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DESIGN OF THE OXYGEN AND SUBSTRATE PATHWAYS

VI. STRUCTURAL BASIS OF INTRACELLULAR SUBSTRATE SUPPLY TO MITOCHONDRIA IN MUSCLE CELLS

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Summary

This paper quantifies the structures involved in the transport and oxidation of carbohydrates and fatty acids within the muscle cell. The structural capacity is measured on whole-body random samples of the musculature of dogs and pygmy goats and compared with maximal rates of oxygen consumption and substrate oxidation. Comparing dogs and goats of the same body size provided a 1.55-fold difference in the maximal rate of oxidation when related to muscle mass. As in previous studies, we found that the volume of mitochondria was approximately proportional to aerobic capacity. The maximal glucose flux from intracellular stores to mitochondria is 1.6 times greater in the dog than in the goat; we find that the amount of glycogen stored in the muscle cells is 4.2 times as great in the dog, but part of the intracellular glycogen pool is used for anaerobic rather than for oxidative metabolism. The maximal fatty acid flux from intracellular stores to mitochondria is 1.5 times larger in the dog, and the amount of lipid stored is 2.3 times as great in the dog. Every lipid droplet is in direct contact with the outer membrane of a mitochondrion and the contact surface area is 3.6 times greater in the dog than in the goat. Additional measurements are needed to investigate the role of structural limitation at this step. The amount of substrates stored intracellularly in the muscle cells of the dog is about twice as much as would match the differences in the maximal rates of utilization. This allows the endurance-specialized dogs to run for longer periods at higher rates of oxidation.

Key words: muscle oxidative metabolism, mitochondria, substrate pathways, lipid droplets, glycogen, symmorphosis, dog, goat.

Introduction

Oxidative metabolism in mitochondria depends on an adequate supply of both O_2 and substrates. While O_2 must be supplied continuously from air *via* the blood, the supply of substrate involves intermediary stores, both organismic (liver for glucose and adipose tissue for fatty acids) and intracellular near the site of combustion (glycogen and lipid droplets in muscle cells). In this series of studies, we ask how structures are designed if they serve more than one function, specifically how the O_2 and substrate pathways are designed in order to supply both O_2 and fuel to the mitochondria at the rate required by the working muscle.

This series extends the scope of previous studies which investigated the design of the pathway for oxygen by comparing animals of different aerobic capacity due to differences in body size or athletic prowess (Weibel *et al.* 1992). These studies had shown consistently that cellular design was closely adjusted to the capacity for oxidative metabolism because the volume of mitochondria was proportional to $\dot{V}_{O_2max}$ (Mathieu *et al.* 1981; Hoppeler *et al.* 1987). This led to the conclusion that a higher aerobic capacity was achieved by building more mitochondria into the muscle cell, which lent strong support to the hypothesis of symmorphosis (Hoppeler and Lindstedt, 1985; Weibel *et al.* 1991, 1992).

Here we extend the concept of symmorphosis from the linear O_2 pathway to the network design of substrate pathways where the substrate supply is partitioned to different substrates (fat and carbohydrate) and to different sources (microvascular and intramuscular). Some of the structural steps are shared by the O_2 and the substrate pathways. We postulate that the design of each step is adjusted to the specific functional demands in order to achieve a balanced function of the entire network (Taylor *et al.* 1996).

In this part of the study, we ask whether the design of intracellular substrate stores, glycogen granules and lipid droplets, is adjusted to variations in $\dot{V}_{O_2max}$ by comparing dogs

and goats exercising at increasing intensities on a treadmill. In the preceding papers of this series, we have shown (1) that the vascular supply of both glucose and fatty acids is limited to approximately the same level in dogs and goats, (2) that it does not increase with exercise intensity (Weber *et al.* 1996*a*,*b*), and (3) that this limitation may be due to the quantitative design of capillaries and sarcolemma (Vock *et al.* 1996). As a consequence, the higher rate of fuel combustion in the athletic dog compared with the goat, as well as the increment in fuel supply to the mitochondria with increasing exercise intensity, must be met from intracellular substrate stores. We therefore ask whether the amount of glycogen and lipid stored in the muscle cells of dogs and goats and the structures limiting their supply are proportional to the different rates at which the fuels must be supplied to the mitochondria, as would be predicted by the hypothesis of symmorphosis.

Model of oxygen and substrate pathways

A model for the fluxes of oxygen and of substrates from their sources to the site of consumption, the mitochondrion, is described in detail in the first paper of this series (Taylor *et al.* 1996). Here we concentrate on the last part of this pathway: the relationship between intracellular substrate pools and the mitochondria (Fig. 1). The reducing equivalents required for terminal oxidation in the respiratory chain of the inner mitochondrial membrane are generated in the Krebs cycle from a pool of acetyl-CoA, which is replenished from the import of pyruvate from glycolysis in the cytoplasm and from β-oxidation of fatty acids that are fed into the mitochondrion through the carnitine shuttle.

The pathway supporting the flux of carbohydrate, $\dot{M}_{CHO}$(ic), begins at the glycogen granules, which are dispersed throughout the sarcoplasm and are even found within myofibrils (Fig. 2A,B). Glycogenolysis liberates glucose, which enters glycolysis to be split into pyruvate, an anaerobic process which generates ATP; pyruvate enters the mitochondria and is converted to acetyl-CoA. The structural correlate of $\dot{M}_{CHO}$(ic) is the size of the pool of glycogen granules; this is difficult to measure using morphometric methods because glycogen granules are incompletely or inconsistently retained in electron microscope preparations. We therefore assessed the (molar) concentration of glycogen glucose in the muscle fibre, C_{gluc}, using biochemical methods.

The structural correlates for $\dot{M}_{FFA}$(ic) are the volume of intracellular lipid droplets, *V*(li), and the contact surface area between the lipid droplets and the mitochondrial outer membranes, *S*(li-om). In muscle cells, all lipid droplets are in direct contact with mitochondrial outer membranes (Fig. 2B). We postulate that the transfer of fatty acids from intracellular stores occurs exclusively at these sites of contact; the relevant measures are then the relative area of lipid contact of the mitochondria, S_S(li-om,om), and the relative mitochondrial contact area of the lipid droplets, S_S(li-om,li).

The goal of this study is to measure the structural parameters that characterize the supply of substrates from intracellular stores to the mitochondria, particularly the total volume of mitochondria, *V*(mt), the volume of lipid droplets, *V*(li), and of their contact surface area with mitochondria, *S*(li-om), as well as the concentration of glycogen deposits, C_{gluc}, in order to relate them to the flux rates determined for the same animals in the physiological companion papers (Weber *et al.* 1996*a*,*b*).

Materials and methods

Animals and physiological studies

The study included three adult female pygmy goats (*Capra hircus*, body mass, M_b, 21.0±0.3 kg) and three adult female

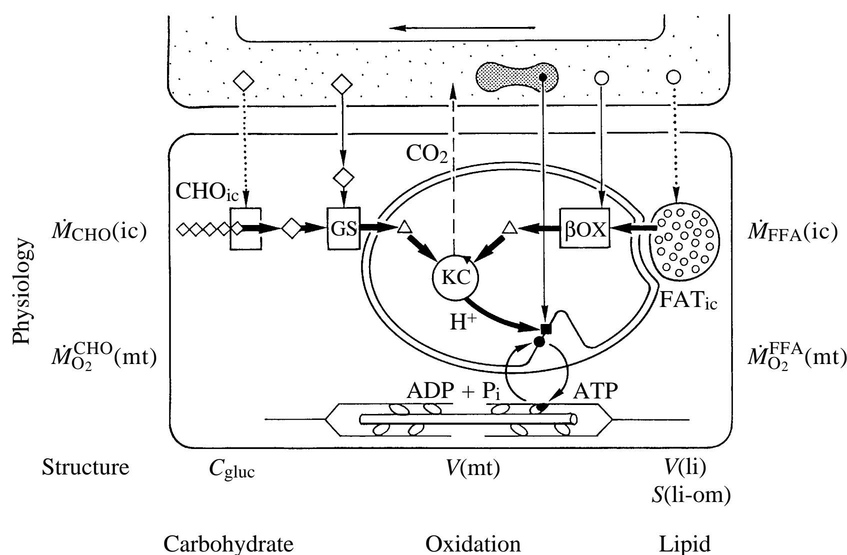

Fig. 1. Model for structure–function relationships of oxygen and intracellular substrate supply to the mitochondria of skeletal muscle cells. Dots indicate oxygen, open circles indicate fatty acids, squares indicate glucose; row of squares indicates polymerized glycogen and triangles indicate acetyl-CoA. The heavy arrows show the pathways of intracellular substrate breakdown from the intracellular stores to the terminal oxidase in the mitochondrial inner membrane (black square). The thin arrows indicate the supply routes of oxygen and substrates from the capillaries, with dotted arrows for the supply route to intracellular stores, which is temporally split from the phase of oxidation. Dashed arrow indicates CO_2 discharge to the blood. The morphometric parameters are listed together with the correlated physiological parameters: *V*(mt) and *V*(li) are the volumes of mitochondria and lipid droplets, respectively; *S*(li-om) is the contact surface area between the lipid droplets and the outer mitochondrial membranes; C_{gluc} represents molar glycogen concentration in the muscle fibres (in glucosyl units); $\dot{M}_{CHO}$(ic) and $\dot{M}_{FFA}$(ic) are molar consumption rates of intracellular carbohydrates and fatty acids; $\dot{M}_{O_2}^{CHO}$(mt) and $\dot{M}_{O_2}^{FFA}$(mt) are molar oxidation rates of total carbohydrates and fatty acids, respectively, in muscle mitochondria in oxygen equivalents. CHO_{ic}, glycogen; FAT_{ic}, fat droplet; GS, glycolysis; βOX, β-oxidation; KC, Krebs cycle; H^+, reducing equivalents.

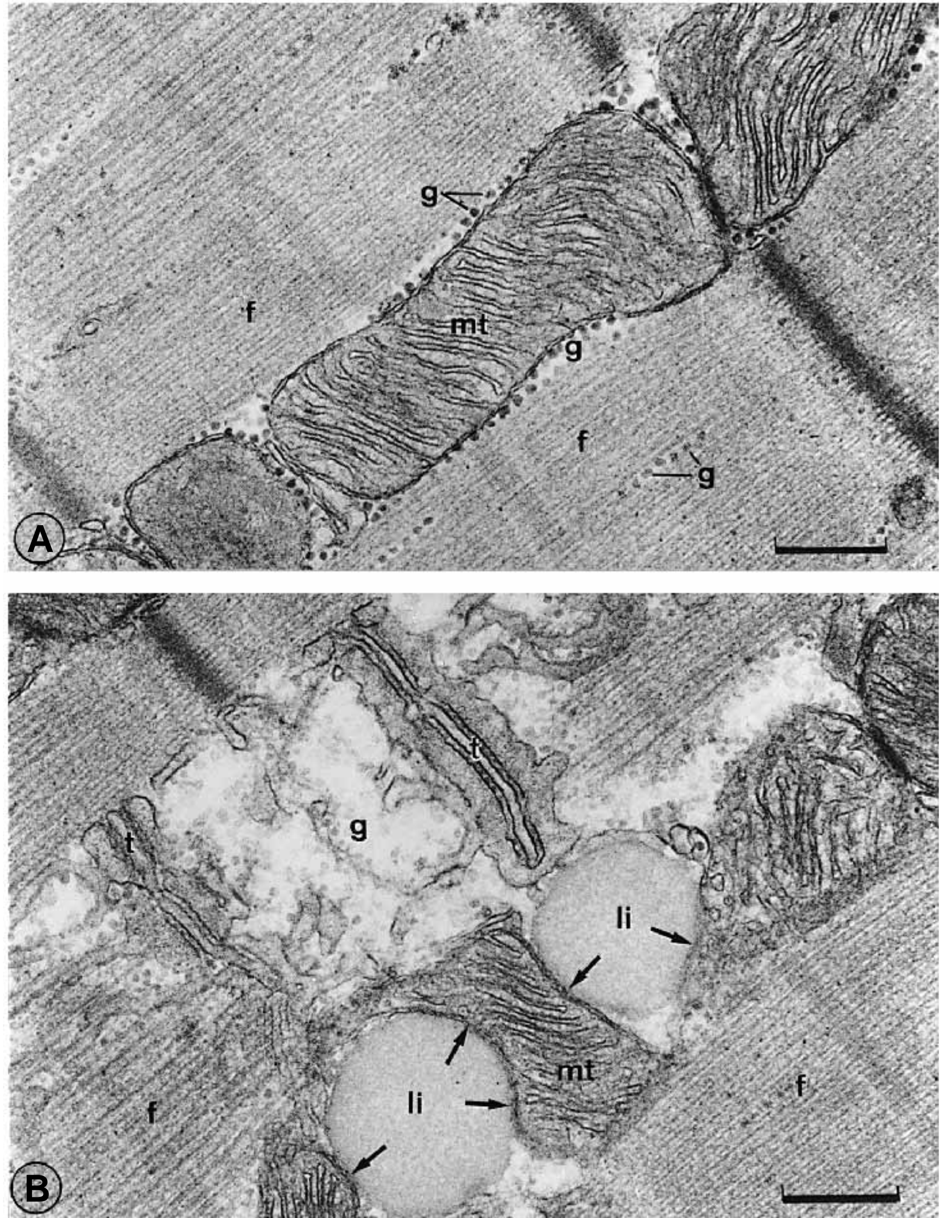

Fig. 2. Transmission electron micrographs from a muscle cell of dog triceps showing myofibrils (f), mitochondria (mt), intracellular lipid droplets (li) and glycogen granules (g). In A, the black glycogen granules are located near the mitochondria and in or around myofibrils. In B, some of the glycogen granules in the region of the tubular system (t) look 'washed out', whereas the lipid droplets are grey and show a dark surface contour. Note the dense contact surface between lipid droplets and the indented mitochondria (arrows). Scale bars, 0.3 μm.

Labrador dogs (M_b 23.7±1.0 kg; means ± S.E.M.) from the physiological studies discussed in the previous papers (Roberts *et al.* 1996; Weber *et al.* 1996*a*,*b*). Surgical procedures, training schedule, measurement of $\dot{M}_{O_2max}$ and exercise protocols of the animals are described in detail in Roberts *et al.* (1996) and are the same as for the preceding paper (Vock *et al.* 1996).

Briefly, the animals were trained to run on an inclined treadmill at different speeds until their $\dot{M}_{O_2}$ was reproducible at each speed. After determination of their individual maximal molar oxygen consumption rates ($\dot{M}_{O_2max}$), the animals were trained for three different exercise protocols, namely 2 h at 40 % $\dot{M}_{O_2max}$, 1 h at 60 % $\dot{M}_{O_2max}$ and 16 min at 85 % $\dot{M}_{O_2max}$. At each exercise intensity, glucose and fat oxidation rates were determined by means of indirect calorimetry (Roberts *et al.* 1996). The rates of glucose and fatty acid consumption from intracellular stores, $\dot{M}_{CHO}$(ic) and $\dot{M}_{FFA}$(ic), were determined using radiolabelled tracers by Weber *et al.* (1996*a*,*b*).

The animals were killed within 24 h of their last exercise trial. Tissue samples from locomotor muscles were taken and processed for morphometric as well as for biochemical studies, as described below. Whole-body muscle mass was determined by carefully dissecting and weighing the entire skeletal musculature of half the carcass of each animal. Head muscles were excluded since they do not serve locomotion.

Muscle tissue sampling, fixation and processing

According to a systematic and volume-weighted random sampling strategy described previously (Hoppeler *et al.* 1984), 15 whole-body random samples were taken from the skeletal musculature of each animal. The actual procedure is described in detail in the preceding paper (Vock *et al.* 1996). The samples

were excised within 90 min *post mortem*. One part of each sample was immediately frozen in isopentane cooled by liquid nitrogen and stored in liquid nitrogen for subsequent biochemical analysis. The remaining part was processed for electron microscopy: thin longitudinal muscle strips were fixed in a 6.25 % solution of glutaraldehyde in 0.1 mol l^{-1} sodium cacodylate buffer (adjusted to 430 mosmol l^{-1} with NaCl); the total osmolarity of the fixative was 1150 mosmol l^{-1}, pH 7.4. The blocks were rinsed overnight in 0.1 mol l^{-1} sodium cacodylate buffer, postfixed for 2 h in a 1 % solution of osmium tetroxide and block-contrasted with 0.5 % uranyl acetate. After dehydration with increasing ethanol concentrations, they were embedded in Epon in a special manner for isotropic uniform random (IUR) sectioning (see Vock *et al.* 1996, for details).

Morphometric analysis

From each of the 15 random samples, IUR sections of approximately 50–70 nm thickness were cut and picked up on 200 mesh copper grids covered with a thin carbon-coated Parlodion film; they were contrasted with lead citrate and uranyl acetate. From each of these sections, 10 micrographs were taken in a Philips 300 transmission electron microscope and recorded on 35 mm film to achieve a final magnification of about 25 000× on the morphometric screen; on each film, a carbon grating replica was recorded for calibration. At this magnification, intracellular parameters, such as the volume and surface densities of mitochondria and intracellular lipid droplets, and the amount of contact surface area between these structures were determined by point and intersection counting using standard procedures (Weibel, 1979). Fibre types were not differentiated.

Individual values for each animal were calculated by means of a morphometric program called STEPone (Humbert *et al.* 1990) whose output consisted of stereological ratios computed from point and intersection counts. All 15 random samples from one animal taken together were regarded as one entity for the stereological calculations.

In this study, we compare all findings on the basis of muscle-mass-specific parameters expressed per kilogram muscle mass rather than body mass. Since the primary morphometric data are volumes or surface areas per unit volume, muscle-mass-specific data are simply obtained by dividing the morphometric estimates by the muscle density of 1.06 g ml^{-1} and multiplying them by 1000.

Biochemical measurement of glycogen concentration

Glycogen content of the muscle fibres was determined as free glucosyl units after acid hydrolysis of muscle tissue homogenates, essentially as described by Passonneau and Lauderdale (1974). Briefly, pieces weighing between 7 and 27 mg were cut from the frozen samples using a razor blade at −28 °C, quickly weighed on a Mettler microbalance without thawing and immediately homogenized as a dilution of 1:20 in 30 mmol l^{-1} HCl. Homogenization was performed by hand in a tight-fitting glass homogenizer on ice. Thereafter, the homogenate was transferred to an Eppendorf tube and heated in boiling water for 5 min. For hydrolysis, 60 μl of homogenate was diluted in 540 μl of 1 mol l^{-1} HCl and heated to 100 °C for 4 h. After neutralization with NaOH, glucose was determined in the hydrolysate using glucose-6-phosphate dehydrogenase and hexokinase as described in Lowry and Passonneau (1972). The concentration of glucosyl residues in the hydrolysate was high enough to be measured using a spectrophotometer.

Statistical analyses

To compare the two species groups, the mean of the three individual values per group and the standard error of the mean, S.E.M., were calculated for each parameter. The statistical analysis consisted of the Mann–Whitney *U*-test, a nonparametric test for group comparisons, and a probability *P* of 0.05 was interpreted as a statistically significant difference. The data were analysed by means of SYSTAT for Windows, version 5 (SYSTAT, Inc. Evanston, IL, USA).

Results

Qualitative findings

We found a wide variation in frequency and distribution of mitochondria since our random samples contained representatives of all fibre types. In general, there was a smaller subsarcolemmal or peripheral population of mitochondria rather concentrated near the site of capillaries and a larger central or interfibrillar population, many of which were in close contact with intracellular lipid droplets. These lipid droplets were clearly identifiable although their aspect was variable, presumably depending on slight differences in tissue preparation: they were either pale grey (Fig. 2B) to white or had an onion-skin appearance. An interesting finding was that all the lipid droplets were situated in the direct neighbourhood of one or several mitochondrial profiles, giving a tight contact surface which was marked by a dent in the mitochondrial surface and a distinct dark line (Fig. 2B).

Although glycogen granules could be seen under the electron microsope, their contrast was irregular and inconsistent (Fig. 2A,B); glycogen seemed to be partly washed out in the course of the preparation procedures. We therefore decided to determine only the lipid stores by morphometry and to measure the glycogen content of the muscle fibres biochemically on the frozen samples, as described in the Materials and methods section.

Morphometric results

Although the animals of the two groups were approximately matched with respect to their body mass, their whole-body skeletal muscle masses, M_m, differed greatly (Table 1) in that the relative muscle mass, M_m/M_b, was larger in the dogs (37 %) than in the goats (26 %), giving a dog:goat ratio of 1.42. Considering that, during exercise, substrate metabolism predominantly occurs in muscle tissue, we decided to relate all our structural parameters to total muscle mass rather than to body mass.

All parameters concerning mitochondria and lipid droplets were approximately twice as large in the dogs as in the goats,

Table 1. *Physiological and morphometric data for the animals used in this study*

Parameters	Dogs	Goats	Dog:goat ratio	Significance
M_b (kg)	23.7±0.98	21.0±0.29	1.13	*
M_m/M_b (%)	36.5±0.9	25.7±1.2	1.42	*
$\dot{V}_{O_2max}/M_b$ (ml O_2 kg^{-1} s^{-1})	2.43±0.05	1.10±0.05	2.21	*
$\dot{V}_{O_2max}/M_m$ (ml O_2 kg^{-1} s^{-1})	6.67±0.17	4.30±0.23	1.55	*
$\dot{V}_V$(mt,f) (%)	8.64±0.20	4.12±0.40	2.10	*
V_V(li,f) (%)	0.462±0.082	0.199±0.040	2.32	*
S_V(om,f) (cm^2 cm^{-3})	8819±400	5157±440	1.71	*
S_V(li,f) (cm^2 cm^{-3})	454±81	210±45	2.17	*
S_S(li-om,om) (%)	2.05±0.31	0.95±0.19	2.16	*
S_S(li-om,li) (%)	40.1±1.0	23.4±1.8	1.71	*

$\dot{V}_{O_2max}$ is the value calculated for the animals included in the morphometric study.

M_b and M_m are body and muscle mass, respectively; V_V and S_V are volume and surface densities of components (mitochondria, mt; intracellular lipid droplets, li; outer mitochondrial membrane, om) per muscle fibre volume (f); S_S(li-om,om) is the percentage of outer mitochondrial membranes in direct contact with lipid deposits; S_S(li-om,li) is the percentage of lipid droplet surface in direct contact with outer mitochondrial membranes.

Values are means ± S.E.M.; N=3 for dogs; N=3 for goats.

Asterisks indicate a significant difference between species (P<0.05).

and all the differences were statistically significant (Tables 1, 2). As shown in Table 2, the muscle-mass-specific mitochondrial volume, V(mt)/M_m, and outer mitochondrial membrane surface area S(om)/M_m were both approximately twofold larger in the dogs than in the goats. The average diameter of the mitochondria is about 20 % larger in the dogs. The muscle-mass-specific volume of the intracellular lipid droplets, V(li)/M_m, was approximately 5 % of the mitochondrial volume, and lipid droplets were of similar size in both species. The specific surface area of lipid droplets inside skeletal muscle cells, S(li)/M_m, was about 2.2 times larger in the dogs than in the goats. Of the lipid droplet surface area, about 40 % was in direct contact with the mitochondrial outer membrane [S_S(li-om,li)] in the dogs compared with 23 % in the goats. Conversely, in the dogs, 2 % of the mitochondrial surface area was in direct contact with lipid deposits compared with 1 % in the goats [S_S(li-om,om)]. Since the surface areas of both mitochondria and lipid droplets are larger in the dogs, the total lipid–mitochondria contact surface area per kilogram muscle mass, S(li-om)/M_m, was 3.61 times larger in the dogs than in the goats (Table 2).

Biochemical determination of glycogen stores

In the biochemical analyses, the random samples showed a more than fourfold higher glycogen concentration in the dogs than in the goats (Table 2). The intracellular carbohydrate stores available for anaerobic glycolysis as well as oxidative metabolism in skeletal muscles are thus about four times higher in the athletic species.

Discussion

Cell structure and the consumption of O_2 and substrates

The physiological part of this study has shown that maximal

Table 2. *Mitochondrial and structural substrate parameters per unit muscle mass calculated from data in Table 1*

Parameters	Dogs	Goats	Dog:goat ratio	Significance
V(mt)/M_m (ml kg^{-1})	81.5±1.98	38.9±3.8	2.10	*
V(li)/M_m (ml kg^{-1})	4.36±0.77	1.88±0.38	2.32	*
S(om)/M_m (m^2 kg^{-1})	832±37.8	487±41.6	1.71	*
S(li)/M_m (m^2 kg^{-1})	42.9±7.6	19.8±4.3	2.17	*
S(li-om)/M_m (m^2 kg^{-1})	17.18±3.04	4.76±1.22	3.61	*
C_{gluc} (mmol kg^{-1})	71.9±8.77	17.1±3.80	4.20	*

V(mt) and V(li) are volumes of mitochondria and lipid droplets, respectively; S(om) and S(li) are the surface areas of the outer mitochondrial membranes and intracellular lipid droplets, respectively; S(li-om) is the contact surface area between both structures; C_{gluc} is the biochemically determined glycogen content of muscle fibres expressed in glucosyl units; M_m, muscle mass.

Values are means ± S.E.M.; N=3 for dogs; N=3 for goats.

Asterisks indicate a significant difference between species (P<0.05).

body-mass-specific O_2 consumption was 2.2 times greater in the dogs than in the goats (Roberts *et al.* 1996), although some of this difference was due to the greater relative muscle mass in the dog. When maximal O_2 consumption was related to muscle mass, it still was 1.55 times greater in the dog. This significant difference correlates with a proportionally larger mitochondrial volume in the dog in accordance with previous findings (Hoppeler *et al.* 1987).

The focus of this study was on the supply of substrates (carbohydrates and fatty acids) from their intracellular stores to the mitochondria of muscle cells. Physiological studies of the same animals (Roberts *et al.* 1996) have shown that the total consumption of both carbohydrates and fatty acids was proportional to O_2 consumption and was thus 1.5 times greater in the dogs than in the goats at their respective maximal fluxes. Furthermore, it was shown that dogs could not supply this difference from the microvasculature but met the demand from intracellular stores (Weber *et al.* 1996*a*,*b*). In the preceding paper of this series (Vock *et al.* 1996), it was shown that the limitations on vascular substrate supply were due to the design of the substrate pathway, in particular of the sarcolemma. It appeared that capillaries are designed for O_2 supply but are inadequate to sustain substrate fluxes at the rates required by the exercising muscle cells.

The morphometric and biochemical study of muscle cells described here revealed that the intracellular substrate pools of glycogen granules and lipid droplets were larger in dogs than in goats, as was the contact surface area between lipid droplets and mitochondria. We conclude that the size of the intracellular substrate pools is adjusted to allow a higher rate of substrate supply to the mitochondria in dogs at all exercise intensities.

Methodological problems and solutions

The rationale used to normalize structural and functional estimates to muscle mass instead of body mass has been discussed in detail in the previous study (Vock *et al.* 1996) and is justified by muscle mass being responsible for most of the substrate turnover during exercise and by dogs having a significantly larger relative muscle mass than goats (37 *versus* 26 %, respectively).

The determination of surface areas of different anisotropic structures such as capillaries and sarcolemma required a refinement of sampling and morphometric methods by preparing isotropic uniform random (IUR) sections (Vock *et al.* 1996). The present study used the same preparations, except that thinner sections were prepared to improve resolution.

The inner mitochondrial membrane area was not determined, since the mitochondrial volume is known to be a good indicator of oxidative capacity in these species and since the surface of inner mitochondrial membranes per mitochondrial volume is relatively invariant (Hoppeler, 1986; Schwerzmann *et al.* 1989). In some species or specialized muscles, such as hummingbird flight muscle, the inner membrane density appears to be greater than in these mammals (Mathieu-Costello *et al.* 1992) so that this simplification cannot be used indiscriminately.

Morphometric characteristics of locomotor muscles

The volume density of mitochondria in muscle fibres shows great variation between different fibre types (Hoppeler *et al.* 1981). In this study, this variation was disregarded because we wished to obtain an unbiased overall estimate of the mitochondrial content of locomotor muscles. Using stratified random samples of muscle from the entire locomotor muscle mass, we obtained an unbiased estimate of mitochondrial volume density, V_V(mt,f), amounting to 8.6 % in the dogs and 4.1 % in the goats (Table 1). This primary estimate of muscle mitochondrial content is quite similar to that previously estimated from 'representative' muscles (M. vastus medialis and semitendinosus) (Hoppeler *et al.* 1987). Considering the methodological refinements introduced in this study, mainly with respect to sampling, we conclude that the morphometric data presented here are acceptable as overall structural parameters for the oxidative capacity of the entire locomotor musculature.

The same can be said for the other morphometric parameters, such as the surface density of outer mitochondrial membranes and the volume and surface area of lipid droplets, for which there are no comparable data for dogs and goats in the literature. The coefficient of variation was around 5–6 % of the mean for mitochondrial surface and up to 20 % for the much rarer lipid droplets; but the differences between dogs and goats were still highly significant (Table 1). When expressed per unit muscle mass, the intracellular lipid stores are 2.3 times greater in the dogs than in the goats, amounting to about 4.4 ml kg^{-1} in the dogs compared with 1.9 ml kg^{-1} in the goats (Table 2).

From the biochemical assays, we estimate the glycogen deposits per unit muscle mass to be approximately 13 g glucose kg^{-1} in the dogs compared with 3 g kg^{-1} in the goats. It should be remembered that this is probably a conservative estimate of the intracellular carbohydrate reserves because the animals had performed an exercise programme within 24 h prior to death for reasons explained above.

Relationships between functional and structural data

The functional data, obtained in the companion studies (Weber *et al.* 1996*a*,*b*) and summarized in Table 3, can now be related to the design of the muscle cell and its mitochondria on the basis of the model of Fig. 1. We first note that the supply of substrates from vascular sources was found to be invariant both with respect to species differences and with respect to the increased substrate requirement with increasing exercise intensity. As a consequence, the exercise-induced increase in substrate utilization must be met from intracellular stores laid down during periods of rest, and dogs must draw more from these stores than goats (Vock *et al.* 1996). The hypothesis of symmorphosis predicts that the different aerobic capacities of dogs and goats should be reflected in differences in the size of the intracellular stores of glycogen and lipid.

In a broad interspecies comparison of animals of different body size and of athletic *versus* sedentary species, we consistently found the ratio $\dot{M}_{O_2max}/V(mt)$ to be invariant (Hoppeler and Lindstedt, 1985; Weibel *et al.* 1992) at about 180–270 μmol O_2 ml^{-1} min^{-1} [$\dot{V}_{O_2max}/V(mt)$

Table 3. *Molar oxidation rates and flux densities for the animals used in this study*

Parameters	Dogs	Goats	Dog:goat ratio
Oxygen at $\dot{M}_{O_2max}$			
$\dot{M}_{O_2}/M_m$ (μmol min^{-1} kg^{-1})	17937	11778	1.52
$\dot{M}_{O_2}/V$(mt) (μmol min^{-1} ml^{-1})	220	303	0.73
Glucose at 85 % $\dot{M}_{O_2max}$			
$\dot{M}_{O_2}^{CHO}$(mt)/M_m (μmol min^{-1} kg^{-1})	12236	8603	1.42
$\dot{M}_{O_2}^{CHO}$(ic)/M_m (μmol min^{-1} kg^{-1})	10616	6622	1.60
$\dot{M}_{CHO}$(ic)/M_m (μmol min^{-1} kg^{-1})	1769	1104	1.60
$\dot{M}_{CHO}$(ic)/C_{gluc} (μmol min^{-1} mol^{-1})	24.6	64.5	0.38
Fatty acids at 40 % $\dot{M}_{O_2max}$			
$\dot{M}_{O_2}^{FFA}$(mt)/M_m (μmol min^{-1} kg^{-1})	4575	3233	1.42
$\dot{M}_{O_2}^{FFA}$(ic)/M_m (μmol min^{-1} kg^{-1})	3501	2354	1.49
$\dot{M}_{FFA}$(ic)/M_m (μmol min^{-1} kg^{-1})	152.2	102.3	1.49
$\dot{M}_{FFA}$(ic)/V(li) (μmol min^{-1} ml^{-1})	35.0	54.4	0.64
$\dot{M}_{FFA}$(ic)/S(li-om) (μmol min^{-1} m^{-2})	8.86	21.5	0.41

Molar oxidation for total mitochondria (mt) and substrate flux rates from intracellular (ic) sources for glucose (CHO) and free fatty acids (FFA) calculated for the exercise intensities at which maximal rates were measured.

Flux rates from intracellular stores are related to glycogen content, C_{gluc}, volume of lipid droplets, V(li), and mitochondria–lipid contact surface area, S(li-om).

Functional data are calculated from Roberts *et al.* (1996), McClelland *et al.* (1994) and Weber *et al.* (1996*a*,*b*) and are expressed per unit muscle mass.

Abbreviations are defined in the text.

4–6 ml O_2 ml^{-1} min^{-1}]. The maximal rates of O_2 consumption per unit volume of mitochondria estimated in this study (Table 3) are close to this range but are about 20 % larger than in previous studies; the values are somewhat higher in the goats than in the dogs, which agrees with the previous findings on these two species where the mitochondrial O_2 consumption rate was also some 20 % greater in goats than in dogs (Hoppeler *et al.* 1987).

Whereas total mitochondrial carbohydrate oxidation, $\dot{M}_{O_2}^{CHO}$(mt)/M_m, increases with increasing exercise intensity and is 1.42 times greater in the dogs than in the goats at 85 % $\dot{M}_{O_2max}$ (Table 3), total fatty acid oxidation, $\dot{M}_{O_2}^{FFA}$(mt)/M_m, is maximal at 40 % $\dot{M}_{O_2max}$ in both species and is also 1.42 times greater in the dogs (Roberts *et al.* 1996; Table 3). The supply of these substrates from vascular sources is invariant so that, as a consequence, the supply of glucose from glycogen stores at 85 % $\dot{M}_{O_2max}$ amounts to 87 % of the total glucose oxidized in the dog and 77 % in the goat; at this exercise intensity, the dog consumes 1.6 times as much glucose derived from intracellular glycogen as the goat (Table 3).

We can now ask whether the size of the intracellular substrate stores is related to the different flux rates of the substrates drawn from them in these two species (Fig. 3). For carbohydrates, we found that the glycogen stores are four times larger in the dogs than in the goats, whereas the maximal flux rate was 1.6 times greater in the dogs. As a result, the dogs extract a smaller fraction of their carbohydrate stores than the goats so that their intracellular reserves last for high-intensity exercise periods about 2–3 times as long (Table 3): at an extraction rate of 1.8 mmol glucose min^{-1} kg^{-1} and with a glycogen store of 72 mmol glucose kg^{-1}, dogs can fuel exercise at an intensity of 85 % $\dot{M}_{O_2max}$ for about 40 min, whereas in goats, with their store of 17 mmol glucose kg^{-1}, this exercise intensity can be fuelled for only 15 min. At 60 % $\dot{M}_{O_2max}$, the reserves last for 90 and 45 min in dogs and goats, respectively. At 40 % $\dot{M}_{O_2max}$, the dog takes most of the glucose needed from intravascular sources so that it can run for hours without depleting its intracellular glycogen stores. We conclude that the dog, as an endurance athlete, builds intracellular carbohydrate reserves that last more than twice as long as those of the goat.

It must be noted that glycogen can be used for anaerobic as well as for aerobic energy production. The physiological value determined here only concerns the oxidative portion of glycogen utilization. The excess glycogen stores may also serve for anaerobic use, for example for burst activity, and may correspond to the interfibrillar portion of glycogen which is localized very close to the myofibrillar ATPase. Some of the products of anaerobic glycolysis can be dissipated into the blood as lactate.

Very similar results are obtained with respect to the lipid stores, which are most important at low exercise intensities (Fig. 3; Table 3). In spite of a 1.5-fold higher rate of free fatty acid combustion from intracellular sources, the 2.3-fold larger triglyceride deposits of the dog can fuel exercise for almost twice as long as in the goat. At 60 % $\dot{M}_{O_2max}$, the lipid stores last twice as long as the carbohydrate stores in both species, namely an estimated 3 and 2 h, respectively.

One of the interesting observations of this study concerns the intimate relationship between the lipid droplet surface and the outer mitochondrial membrane. We have found that the relative contact surface area is about twice as large in the dogs as in the goats (Table 1). However, because of the greater mitochondrial and lipid content of dog muscle fibres, the total lipid–mitochondria contact surface area related to muscle mass [S(li-om)/M_m] is 3.6 times larger in the dogs than in the goats, and the absolute contact surface area of an animal represents an area of 149 m^2 for a dog and 26 m^2 for a goat with its smaller muscle mass. Thus, the interface between the intracellular lipid deposits and the site of their oxidation is not only very tight but also of substantial size. Calculating the flux density of free fatty acids across the unit contact surface area, we find that in the dogs it is less than half that of the goats (Table 3). Since the liberation of fatty acids from triglyceride droplets involves a lipase whose location and density is, to our knowledge, unknown, this observation cannot be further interpreted.

Conclusions

In this study, we asked whether the structural design of muscle cells reflects the different requirements for oxidative phosphorylation and for substrate supply from intracellular

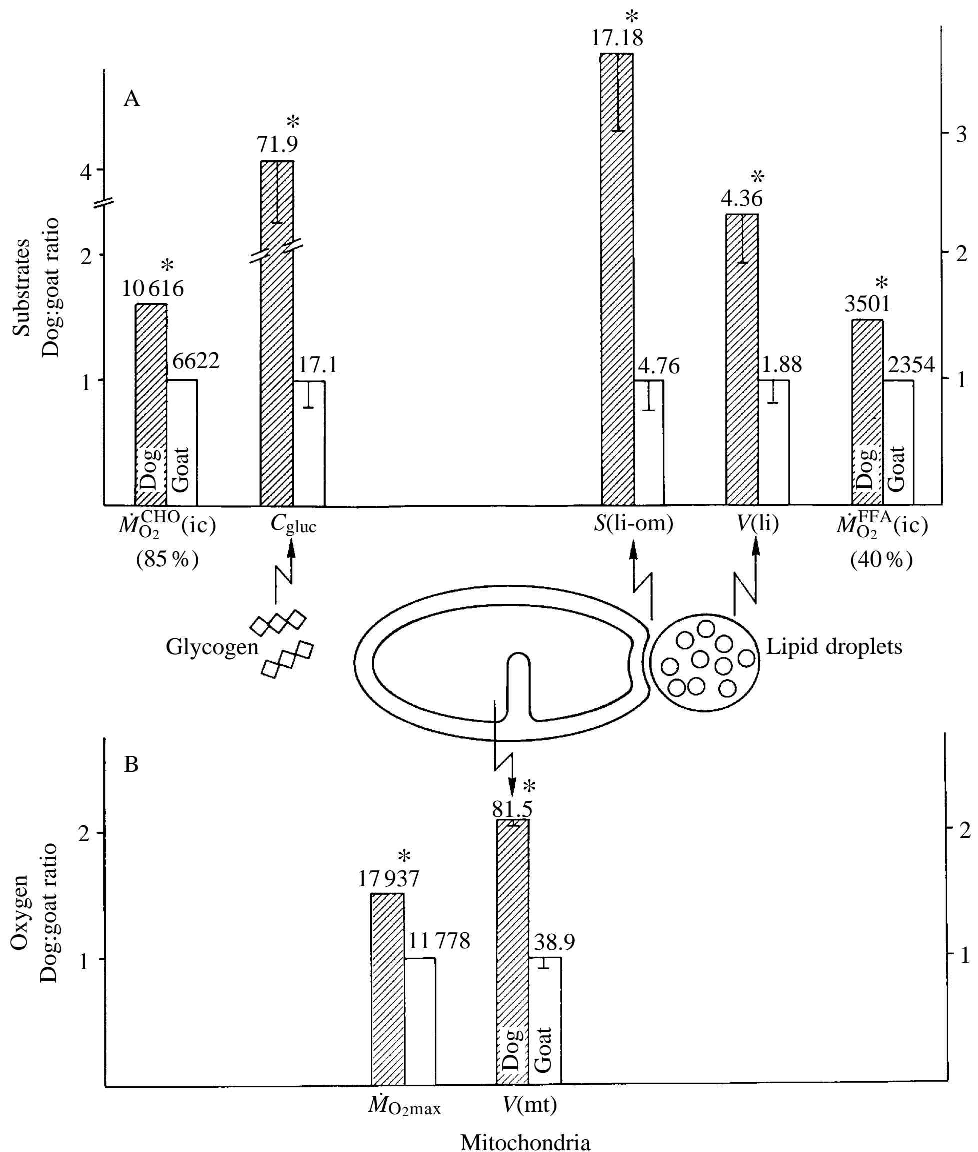

Fig. 3. Comparison of physiological data (from Table 3) expressing intracellular substrate mobilization (A) and maximal oxygen consumption (B) in muscle cells with related morphometric and biochemical parameters for intracellular stores of lipid, *V*(li), and glycogen, C_{gluc}, and the mitochondrial volume, *V*(mt). All data are expressed per kilogram muscle mass and plotted as dog:goat ratios; absolute values are given at the top of each column. *N*=3 animals per group. For symbols and units, see Tables 2 and 3.

stores by comparing dogs as endurance athletes with more sedentary goats. We find that this is the case.

(1) We find that the quantity of mitochondria built into muscle cells is approximately proportional to maximal aerobic capacity, $\dot{M}_{O_2max}$, with a slight excess in dogs, confirming previous studies.

(2) An intracellular substrate pool of adequate size is critical because the supply of substrates from the microcirculation is strictly limited.

(3) The intracellular stores of glucose in the form of glycogen granules are four times larger in the dogs than in the goats. When related to the 1.6-fold greater glucose flux rates from intracellular stores in the dogs, this allows them to sustain high aerobic exercise intensities for twice as long as goats. Because the glycogen pool also provides extra fuel for anaerobic metabolism, e.g. during burst activity, it appears in two functional compartments: perimitochondrial as well as inter- and even intrafibrillar.

(4) The intracellular stores of fatty acids in the form of triglyceride droplets are 2.3 times greater in the dogs and have a larger contact surface area with mitochondrial outer membranes. Here again, the dog has twice the substrate reserves of the goat, which endows it with the capacity to run for longer.

(5) The dog, as an endurance athlete, is able to run for longer and at higher intensities than the goat. The substrate pools built into the muscle cells account for this. Their size is therefore adjusted to the functional capacity in agreement with the hypothesis of symmorphosis, extended to network structures.

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