Endothelial nitric oxide synthase (NOS) deficiency affects energy metabolism pattern in murine oxidative skeletal muscle

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Oxidative capacity of muscles correlates with capillary density and with microcirculation, which in turn depend on various regulatory factors, including NO generated by endothelial nitric oxide synthase (eNOS). To determine the role of eNOS in patterns of regulation of energy metabolism in various muscles, we studied mitochondrial respiration *in situ* in saponin-permeabilized fibres as well as the energy metabolism enzyme profile in the cardiac, soleus (oxidative) and gastrocnemius (glycolytic) muscles isolated from mice lacking eNOS (eNOS^{-/-}). In soleus muscle, the absence of eNOS induced a marked decrease in both basal mitochondrial respiration without ADP (–32%; $P < 0.05$) and maximal respiration in the presence of ADP (–29%; $P < 0.05$). Furthermore, the eNOS^{-/-} soleus muscle showed a decrease in total creatine kinase (–29%; $P < 0.05$), citrate synthase (–31%; $P < 0.01$), adenylate kinase (–27%; $P < 0.05$), glyceraldehyde-3-phosphate dehydrogenase (–43%; $P < 0.01$) and pyruvate kinase (–26%; $P < 0.05$) activities. The percen-

tage of myosin heavy chains I (slow isoform) was significantly increased from $24.3 \pm 1.5\%$ in control to $30.1 \pm 1.1\%$ in eNOS^{-/-} soleus muscle ($P < 0.05$) at the expense of a slight non-significant decrease in the three other (fast) isoforms. Besides, eNOS^{-/-} soleus showed a 28% loss of weight. Interestingly, we did not find differences in any parameters in cardiac and gastrocnemius muscles compared with respective controls. These results show that eNOS knockout has an important effect on muscle oxidative capacity as well on the activities of energy metabolism enzymes in oxidative (soleus) muscle. The absence of such effects in cardiac and glycolytic (gastrocnemius) muscle suggests a specific role for eNOS-produced NO in oxidative skeletal muscle.

Key words: creatine kinase, glycolytic muscle, mitochondrial respiration, oxidative muscle.

INTRODUCTION

NO produced from arginine and oxygen in a variety of mammalian tissues mediates many physiological responses. NO is synthesized by a family of three nitric oxide synthases (NOSs): two constitutively transcribed calcium-sensitive forms [neuronal NOS and endothelial NOS (eNOS)] and an inducible form (inducible NOS). NO produced by these NOS isoforms, which are expressed in specific cell types, is thought to exert distinct effects on various physiological and pathological processes.

Endothelial NOS is the major isoform expressed in endothelial cells throughout the vascular bed. It is generally accepted that endothelium-derived NO is an important factor in the control of basal vascular tone [1]. Transgenic mice overexpressing eNOS in the vascular wall demonstrate a lower blood pressure and a reduced vascular reactivity to NO-mediated vasodilators [2]. Administration of NOS inhibitors results in elevated blood pressure and increased peripheral resistance [3,4]. Mice that are deficient in eNOS have a significant increase in blood pressure [5,6], probably due to the lack of the local vasorelaxing pathway as well as due to an activation of the renin/angiotensin system. Interestingly, mice lacking a functional inducible NOS gene have normal blood pressure [5].

Thus, NO produced by eNOS seems to play a pivotal role in the regulation of vascular tone and the matching of tissue perfusion to demand (for a review, see [7]). In addition, this enzyme appears able to control blood supply in peripheral tissues

by modulating angiogenesis. Mice lacking the eNOS gene show impaired hindlimb angiogenesis [8]. Endothelial NOS seems to mediate the effect of the vascular endothelial growth factor on the development of the vascular bed [9].

These results strongly suggest that NO produced by eNOS may control haemodynamics in peripheral tissues. Such a regulation may have an important impact on the properties of muscles. It has been demonstrated that the microcirculation is a determinant of oxidative capacity in skeletal muscles [10–12]. Severe reductions in blood flow have been shown to deleteriously affect muscle oxidative capacity and related enzyme activities [13,14]. Tissue capillarization might also regulate muscle energy metabolism. The capillary-to-fibre ratio has been reported to be tightly coupled to muscle mitochondrial volume density in stimulated rat muscle [12,15]. Increased capillarization in muscle induced by muscle stimulation appears to be an important factor contributing to the elevated mitochondrial enzyme activities [16]. It cannot be excluded that eNOS-derived NO might also have a direct muscular effect that is not related to modulation of the microcirculation or angiogenesis. NO has been shown to regulate several skeletal muscle functions (for recent review, see [17]) and inhibition of NOS in rats with *N*^ω-nitro-L-arginine methyl ester (L-NAME) results in a progressive, severe reduction in muscular activity [18].

The possible role of NO-mediated regulation of mitochondrial oxidative capacity may have an important effect on muscle function in pathological states like heart failure. It is well known

Abbreviations used: CK, creatine kinase; eNOS, endothelial NOS; eNOS^{-/-}, eNOS knockout; LDH, lactate dehydrogenase; MHC, myosin heavy chain; NOS, nitric oxide synthase; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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that heart failure is associated with impairments in the skeletal muscle function. Indeed, we have recently shown that failing rats demonstrate a broad metabolic myopathy [decrease in oxidative capacities, altered regulation of respiration by creatine kinase (CK) and adenylate kinase] in different types of muscle, including myocardium, oxidative and glycolytic skeletal muscles as well as diaphragm [19–21]. Failing hearts have been shown to have altered expression and activity of eNOS, although some conflicting observations are reported (see [22] and references therein). Treatment with both angiotensin-converting enzyme inhibitors and β -blockers seems to up-regulate eNOS activity in the myocardium [23]. In skeletal muscle of rats with heart failure, eNOS mRNA expression was shown to be reduced, and this could be prevented by exercise [24], which usually increased the muscle oxidative capacity.

The purpose of the present study, therefore, was to investigate the role of eNOS in the regulation of the energy metabolism phenotype in various muscles. We used eNOS-deficient (eNOS^{-/-}) mice to estimate the mitochondrial function *in situ*, in permeabilized muscle fibres. We also determined the activity of enzymes controlling energy metabolism. We demonstrate that eNOS deficiency has an important effect on the muscle oxidative capacity as well on the related enzyme activities in oxidative (soleus) muscle. The absence of such effects in cardiac and glycolytic (gastrocnemius) muscle suggests a specific role of eNOS-produced NO in oxidative skeletal muscle.

EXPERIMENTAL

Male C57BL wild-type and eNOS-knockout (eNOS^{-/-}) [5] mice (18 months old) that had been matched for age were studied. Taking into consideration that the NO/cGMP pathway is up-regulated in aged myocardium [25] and also that the role of eNOS in the control of the microvasculature seems to increase with age [26], we used relatively aged animals in our study. The investigation was carried out in accordance with the Helsinki Recommendations for Humane Treatment of Animals. Animals were anaesthetized with an intraperitoneal injection of pentothal, and then the heart as well as soleus (slow oxidative), plantaris (mixed) and superficial portions of gastrocnemius (rapid glycolytic) muscles were isolated. A portion of the muscle tissues were rapidly frozen for biochemical determinations.

Functional properties of mitochondria and bound CK

Respiratory parameters of the total mitochondrial population were studied *in situ* in saponin-permeabilized fibres using the method described earlier [27] with minor changes [28]. Thin fibre bundles (100–250 μ m in diameter) were excised from ventricular and skeletal muscles. The bundles were incubated with intense shaking for 30 min in solution S (see below) containing 50 μ g/ml saponin to selectively destroy the integrity of the sarcolemma. Then the bundles were transferred into solution R (see below) twice for 10 min to wash out adenine nucleotides and phosphocreatine. All procedures were carried out at 4 °C.

Solutions S and R contained 10 mM EGTA/Ca-EGTA buffer (free Ca²⁺ concentration, 100 nM), 3 mM free Mg²⁺, 20 mM taurine, 0.5 mM dithiothreitol and 20 mM imidazole. Ionic strength was adjusted to 0.16 M by addition of potassium methanesulphonate. Solution S (pH 7.1) also contained 5 mM MgATP and 15 mM phosphocreatine. Solution R (pH 7.1) contained 5 mM glutamate, 2 mM malate, 3 mM phosphate and 2 mg/ml fatty acid-free BSA, instead of high-energy phosphates.

Respiratory rates were determined by a Clark electrode (Strathkelvin Instruments, Glasgow, U.K.) in an oxygraphic cell containing 15–20 fibre bundles in 3 ml of solution R, at 22 °C with continuous stirring. The solubility of oxygen was taken to be 230 mmol of O₂/l. After measurement, the bundles were removed and dried. Respiration rates were expressed as μ mol of O₂/min per g of dry weight. The ratio wet weight/dry weight was determined to be 3.80 ± 0.06 for ventricle, 3.62 ± 0.09 for soleus and 3.29 ± 0.10 for gastrocnemius muscle, with no differences between knockout and control tissues.

To obtain respiratory parameters at various ADP concentrations, permeabilized fibres were exposed to increasing ADP concentrations in the absence or presence (20 mM) of creatine to assess the functional activity of mitochondrial CK. ADP-stimulated respiration above basal oxygen consumption was plotted to determine the apparent K_m for ADP and V_{max} using a non-linear fitting of the Michaelis–Menten equation.

Enzyme analysis

Tissue samples were weighed, homogenized in ice-cold buffer (50 mg of wet weight/1 ml) containing 5 mM Hepes (pH 8.7), 1 mM EGTA, 1 mM dithiothreitol and 0.1 % Triton X-100, and incubated for 60 min at 4 °C for complete enzyme extraction. Enzyme activities were determined as described previously [20]. The total activities of CK, adenylate kinase, lactate dehydrogenase (LDH) and citrate synthase were assayed (30 °C, pH 7.5) with coupled enzyme systems. CK and LDH isoenzymes were separated using agarose (1 %) gel electrophoresis performed at 250 V (for CK) or 200 V (for LDH) for 90 min. Individual isoenzymes were detected either through incubation of the gels with a coupled enzyme system (CK) or a commercial revelation kit (Sigma LDH reagent kit).

Activity of CK and LDH isoenzymes was quantified by multiplying each percentage by total activity, as determined spectrophotometrically. Four different isoforms of CK are expressed in a tissue-specific manner. A major part of muscle CK exists as dimers composed of two subunits, M and B, giving three isoenzymes, MM, BB and MB. In addition, there is a fourth isoenzyme in the mitochondria, mitochondrial CK, which can form both octameric and dimeric structures. LDH is a tetramer made up of two types of subunit: M (found mainly in skeletal muscle) and H (found mainly in the heart). The two homo-(H₄ and M₄) and three hetero-(H₃M, H₂M₂ and HM₃) tetramers of LDH were detected. The H subunit of LDH was calculated as follows: H-LDH = $H_4 + 0.75 \cdot H_3M + 0.5 \cdot H_2M_2 + 0.25 \cdot HM_3$.

Myosin heavy chains (MHCs)

Myosin was extracted in a high-ionic-strength buffer as described previously [29]. MHCs were separated in 8 % acrylamide/bisacrylamide (50:1) slab gels at constant voltage (70 V) for 31 h and then silver stained. The MHC protein isoform bands were scanned and quantified by using a densitometer system equipped with an integrator (GS-700, Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Statistical analysis

All data are expressed as means $\pm$ S.E.M. Data were statistically evaluated with a Student's *t* test between wild-type and eNOS^{-/-} mice. Values of $P \leq 0.05$ were considered statistically significant.

RESULTS

Anatomical data

eNOS-knockout and control mice showed similar absolute body weights (Table 1). Transgenic animals, however, had decreased skeletal growth on the basis of lower tibia length. We did not observe absolute or relative changes in the heart weight of the eNOS^{-/-} mice, although skeletal muscle weights decreased significantly, by 28 % in soleus and by 11 % in plantaris muscle. This muscular atrophy in knockout mice was not followed by a general weight loss, because weights of internal organs (liver, lung, kidney) were not different from control values.

Mitochondrial function and CK

Oxidative capacities of three different muscle types in the presence of glutamate/malate as oxidative substrates are presented in Table 2. As creatine did not change the basal (in the absence of ADP) and maximal (at a saturating ADP concentration, 2 mM) respiration rates, values for these rates were pooled for experiments in the presence and absence of creatine. The basal respiration (V_o) of the permeabilized fibres of ventricular and gastrocnemius knockout muscles was slightly but not significantly lower than the respiration of the respective control fibres. However, basal respiration of eNOS^{-/-} soleus fibres was significantly diminished by one-third. Similar results were obtained for the maximal oxygen-consumption rate, V_{max} . As ADP concentration increased, respiration rate reached a plateau that was significantly lower in knockout than in control soleus muscle. Such a decrease in the maximal respiration activity was observed only in oxidative skeletal muscle, since both ventricular and

Table 3 Mitochondrial respiration with 2 mM ADP and various oxidative substrates in saponin-permeabilized fibres from soleus muscles isolated from control and eNOS^{-/-} mice

Respiration is expressed in $\mu\text{mol of O}_2/\text{min per g of dry weight}$. The fibres were incubated with 1 mM pyruvate + 2 mM malate, 10 mM succinate + 1 mM amytal or 0.5 mM TMPD + 0.5 mM ascorbate + 37 μM antimycin. Data are means $\pm$ S.E.M. Statistical significance from the control group is shown at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

	Pyruvate/malate ($n = 8-11$)	Succinate ($n = 11$)	TMPD/ascorbate ($n = 11$)
Control	22.0 $\pm$ 3.5	14.8 $\pm$ 1.4	35.8 $\pm$ 3.3
eNOS ^{-/-}	10.9 $\pm$ 1.4*	7.59 $\pm$ 0.85***	23.8 $\pm$ 1.8**

gastrocnemius muscles isolated from eNOS^{-/-} mice demonstrated completely normal oxygen consumption.

The relationship between respiration rate and ADP concentration could be reasonably fitted by the Michaelis–Menten equation. This analysis allowed us, by calculating the apparent K_m , to estimate the sensitivity of the mitochondrial respiration to ADP. The averaged results are presented in Table 2. In the absence of creatine, the K_m for ADP was much higher in oxidative skeletal and ventricular muscles compared with the glycolytic skeletal muscle. We did not find significant differences in the ADP sensitivity between knockout and control mitochondria in any muscle.

In muscles having low ADP sensitivity (ventricle and soleus), the addition of creatine shifted the relationship of respiration/[ADP] towards lower ADP concentrations, due to local ADP regeneration catalysed by mitochondrial CK. This was manifested by a marked decrease in the apparent K_m for ADP. This shift was similar in eNOS^{-/-} and control fibres, showing preserved function of mitochondrial CK.

For gastrocnemius muscle (glycolytic), the apparent K_m value was very low, indicating a high mitochondrial ADP sensitivity. In contrast with oxidative muscles, addition of creatine did not decrease the apparent K_m for ADP. There were no differences in ADP sensitivity between control and eNOS^{-/-} fibres.

To estimate possible contribution of different steps of mitochondrial oxidation in the diminished oxidative capacity of soleus muscle, we measured respiratory activity of the permeabilized fibres in the presence of various substrates. The maximal oxygen consumption rate stimulated by 2 mM ADP was determined in the presence of pyruvate/malate, succinate or *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD)/ascorbate (Table 3). As can be seen, the knockout fibres demonstrated significantly lower respiration rates whatever substrate was used.

Table 1 Anatomical data

Values are means $\pm$ S.E.M. Statistical significance from the control group at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ is indicated.

Physical parameter	Control ($n = 6$)	eNOS ^{-/-} ($n = 7$)
Body weight (g)	31.8 $\pm$ 0.5	35.8 $\pm$ 1.9
Tibia length (cm)	1.84 $\pm$ 0.01	1.78 $\pm$ 0.01**
Heart weight (g)	0.18 $\pm$ 0.01	0.18 $\pm$ 0.01
Heart weight/body weight (mg/g)	5.6 $\pm$ 0.2	5.2 $\pm$ 0.3
Soleus weight (mg)	12.0 $\pm$ 0.5	8.7 $\pm$ 0.5***
Plantaris weight (mg)	20.3 $\pm$ 0.5	18.1 $\pm$ 0.5*
Lung weight (g)	0.19 $\pm$ 0.01	0.17 $\pm$ 0.01
Liver weight (g)	1.59 $\pm$ 0.05	1.58 $\pm$ 0.15
Kidney weight (g)	0.2 $\pm$ 0.01	0.22 $\pm$ 0.02

Table 2 Parameters of mitochondrial function in saponin-permeabilized fibres from different muscles

Glutamate (5 mM) and malate (2 mM) were used as oxidative substrates. V_o and V_{max} are oxygen-consumption rates in the absence and presence of 2 mM ADP respectively, and are shown as $\mu\text{mol of O}_2/\text{min per g of dry weight}$. K_m (μM) is the Michaelis–Menten constant. Data are means $\pm$ S.E.M. ** $P \leq 0.01$, statistical significance from respective control group.

	Ventricle ($n = 24-30$; $n = 12-14$ for K_m)		Soleus ($n = 11-16$; $n = 4-7$ for K_m)		Gastrocnemius ($n = 18-21$; $n = 9-11$ for K_m)	
	Control	eNOS ^{-/-}	Control	eNOS ^{-/-}	Control	eNOS ^{-/-}
V_o	9.95 $\pm$ 0.57	8.78 $\pm$ 0.32	3.91 $\pm$ 0.30	2.55 $\pm$ 0.36**	3.60 $\pm$ 0.35	3.08 $\pm$ 0.33
V_{max}	25.5 $\pm$ 2.2	26.0 $\pm$ 1.4	21.5 $\pm$ 1.9	14.8 $\pm$ 1.3**	8.44 $\pm$ 0.47	9.23 $\pm$ 1.64
K_m for ADP						
Without creatine	209 $\pm$ 16	248 $\pm$ 29	277 $\pm$ 48	329 $\pm$ 42	14 $\pm$ 3	14 $\pm$ 3
With creatine	40 $\pm$ 5	45 $\pm$ 4	71 $\pm$ 12	67 $\pm$ 13	27 $\pm$ 5	25 $\pm$ 6

Table 4 Activities of the energy metabolism enzymes

Values are means $\pm$ S.E.M. Except for protein and the LDH isoform H/M ratio, all values are shown in international units/mg of protein. Statistical significance from respective control groups is shown at * $P \leq 0.05$ and ** $P \leq 0.01$. n.d., not determined.

	Ventricle		Soleus		Gastrocnemius	
	Control ($n = 6$)	eNOS ^{-/-} ($n = 7$)	Control ($n = 6$)	eNOS ^{-/-} ($n = 7$)	Control ($n = 6$)	eNOS ^{-/-} ($n = 7$)
Protein (g of protein/g of wet weight)	0.185 $\pm$ 0.002	0.184 $\pm$ 0.008	0.122 $\pm$ 0.008	0.111 $\pm$ 0.006	0.131 $\pm$ 0.013	0.126 $\pm$ 0.013
Total CK	2.82 $\pm$ 0.14	2.58 $\pm$ 0.11	6.83 $\pm$ 0.53	5.14 $\pm$ 0.4*	26.4 $\pm$ 1.3	26.4 $\pm$ 2.4
MM CK	2.07 $\pm$ 0.08	1.98 $\pm$ 0.10	6.41 $\pm$ 0.49	4.69 $\pm$ 0.35*	18.2 $\pm$ 1.5	18.1 $\pm$ 1.8
MB CK	0.1 $\pm$ 0.01	0.09 $\pm$ 0.01	0.04 $\pm$ 0.01	0.06 $\pm$ 0.01	0.09 $\pm$ 0.01	0.08 $\pm$ 0.01
BB CK	0.02 $\pm$ 0.01	0.03 $\pm$ 0.01	n.d.	n.d.	n.d.	n.d.
Mitochondrial CK	0.63 $\pm$ 0.07	0.49 $\pm$ 0.08	0.36 $\pm$ 0.03	0.38 $\pm$ 0.08	0.03 $\pm$ 0.01	0.04 $\pm$ 0.02
Adenylate kinase	1.62 $\pm$ 0.11	1.8 $\pm$ 0.08	3.29 $\pm$ 0.22	2.41 $\pm$ 0.14**	7.53 $\pm$ 0.39	7.66 $\pm$ 0.69
Citrate synthase	1.02 $\pm$ 0.07	0.98 $\pm$ 0.08	0.46 $\pm$ 0.03	0.31 $\pm$ 0.02**	0.13 $\pm$ 0.01	0.14 $\pm$ 0.02
Glyceraldehyde-3-phosphate dehydrogenase	0.5 $\pm$ 0.03	0.42 $\pm$ 0.1	1.06 $\pm$ 0.12	0.6 $\pm$ 0.08**	0.21 $\pm$ 0.03	0.2 $\pm$ 0.02
Pyruvate kinase	0.47 $\pm$ 0.04	0.46 $\pm$ 0.03	0.43 $\pm$ 0.03	0.32 $\pm$ 0.03*	1.42 $\pm$ 0.09	1.58 $\pm$ 0.16
LDH	1.09 $\pm$ 0.05	1.22 $\pm$ 0.08	1.31 $\pm$ 0.08	1.26 $\pm$ 0.26	2.81 $\pm$ 0.17	3.33 $\pm$ 0.41
LDH isoform H/M ratio	1.55 $\pm$ 0.05	1.49 $\pm$ 0.05	1.65 $\pm$ 0.16	1.05 $\pm$ 0.06**	0.03 $\pm$ 0.01	0.14 $\pm$ 0.04*

Energy metabolism enzymes

Activities of several enzymes involved in energy metabolism are presented in Table 4. CK activity was unchanged in ventricular and gastrocnemius transgenic muscles compared with relative controls. However, knockout soleus muscles had a significantly diminished CK activity (by approx. 30%). We also determined activities of the main CK isoforms. Ventricular and soleus muscles demonstrated a typical oxidative profile, as indicated by relatively low activity of the MM isoform and relatively high activity of the mitochondrial form. In contrast, glycolytic gastrocnemius muscle had a very high MM-CK activity, whereas mitochondrial CK activity was negligible. Activities of both isoforms were similar in control and eNOS^{-/-} muscles except for soleus muscle. Transgenic soleus showed a significant decrease in MM-CK activity by 31%. Such a decrease was not accompanied by a significant diminution in the specific activity of the mitochondrial form of CK.

We also measured tissue activities of adenylate kinase (a phospho-transfer enzyme), citrate synthase (a marker of the citric acid cycle) and several glycolytic enzymes (glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase and LDH) in all three types of muscle (Table 4). In ventricle and gastrocnemius muscle, adenylate kinase activity was similar in control and knockout mice; however, soleus eNOS^{-/-} muscle demonstrated a considerable decrease in enzyme activity. The same was shown for citrate synthase activity, which was unchanged in ventricle and gastrocnemius muscle but significantly lower in knockout soleus muscle. Thus the enzyme activities correlate qualitatively with changes in respiratory capacities in muscles.

Transgenic soleus muscle also showed decreased activities of glycolytic enzymes. Glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase activities were diminished by 43% and 26% compared with respective control values. In ventricle and gastrocnemius muscle, these activities were similar in control and eNOS^{-/-} animals. LDH total activities in eNOS^{-/-} mice were unchanged in all muscles. However, examination of LDH subunits revealed that the balance between H (cardiac aerobic) and M (muscular anaerobic) isoforms was switched in knockout skeletal muscles. The H/M ratio was significantly decreased in eNOS^{-/-} soleus muscle. Transgenic gastrocnemius muscle, on the other hand, showed an increase in this ratio, due to appearance of the H subunit, which was almost absent in control gastrocnemius muscle (results not shown).

Table 5 MHC distribution in soleus muscles of control ($n = 4$) and eNOS^{-/-} ($n = 4$) mice

Data are means $\pm$ S.E.M. Statistical significance from the control group is shown at * $P < 0.05$.

	MHC distribution (%)			
	MHC I	MHC IIa	MHC IIb	MHC IIx
Control	24.3 $\pm$ 1.5	47.8 $\pm$ 2.8	13.4 $\pm$ 2.5	14.5 $\pm$ 1.1
eNOS ^{-/-}	30.2 $\pm$ 1.2*	44.9 $\pm$ 3.0	11.3 $\pm$ 1.2	13.7 $\pm$ 2.8

MHC distribution

As the metabolic phenotype in muscles is often related to the pattern of myosin isoforms, we analysed the MHC distribution in soleus muscle, where energetic metabolism was essentially altered. Table 5 demonstrates the distribution of four isoforms of MHC in control and knockout muscle. The percentage of MHC I (slow isoform) was significantly increased by 24% in eNOS^{-/-} soleus muscle at the expense of a slight non-significant decrease in the three other (fast) isoforms.

DISCUSSION

This study examined whether alterations in eNOS-derived NO may contribute to the determination of intrinsic oxidative capacity and energy metabolism in various muscles. The main result of the work is that deletion of eNOS is followed by modulation of the energetic profile of oxidative skeletal muscle, whereas the profiles of cardiac and glycolytic skeletal muscles remain almost unchanged. These data directly support eNOS-derived NO as a regulator of the pattern of energy metabolism in slow-twitch skeletal muscles with high energy-turnover rates.

The disruption of the *eNOS* gene induces some anatomical alterations in mice. Total body weight remains unchanged but knockout animals have a significantly decreased tibia length that suggests diminished skeletal growth. This result is in line with very recent data showing that the eNOS is implicated in the local regulation of bone metabolism and bone growth by controlling osteoblast function [30,31]. We did not find an increase in heart weight in knockout mice, although these animals have been shown to have elevated blood pressure [5,6]. Data in the literature

concerning ventricular hypertrophy in eNOS^{-/-} mice are rather conflicting. Yang et al. [32] reported an increase in left ventricle wall thickness and mass, whereas Vandecasteele et al. [33] observed no significant difference in absolute weight of the ventricles. The latter group found, however, a 10 % significant increase in the ratio of ventricular weight to body weight. This was not the case in the present work. These discrepancies can probably be explained by differences in the age and gender of the animals.

An interesting finding in this work was muscular atrophy in eNOS-knockout mice. Such an atrophy was more pronounced in oxidative muscle, as soleus muscle lost up to 28 % in weight. Mixed plantaris muscle also demonstrated significant but much lesser atrophy. This atrophy was specific for skeletal muscle because internal organs such as liver, lung and kidney had normal weights.

Studies of mitochondrial respiration in skinned fibres offer the unique opportunity to assess intrinsic oxidative capacities in muscles with non-limiting amounts of substrates and oxygen. A new and very important result obtained using this technique is a marked decrease in mitochondrial respiratory capacity which occurs specifically in oxidative skeletal muscle (soleus). We found a diminished oxygen-consumption rate in this tissue in both the presence and absence of ADP. The degree of this decrease was similar for both parameters (about 30 %) so that ratio of maximal to basal respiration (acceptor control ratio) was the same for knockout and control mice. This result suggests that the coupling between oxidation and phosphorylation is well preserved in eNOS^{-/-} mitochondria. The reduction of oxidative capacity expressed per unit of tissue weight could be explained by a decrease in the amount of mitochondria in muscle. This conclusion has been confirmed by decreased respiratory activities in the presence of different oxidative substrates. Indeed, the respiration on pyruvate/malate, succinate or TMPD/ascorbate was significantly diminished in knockout soleus muscle. These data suggest that eNOS^{-/-} soleus has a generalized decrease in the number of respiratory units rather than an isolated defect in a Krebs cycle step or an impairment of some respiratory chain complex. Furthermore, we found a 40 % decrease in citrate synthase activity in soleus muscle that supports the notion that the absence of eNOS induced a dramatic mitochondrial depression in oxidative skeletal muscle. This depression does not occur in other types of muscle: ventricle or glycolytic skeletal muscle. Cardiac muscle and gastrocnemius muscle of knockout mice have normal mitochondrial respiration *in situ* in both the presence and absence of ADP. These data are in line with the normal citrate synthase activity observed in these eNOS^{-/-} muscles.

It is well known that skeletal muscle fibres having different contractile phenotypes possess different oxidative capacities. We tested the possibility that the diminished oxidative capacity of soleus muscle found in our work was associated with a redistribution of MHC isoforms. Indeed, we have shown a slight increase in the proportion of type I MHC (present in slow oxidative fibres). This shift could theoretically contribute to the decrease in oxidative capacity if the proportion of type IIa MHC (present in fast oxidative and glycolytic fibres) fell, because type IIa fibres are known to have higher oxidative potential than type I fibres [34]. However, this was not the case in the present study where the percentage of type IIa MHC decreased only very slightly; moreover, decreases of similar amplitude occurred for both types IIb and IIx MHC isoforms, which normally are present in fast glycolytic fibres with low oxidative capacity.

The diminished ability of oxidative skeletal muscle mitochondria to respire is not followed by changes in a very important

kinetic mitochondrial parameter, ADP sensitivity *in situ*. The sensitivity (estimated by apparent K_m for ADP) normally is very high (low K_m) in glycolytic muscle and low (high K_m) in oxidative muscles. Various chronic states where muscular oxidative capacity is impaired (CK knockout, creatine substitution with β -guanidinopropionic acid, food restriction, hypothyroidism) are usually associated with a decrease in K_m for ADP in cardiac or oxidative skeletal muscle [28,35–37]. Such an increase in ADP sensitivity probably results from a rearrangement of the cellular architecture so that ADP generated by cellular ATPases is more accessible to the mitochondria. Such a rearrangement theoretically facilitates the adenine nucleotide exchange between mitochondria and cellular ATPases. This does not seem to be the case in eNOS^{-/-} oxidative skeletal muscle and these results imply that the internal architecture of mitochondrial arrangement in the muscle cells is not altered. Unchanged ADP sensitivity of knockout mitochondria suggests that the reduction in the oxidative capacity could not be compensated *in vivo* by facilitated intracellular adenine nucleotide exchange/transport.

In cardiac and oxidative skeletal muscles, energy transport between the mitochondria and sites of energy utilization is also regulated by the CK system. We have found a marked decrease in total CK activity, specifically in soleus muscle. This decrease resulted from a considerable reduction in the main CK isoform, MM-CK. Normally, it is the type I fibres which have lower MM-CK activity compared with other types. Probably, the increase in the percentage of type I MHC found in this study might contribute to this alteration but the absolute change in the MHC distribution pattern is too small to explain such a significant decrease in MM-CK activity. Although in various physiological states, muscles show a correlation between the proportion of fast fibres or fast MHC isoforms and MM-CK activity, in pathology this correlation may disappear. For example, in experimental heart failure, a decrease in MM-CK activity in soleus but not in gastrocnemius muscle (as in the eNOS^{-/-} muscles) was demonstrated. This decrease, however, was associated with an increase in fast-type IIa MHC expression [20]. It cannot be excluded that the decrease in MM-CK in failing slow oxidative skeletal muscles could be at least partially induced by alterations in NO production.

It is noteworthy that different pathological states (heart failure, ischaemia, various cardiomyopathies) associated with a diminished mitochondrial function are characterized by a decrease in mitochondrial CK activity in heart and muscles [19,20,38]. However, in eNOS^{-/-} soleus (as in other muscles) this isoform was unchanged in spite of decreased mitochondrial respiration. Thus one may suggest that mechanisms implicated in a simultaneous decrease in mitochondrial function and mitochondrial CK activity in cardiac pathologies are different from mechanisms that condition the mitochondrial modifications in eNOS deficiency.

Other enzymic systems participating in energy metabolism are also specifically modified in oxidative muscle of knockout mice. We found a marked decrease in tissue activity of adenylate kinase, an enzyme which contributes to the intracellular ATP/ADP/AMP balance and seems to participate in intracellular energy transport [39]. Thus two alternative systems of energy transport (CK and adenylate kinase) appear to be dependent on NO production and are impaired if this production is blocked.

Deletion of eNOS seems to down-regulate not only oxidative metabolism in slow-twitch muscle but also the glycolytic pathway. Two enzymes contributing to the control of glycolysis in skeletal muscle, glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase, have significantly lower activities in eNOS^{-/-} soleus. Interestingly, LDH is unchanged in soleus knockout mice, but the

proportion between two subunits is significantly shifted in favour of the glycolytic M form at the expense of oxidative H form. Similar changes were found in soleus muscles after removal of weight-bearing activity [29]. In the present study the impairment of microcirculation probably resulted from a lack of eNOS induction, such a shift towards a more anaerobic energy profile.

In general, our data show that the deficiency in eNOS expression induces important modifications, specifically in oxidative skeletal muscle, while cardiac and glycolytic muscles remain normal. Keeping in mind that alterations in blood delivery could change the metabolic profile and oxidative capacity of skeletal muscle, the modifications found in knockout soleus muscle seem to be induced by a de-regulation of microvascular flow when the NO production is blocked. However, the data obtained in the present study pose the question of why these multiple changes in mitochondrial activity and metabolic profile in eNOS^{-/-} mice occur only in slow-twitch oxidative muscle and not in cardiac or glycolytic muscle? Probably, this may be related to a different impact of eNOS-derived NO on the blood perfusion of different muscles. As to cardiac muscle, it has been shown that inhibition of NO formation in eNOS^{-/-} mice induces no changes in basal coronary flow and reactive hyperaemia [40]. Substantial redundancy appears to exist in regulation of the coronary system because even NOS inhibition combined with adenosine receptor blockade does not influence either resting or exercise coronary flow unless ATP-dependent K⁺ channels are intact [41]. As to specific effects of eNOS deficiency in slow-twitch muscle, they are in accord with differential expression of this isoform of the enzyme in various skeletal muscles. eNOS expression in murine fast-twitch muscles (extensor digitorum longus and cremaster) was found to be about only 40% of that in soleus, whereas neuronal NOS was much more abundant in the fast-twitch muscles; gene ablation of eNOS did not alter expression of nNOS in muscles [42]. Mechanisms of the blood flow regulation might be different in oxidative and glycolytic skeletal muscles. For example, exercise increases the blood flow considerably in red gastrocnemius muscle but in muscles with high populations of fast-twitch glycolytic fibres this increase is weak or diminished blood flow may even occur [43,44]. The blood perfusion in fast-twitch glycolytic skeletal muscle seems to be rather insensitive to NO production. NOS inhibition induces a reduction in blood flow to rat skeletal muscle but this reduction is strictly proportional to the percentage of oxidative fibres in various muscles [45]. Thus the higher eNOS expression in soleus and the relative sensitivity of skeletal oxidative fibre blood perfusion to NO production may explain the specificity of the alterations of the energy metabolism phenotype in eNOS^{-/-} mice.

The mechanism of such a down-regulation of energy metabolism in oxidative skeletal muscle is not clear. The reduction of the number of respiratory units could be induced by either accelerated mitochondrial degradation or slowed mitochondrial biogenesis. Inadequate oxygen supply due to altered vascular responsiveness in the absence of eNOS-produced NO might shift the equilibrium between formation of mitochondria and their degradation. One possible mechanism could be the increased autophagy of mitochondria mediated by a membrane depolarization, as proposed by Lemasters et al. [46]. However, this mechanism does not explain the generalized down-regulation of almost all enzymes implicated in energy metabolism.

Another and perhaps more probable possibility is the activation of apoptotic processes. Our results show a very significant decrease in total muscular mass, which is a well-known characteristic of apoptosis. Importantly, eNOS seems to be implicated in the regulation of programmed cell death. Recent studies show that eNOS-produced NO is an inhibitor of caspase 3, a key

mediator of apoptosis [47], whereas inhibition of NO synthase potentiates apoptosis [48]. Furthermore, inhibition of NO production may lead to inactivation of the antioxidant status in muscles [49], thus promoting oxidative stress. This also might contribute to induction of apoptosis in muscle. Keeping in mind the important role of mitochondria in apoptosis, one might speculate that oxidative muscle having a high mitochondrial density is more susceptible to apoptosis than glycolytic muscle.

This possible anti-apoptotic role of eNOS in slow-twitch muscle may have an important physiological aspect concerning exercise or muscle disuse. Exercise increases NOS expression and activity in skeletal muscle [24,50], whereas muscle immobilization, for example hindlimb unweighting, decreases eNOS expression [51]. Very interestingly, this decrease occurs specifically in soleus muscle but not in gastrocnemius [52]. This more profound down-regulation of eNOS is associated with muscle atrophy, being in general much more prominent in slow-twitch than in fast-twitch muscle. Thus it would be interesting to investigate whether modulation of eNOS expression in skeletal (mostly oxidative) muscle by training or immobilization contributes to the well-known changes in patterns of energy metabolism.

The extensive inhibition of the energy metabolism found in the present work resembles that described for heart failure [19,20], but in the latter case such a metabolic myopathy was observed in all types of muscle. Therefore these generalized alterations in oxidative metabolism associated with heart failure cannot be ascribed only to an impairment in eNOS expression. To explain these alterations, other central mechanisms or systemic factors should be taken in consideration. These could be, for example, changes in the neurohumoral system or circulating factors such as cytokines or tissue necrosis factor. Finally, the possible role of alterations in inducible NOS also deserves more detailed study.

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