibitory effect on respiration without penetrating into intermembrane space of mitochondria in skinned fibers.

To determine whether the effects of IgG are related to reversible or irreversible binding, in several experiments the skinned fibers were washed during 30 min to remove the unbound IgG after registration of its inhibitory effect on respiration. It was found that this procedure had no effect, as the respiration rate remained essentially the same (5.36 ± 0.51 and 5.40 ± 0.68 ng-atoms O mg^{-1} min^{-1}, n = 5) before and after PBC-IgG washout, respectively. Hence, irreversible binding of antibodies to intracellular sites in cardiac cells can be assumed.

Table 1 presents the mean values of the effects of IgG on respiration in different groups of IgG preparations. It can be seen that IgG from healthy donors significantly decreased the rate of oxidative phosphorylation in skinned cardiac fibers, relatively by 13% of the preincubation level. In contrast, IgG isolated from patients with PBC or CH equally suppressed this parameter to the markedly larger extent (by 42–44%). Notably, no qualitative differences between the two disease groups, with respect their effects on parameters shown in Fig. 1, were detected. When the relative amounts of antibodies to PDH complex or desmin were plotted against relative inhibition of respiration for corresponding individual serum, no correlation was observed as well (result not shown). Figure 2 shows that IgG from the sera of patients with PBC and CH (including AIH) inhibited oxidative phosphorylation to the similar extent in skinned fibers of heart and skeletal muscle (*M. soleus*). However, no inhibition occurred when the same immunoglobulin fractions were applied to glycolytic muscles. It appears therefore, that the glycolytic muscles either lack the autoantigens capable to react with IgG, or these autoantigens, though present, expose different epitopes compared to that in oxidative muscles.

Structural evidence of binding of IgG to sarcomeric structures of skinned cardiac fibers as revealed by confocal microscopy

In general, Figs 3A–3D demonstrates fairly preserved intracellular structures in skinned cardiac fibers from normal rat. This is evident from parallel rows of mitochondria separated by myofibrils (seen as dark lines between the mitochondrial rows). Intensive and uniform staining of mitochondrial membranes with Mitotracker Red indicates that all mitochondria were functionally normal, since maintenance of high membrane potential ($\Delta\psi$) is conditional for effective transport into

Table 1. The effect of IgG from the sera of healthy donors, patients with PBC and CH on ADP-activated respiration in skinned fibers from rat ventricular myocardium

IgG donors	ADP-activated respiration ng-atoms O mg^{-1} min^{-1}	ADP-activated respiration in the presence of IgG, ng-atoms O mg^{-1} min^{-1}	Inhibition by IgG, %
Healthy controls (7)	9.64 ± 0.72	8.27 ± 0.51*	13 ± 4
Patients with PBC (18)	11.36 ± 0.63	6.69 ± 0.46***	44 ± 3‴
Patients with CH (8)	9.15 ± 1.0	5.43 ± 0.57***	42 ± 3‴

*, ***$p < 0.05$ and 0.001 respectively compared to corresponding value without IgG. ‴$p < 0.001$ compared to inhibition (in %) in healthy controls.

and binding of this staining agent to mitochondria. When the skinned fibers were incubated with IgG from healthy subjects (Fig. 3A), only a little amount of immunoglobulins was bound, compatibly with negligible inhibition of respiration (Table 1). One can also see that IgG was specifically localized between the mitochondria, thereby forming transversal fluorescent lines crossing the whole specimen. The positions of these parallel lines obviously corresponded to the Z-disks

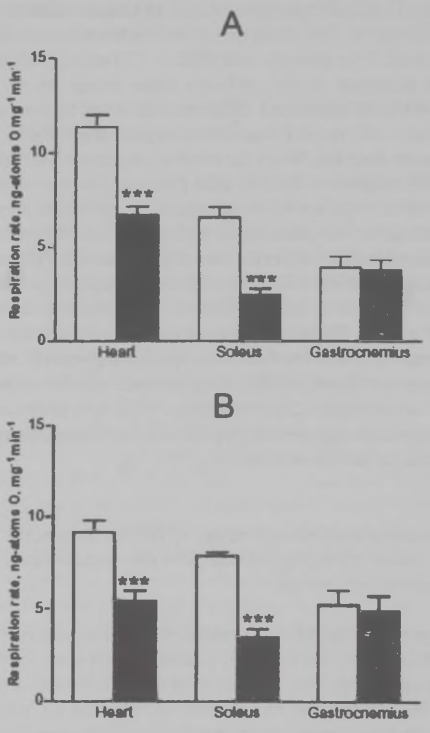

Fig. 2. Effects of PBC-IgG (A) and CH-IgG (B) (black columns) on ADP-activated respiration in oxidative (heart, *M. soleus*) and glycolytic (*M. gastrocnemius*) muscles of rat. ***$p < 0.001$ compared to respiration without IgG (white columns).

of the sarcomere. This is supported by other analyses of the localization of mitochondria with respect to sarcomere, both by laser confocal microscopy [5] and electron microscopy [4]. Notably, the distance between neighbouring transversal green lines was about 2 μm, characteristic of relaxed state of the sarcomere due to low free Ca^{2+} content (0.1 μM) in the medium. In contrast to Z-disks, only negligible amounts of IgG were detected in the sarcomeric space between the Z-disks, and colocalization of IgG with mitochondrial membranes was not seen. Compared to binding of normal IgG, the IgG from PBC group appeared to accumulate in larger quantities, predominantly on Z-disks and less over sarcomeric area, seen there as green grains and fine lines on dark myofibrillal background. Like in experiments with IgG from healthy persons, IgG from PBC patients did not bind to the outer membrane of mitochondria (Fig. 3B).

Figures 3C and 3D localizes IgG from AIH patient on the Z-disks and between them. In addition, and contrasting to PBC-IgG, these antibodies tended to form the transversal lines exactly between the Z-disks, which likely correspond to the M-line of sarcomere. Also, intensive accumulation of IgG between the cells and in the regions of intercalated disks where the proteins connecting the cardiomyocytes end-to-end localize could be observed (Fig. 3D). This feature indicates once again effective removal of sarcolemma between the cardiomyocytes, this allowing IgG easily to penetrate to all intercellular spaces, and from there, into the intracellular cytoplasmic compartments of each individual cardiomyocytes.

Localization of IgG between the mitochondria, within the Z-disk (Figs 3A–3D) and between the Z-lines (Figs 3B–3D) would occur if desmin serves as an autoantigen for IgG. The relevant control experiment was done by assessing the binding of IgG from AIH patient with subcellular structures in skinned cardiac fibers of desmin knockout mouse (Figs 3E and 3F). In accordance with earlier studies [5], lack of desmin resulted in deterioration of the organization of myofibrils and mitochondria in the cardiomyocytes, with large mitochondrial accumulations between separated from each other myofibrils (Figs 3E and 3F). Notwithstanding the absence of desmin, the IgG still bound to Z-disks, sarcomeric space, M-lines and intercalated disks. These observations exclude the desmin for being a predominant antigen for binding of IgG to the sarcomeric structures.

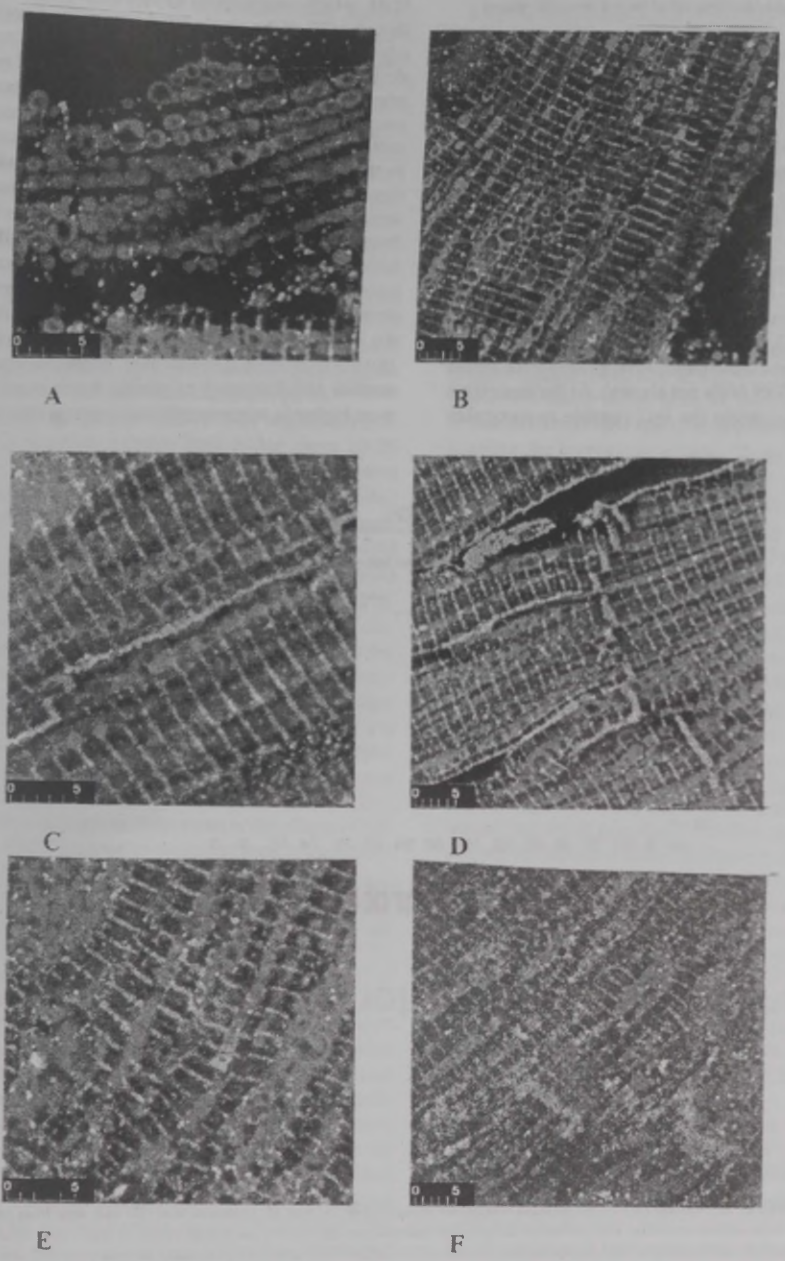

Fig. 3. Double labelling immunofluorescence confocal microscopy of the skinned ventricular fibers from rat incubated with IgG from healthy control (A), with IgG from PBC patient (B), and with IgG from AIH patient (C and D). (E, F) Skinned fibers of the desmin knockout mice, incubated with IgG from AIH patient, as in panels C and D. The red colour is Mitotracker Red associated with mitochondrial membranes. The green colour corresponds to anti-human FITC-conjugated rabbit immunoglobulins. The bars are given in micrometers.

SDS-PAGE and immunoblotting of skinned muscle fibers

To further characterize the protein targets for serum IgG in skinned muscle fibers and to relate these reactivities to IgG mediated inhibition of respiration in slow-twitch muscles, the immunoblotting of skinned fibers from heart muscle and *M. gastrocnemius* was performed with 14 sera. As seen from representative immunoblot of skinned cardiac muscle fibers in Fig. 4A, the IgG of different sera was bound to a variety of proteins ranging from 22–185 kD. Some reactivities were common to all (at 185 kD) or most (at 100 kD) sera within all groups, others being more restricted to one or another of liver diseases (PBC – 63, 54, 43 kD; CH – 84 kD) or occurring only in few or single cases. Not surprisingly, PBC serum reactivities at 63, 54 and 43 kD were directed against PDH components since these bands disappeared after serum preadsorption with PDH (data not shown). At the same time the CH sera did not contain the IgG capable to recognize PDH, which corresponds to earlier observations [15]. Although none of the single band intensities significantly correlated with the IgG mediated inhibition of respiration in skinned cardiac muscle fibers, the number of reacting proteins was generally higher in patients with increased inhibitory properties of purified IgG. Presumably, many immunoreactive protein bands in skinned cardiac muscle immunoblot might be the components of sarcomeric or other cytoskeletal structures, to which IgG was bound in native skinned fibers as shown by laser scanning microscopy (Fig. 3). For example, the frequently occurring serum reactivity at 100 kD comigrated with α-actinin band as revealed by rabbit antisera to α-actinin, and IgG binding to protein bands corresponding to desmin at 56 kD could be detected in several sera. Although the immunoreactivities with some proteins in range of 70–150 kD were more frequent in *M. gastrocnemius*, the overall number and frequency of protein bands reacting with IgG were higher in heart muscle compared to that in fast-twitch

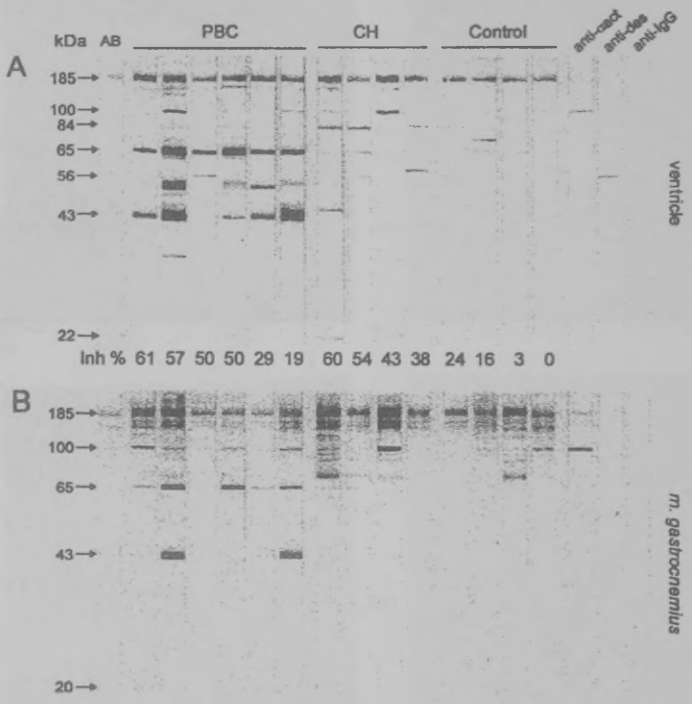

Fig. 4. Immunoblot analysis of rat skinned muscle fibers from ventricular myocardium (A) and *M. gastrocnemius* (B) with sera from patients with liver diseases (PBC, CH) and from healthy controls. The molecular weights are indicated on the left and individual values of respiration inhibition in skinned ventricular fibers are presented below each lane (A). Lanes incubated with rabbit antibodies to α-actinin (anti-αact), desmin (anti-des) and anti-human IgG secondary antibody (anti-IgG) (negative control) are shown on the right side. AB – total protein stained with amidoblack. Characterization of the autoantibodies in isolated IgG fractions.

muscle fibers (Fig. 4B). At the same time many antibody reactivities detected within heart muscle proteins were absent in *M. gastrocnemius* immunoblot. These heart-specific antigens could be responsible for the inhibition of mitochondrial respiration in skinned oxidative muscle fibers. However, none of these reactivities could be selected as definite candidate for causing inhibition of respiration, since they were not present in all individual sera exerting the inhibitory effect.

Discussion

This is the first study to demonstrate that IgG isolated from the sera of healthy persons and patients with PBC and CH inhibit mitochondrial respiration in saponin-skinned fibers of oxidative striated muscles. IgG from patients exerted markedly stronger effect than IgG from healthy controls.

In the presence of IgG the inhibition of respiration progressed slowly, reaching a steady state during about 10–20 min, which is by far more longer than corresponding period for adenine nucleotides (Fig. 1). Principally, such a retarded dynamics of the effect can reflect the slow diffusion of macromolecules, as for them the diffusion rate is at least 10-fold less than for smaller molecules (including adenine nucleotides) in oxidative muscle cells [24]. Laser confocal microscopy (Fig. 3) indicates that in spite of low diffusivity the uniform distribution of IgG in the skinned fibers was achieved. On the other hand, the IgG did not inhibit the mitochondrial function in the glycolytic muscle, *M. gastrocnemius* (Fig. 2). That IgG could not reach the core part of the these muscle cells is unlikely, as the macromolecules diffuse faster in glycolytic than in oxidative fibers, due to thinner Z-disks and narrower M-lines, creating less hindrance for their movement [24]. Thus, muscle type-dependent differences in the effects of IgG on respiration (Fig. 2) cannot be explained merely by distinct diffusional properties of the muscles.

It can be argued that autoantibodies from PBC patients suppress respiration of isolated mitochondrial preparations due to inhibition of PDH complex, as they directly affect the relevant mitochondrial enzyme activities *in vitro* [25–27] or colocalise intracellularly with E2 subunit of pyruvate dehydrogenase complex [28]. A number of arguments allow us to exclude the role of anti-PDH in inhibition of mitochondrial respiration in our test system. (i) PDH complex could not limit the respiration rate because glutamate/malate pair of substrates was used instead of pyruvate to feed the respiratory chain in our experiments. (ii) The extent of inhibition did not correlate with the level of antibodies to PDH (data not shown). (iii) Autoantibodies did not inhibit the respiration in the cells of *M. gastrocnemius*, despite their significant content of mitochondria and PDH. (iv) Similar inhibition of respiration was achieved by IgG of PBC and CH patients, while in CH patients' sera anti-PDH is absent. At the same time the observations that γ-globulin fraction of PBC sera did not inhibit the respiration of isolated mitochondria [29], that IgG of PBC and CH patients shared the property to recognize cytoskeletal proteins (Figs 3 and 4), and that in both cases immunostaining localized predominantly in sites which may form the boundaries of hypothetical ICEUs, i.e. along the lines linking the neighbouring Z-disks and leaving mitochondria between them, allow the effects of IgG on mitochondrial respiration to be interpreted in terms of their influence on the function of ICEUs (Fig. 5). In Fig. 5 the bulk cytoplasmic phase is presented by the solution inside and around the cavities left after disruption of T-tubules with saponin, being in equilibrium with the oxygraphic medium in respiration experiments. On the other hand, the proteins occurring in parallel to T-tubules and between mitochondria at the level of Z-lines (green lines, like in Fig. 3) form a border to separate cytoplasmic phase from its intra-ICEU counterpart. It is not excluded that the Z-disks itself also participate in forming the barriers described. These barriers not only tie mitochondria with adjacent ATPases, but also isolate some amount adenine nucleotides from their cytoplasmic pool, so that adenine nucleotides cannot easily equilibrate between intra-ICEU- and extra-ICEU-spaces [3]. In these conditions, adhering of immunoglobulins to the full length of the barrier forming structures (Figs 3 and 5), would significally restrict diffusion of ADP from outside the cell and cytoplasm into ICEU. Consequently, less ADP can reach ANT to stimulate oxidative phosphorylation, that explains the decreased rate of respiration (Table 1). This type of hindering effect of IgG could be potentiated by virtue of relatively low concentrations of exogenous ADP (0.2 mM) applied, and structural properties of cardiac muscle. Namely, the observation that the effect of IgG manifested in oxidative muscles (heart and *M. soleus*) but not in glycolytic muscles can presumably be explained by thicker Z-disks (112 nm) in former muscle group than in latter one (64 nm) [24, 30]. These differences are due to different sets of α-actinin cross-links between anti-parallel actin molecules, as the white fast muscle disks have two sets of α-actinin links, whereas mammalian slow muscle Z-disks have six [30]. Obviously binding of IgG to thicker Z-disk would result in greater hindrance for diffusion of exogenous ADP than in case of thinner Z-disk. On the other hand, lack of inhibition of respiration by IgG in *M. gastrocnemius* may be explained simply by absence of ICEU, i.e. the proteins forming its structure [1, 3].

The specific subcellular localization of IgG binding (Fig. 3) leads to seek the possible candidates for the target proteins within the sarcomere. In oxidative muscle the Z-disk comprises 5 proteins – α-actinin, γ-filamin, C-terminus of nebulette, N-terminus of titin and CapZ protein [30–33]. The Z-disks of the same sarcomere are connected with intermediate filament protein desmin, which also cross-links individual myofibrils laterally at their Z-disks. It is known that desmin filaments

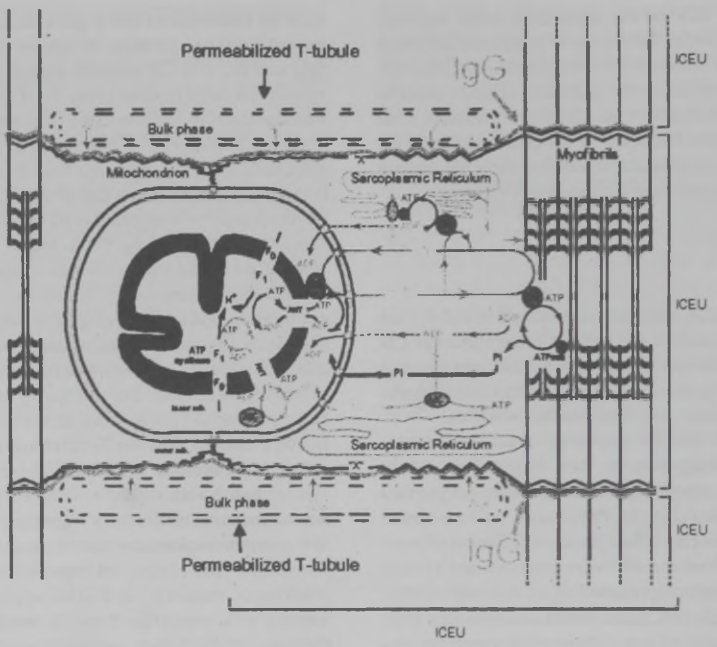

Fig. 5. Scheme of ICEU in the cells of oxidative muscle. It is hypothetized that the protein(s) of unknown nature (X), most probably connected to cytoskeleton, fix the position of mitochondria adjacent to myofibrillar and SR ATPases, and surround these structures into the complexes (ICEUs) isolated from the cytoplasmic bulk phase [2, 3]. As a result, the exogenous ADP added to the skinned fibers cannot reach easily mitochondria and adenine nucleotides within ICEU do not equilibrate rapidly with their cytoplasmic counterparts. Binding of IgG (green lines, as in Fig. 3) with protein X increases the restriction for diffusion of adenine nucleotides from the cytoplasm to ICEU, thus manifesting as inhibition of ADP- stimulated respiration.

colocalize with a cytolinker protein plectin, which, in turn, binds to mitochondria. Therefore the tandem of proteins – desmin and plectin is considered to play an important role in positioning of mitochondria in the intermyofibrillar space exactly at the level between the two Z-disks [34]. In fact, Fig. 3, together with our earlier results [3] fully supports this concept. Firstly, Fig. 3 clearly shows that the mitochondria are fairly localized close to the neighbouring sarcomeres. Secondly, Figs 3E and 3F indicates that in desmin-deficient mice heart, the positions of Z-disks in the neighbouring myofibrils became shifted and the intermitochondrial fibers were lost compared to normal myocardium (Figs 3A–3D) due to disintegration of the cellular structure. At the same time, our results (Fig. 3) clearly show that desmin is not the only candidate for binding IgG with sarcomeric structures. It would be more correct to assume that besides desmin, many other proteins such as actin, myosin, tubulin, troponin, α-actinin and tropomyosin found in patients with PBC and CH [15, 16, 35] play a role of potential antigens in this process. Consistently, the results of immunoblotting revealed serum IgG binding to a number of different proteins in skinned heart muscle, including those comigrating with α-actinin and desmin. In general, there was more proteins capable to bind IgG in cardiac muscle than in *M. gastrocnemius*. Probably the immunoreactive bands that manifest exclusively in the myocardium can be attributed to the proteins participating in control of mitochondrial respiration within the ICEU, and, therefore, conferring sensitivity of this unit to inhibitory action of IgG. At the same time the inhibition did not correlate with single band reactivity on immunoblots, suggesting that the effect on respiration could be mediated by binding of IgG to different molecules forming ICEU. On the other hand, one should consider that the antibody epitopes on native cytoskeletal structures can be lost and neoepitopes be emerged after denaturing SDS-PAGE and immunoblotting as shown earlier [16], and thereby this method may not reflect exactly the IgG binding in skinned muscle fibers. Interestingly, careful inspection of laser confocal image presented in Figs 3C and 3D reveals that IgG of CH patient appears to bind to M-line structures. At present, the nature of corresponding autoantigen(s) is unclear. It is known that M-line that links the parallel myosin molecules, is composed of a number of pro-

teins known as creatine kinase [36], myomesin [37, 38], M-protein [39], skelemin [38, 40], and C-terminus of titin [31, 32]. Intensive accumulation of IgG was observed also in the regions of intercalated disks that connect the adjacent cardiomyocytes. In this region, different proteins such as vinculin, filamin, calspectin with different functions (reviewed by Opie, [41]), [42] exist. None of these proteins have been yet shown to react with IgG from patients with PBC or CH. The observation that IgG circulating in healthy individuals and in PBC and CH patients resulted in qualitatively similar effect – inhibition of respiration – suggests that the antibodies capable to bind to sarcomeric elements may be common among so called 'natural autoantibodies' [43] and frequently detected in liver diseases and even in healthy persons.

Whatever the nature of autoantigens is, this study rises a question whether the observed phenomenon – inhibition of oxidative phosphorylation by circulating autoantibodies - may take place in the living cells *in vivo*, i.e. in the cells with intact sarcolemma? In this regard the important findings are those demonstrating that different autoantibodies can penetrate into the cells [28, 44–47]. For example, Koscec *et al.* [46] have demonstrated a transfer of antibodies to ribosomal P protein into the hepatocytes, followed by cellular dysfunction manifested as intracellular accumulation of cholesterol and lipid droplets. Likewise, autoantibodies to ANT elaborated during autoimmunization of guinea-pigs can reach the intracellular sites in the cardiomyocytes, colocalize with the mitochondrial membranes and disturb the cellular energy metabolism *in vivo* [48]. Internalization of autoantibodies seems to be processed by receptor mediated endocytosis [49, 50], or by recruitment of the properties of F(ab') 2 fragments of IgG [46]. Thus, at least part of pathogenicity of circulating autoantibodies may involve the mechanisms of their intracellular action. Moreover, these mechanisms may cause similar disturbances in different tissues. This is apparent from observations that mitochondrial ANT is an antigen not only in dilated cardiomyopathy but also in PBC [11] and that the liver cirrhosis frequently leads to specific type of cardiomyopathy, which may manifest as congestive heart failure after liver transplantation [51, 52]. Besides the myocardium, the skeletal muscles may be also affected, as development of muscle fatigue [53] or decreased exercise capacity [54–56] in patients with liver cirrhosis has been reported. At present the mechanism underlying muscle dysfunction in these patients is not clear. Rat models of cirrhotic cardiomyopathy have revealed decreased L-Ca^{2+} current [57] and blunted responsiveness to muscarinic M2 receptor [58] and β-adrenoceptors [59], all these changes suggesting altered signalling systems. On the other hand, together with observations made by Schultheiss group [11, 48], several other findings point to involvement of mitochondria. Oliveira *et al.* [60] have shown decreased capacity of mitochondria to accumulate Ca^{2+} resulting in opening of permeability transition pore in experimental acute cholestasis. In cirrhotic patients the subsarcolemmal aggregation of abnormal mitochondria without significant inflammation [53] and reduced mitochondrial capacity to synthesize ATP [54, 55] have been revealed. The results of the present study offer another clue to understand the impaired mitochondrial function: inhibition of oxidative phosphorylation by circulating autoantibodies may be caused through their specific interaction with the components of ICEU in the oxidative muscle cells.

In conclusion, the results show that the IgG isolated from the sera of normal healthy persons and patients with PBC or CH inhibit respiration of mitochondria in oxidative but not in glycolytic muscle cells. IgG from patients with liver diseases was significantly stronger inhibitor of the oxidative phosphorylation than normal IgG. The inhibitory effects of IgG in oxidative phosphorylation is related not to its binding with mitochondrial outer membrane, but rather to their specific interactions with sarcomeric structures, predominantly with the proteins projecting at the Z-disk and M-line areas. These proteins may play a principal role in controlling exchange of adenine nucleotides between the ICEUs and surrounding cytoplasm.

Acknowledgements

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CURRICULUM VITAE

LUMME KADAJA

Born: August 17, 1952 in Estonia

Education

1959–1967	Primary School in Ingliste, Rapla district
1967–1970	Rapla Secondary School
1971–1978	Faculty of Physics and Chemistry, University of Tartu
1992–1993	Master's studies (Biomedicine), Faculty of Medicine, University of Tartu
30.03.1993	Master's thesis and Master's degree
2003–2004	Doctoral studies (external student), Faculty of Medicine, University of Tartu

Professional Employment

1981–1983	Senior laboratory assistant, Institute of General and Molecular Pathology, University of Tartu
1984–1987	Engineer, Institute of General and Molecular Pathology, University of Tartu
1988–1991	Research Fellow, Institute of General and Molecular Pathology, University of Tartu
1993–2003	Research Fellow, Department of Pathophysiology, Institute of General and Molecular Pathology, University of Tartu

Special Courses

1. The Course for Nordic and Baltic Research workers in: Laboratory Animal Science in Modern Biomedical Research, 7-th–14-th August, 1993 held at the Norwegian College of Veterinary Medicine, Oslo.
2. The scientist competence course in: Laboratory Animals in Biomedical Research, held at the University of Tartu, Estonia, 1–11 May, 1994.
3. EMBO Practical Course "Advanced Techniques in Molecular Medicine", Uppsala University, Sweden, August 24–30, 2000.

Scientific work

The research themes have been the regulation of cardiac energy metabolism by thyroid hormones, intracellular energy transport in health and disease, energy networks in muscle cells, regulation of mitochondrial respiration in relation to gene expression and the diseases.

Publications

10 research papers published in international journals.

ELULOOKIRJELDUS

LUMME KADAJA

Sünd. 17. augustil 1952 Eestis

Haridus

1959–1967 Ingliste põhikool Rapla rajoonis
1967–1970 Rapla Keskkool
1971–1978 Tartu Ülikooli füüsika-keemiateaduskond
1992–1993 Magistratuur (Biomeditsiin) Tartu Ülikooli arstiteaduskonnas
30.03.1993 Magistritöö kaitsmine, magistrikraad
2003–2004 Doktoriõppes (eksternina) Tartu Ülikooli arstiteaduskonnas

Kutsealane teenistuskäik

1981–1983 Tartu Ülikool, ÜMPI hormonaalse regulatsiooni labor, vanemlaborant
1984–1987 Tartu Ülikool, ÜMPI hormonaalse regulatsiooni labor, insener
1988–1991 Tartu Ülikool, arstiteaduskond, ÜMPI, teadur
1993–2004 Tartu Ülikool, arstiteaduskond, ÜMPI, patofüsioloogia teadur

Erialane enesetäiendus

1. Põhja- ja Baltimaade teadustöötajate kursus "Katseloomad kaasaegsetes Biomeditsiinilistes uuringutes" Norra Veterinaarmeditsiini Kolledzh, Oslo, 7.–14. aug., 1993.
2. Rahvusvahelised kursused "Katseloomad biomeditsiinilises uurimistöös" Tartu Ülikool, 1.–11. mai, 1994.
3. EMBO (Euroopa Molekulaarbioloogia Organisatsioon) praktiline kursus "Kaasaegsed meetodid molekulaarmeditsiinis", Uppsala Ülikool, Rootsi, 24.–30. aug., 2000.

Teadustegevus

Peamiseks uurimisaineks on olnud türeoidhormoonide toime südame energeetilisele metabolismile, rakusisene energiatransport normis ja patoloogias, energiatranspordi võrgud lihasrakkudes, mitokondrite hingamise regulatsioon *in vivo* seoses koespetsiifilise geeniekspressiooni ja haigustega.

Publikatsioonid

10 artiklit publitseeritud rahvusvahelistes teadusajakirjades

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