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Research Article

Heterogeneous bioenergetic behaviour of subsarcolemmal and intermyofibrillar mitochondria in fed and fasted rats

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Abstract. This study was designed to examine energetic behaviour of skeletal muscle subsarcolemmal and intermyofibrillar mitochondrial populations. The data show that subsarcolemmal mitochondria exhibited a lower degree of coupling and efficiency than intermyofibrillar ones, and can therefore be considered less efficient at producing ATP. In addition, subsarcolemmal mitochondria showed an increased sensitivity to palmitate-induced uncoupling, in line with high adenine nucleotide translocator content and decreased oxidative damage. We then determined the effect of 24 h fasting on energetic characteristics of skeletal muscle mitochondrial populations. We found that fasting enhanced proton leak and decreased the degree of coupling and efficiency, both in the absence and in the presence of palmitate only in subsarcolemmal mitochondria. Moreover, this mitochondrial population showed lower oxidative damage, probably due to a counter-regulatory mechanism mediated by uncoupling protein 3. Subsarcolemmal and intermyofibrillar mitochondria appear to exhibit different energetic characteristics and can be differently affected by physiological stimuli.

Key words. Subsarcolemmal mitochondria; intermyofibrillar mitochondria; mitochondrial efficiency; palmitate-induced proton leak; oxidative damage; fed-fasting transition.

Mitochondria play a central role in the cell, by providing the energy required for almost all cellular processes. In skeletal muscle, mitochondria are heterogeneously distributed according to their metabolic function. Intermyofibrillar (IMF) mitochondria produce ATP to support muscle contraction and are located near the myofibrils, while subsarcolemmal (SS) mitochondria produce ATP for membrane transport and cytoplasmic reactions, and are located beneath the sarcolemmal membrane. Biochemical characterisation of these two mitochondrial populations has shown that SS and IMF mitochondria display marked differences in oxygen consumption and oxidative enzyme rates [1, 2]. In addition, physiological stimuli, such as acute exercise [3], endurance training [4], ageing [5] and a high-fat diet [2] differentially affect SS and IMF mitochondrial function. As for bioenergetic features of these two mitochondrial populations, we have recently shown a lower basal proton leak in SS than in IMF mitochondria [6]. This result is of relevance because the most important factor that affects the degree of coupling of oxidative phosphorylation, and hence mitochondrial efficiency, is the permeability of the inner mitochondrial membrane to H^+ ions [7]. It is now well known that protons can reenter the mitochondrial matrix through a non-specific leak pathway, called the basal proton leak, which is present in the inner membrane of all mitochondria and has been estimated to account for about 20% of the rat standard metabolic rate [8]. In addition, fatty acids are known to be responsible for 'mild uncoupling' of oxidative phosphorylation [9]. This phenomenon is a function of the amount of unbound fatty acids in the cell, and is mediated by proteins such as adenine nucleotide translocator (ANT) and uncoupling proteins (UCPs) [10, 11].

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Therefore, as a follow-up to our previous characterisation of SS and IMF mitochondria bioenergetic behaviour [6], we investigated fatty acid-mediated proton leak, as well as parameters of thermodynamic coupling and efficiency. In addition, markers of mitochondrial oxidative damage and antioxidant defence were also assessed, since the degree of mitochondrial coupling is an important determinant of reactive oxygen species (ROS) production by the respiratory chain [12]. All the above parameters were assessed in fed and 24-hour fasting rats.

Materials and methods

Animals. Two groups of male Wistar rats (Charles River, Calco, Como, Italy) aged 60 days were used for this study. They were kept at 24 °C under an artificial circadian 12-h light/12-h darkness cycle, with ad libitum access to water and a standard stock diet (Mucedola 4RF21; Settimo Milanese, Milan, Italy). Treatment, housing and killing met the guidelines of the Italian Health Ministry.

At the time of the experiments, one group of rats was sacrificed without any previous food deprivation (fed rats), the other group after 24 h fasting (fasted rats). The rats were anaesthetised with chloral hydrate (40 mg per 100 g body weight) and killed by decapitation. Hind leg skeletal muscles were rapidly removed and used for preparation of isolated SS and IMF mitochondria, as previously reported [2, 6]. Briefly, skeletal muscles were homogenised in an isolation medium containing 100 mM KCl, 50 mM Tris, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM EGTA and 0.1% (w/v) fatty acid-free bovine serum albumin (BSA), and then the homogenate was centrifuged at 500 g_{av} for 10 min. The homogenate supernatant was centrifuged at 3000 g_{av} for 10 min and the resulting pellet, containing SS mitochondria, was washed twice, and resuspended in a suspension medium containing 250 mM sucrose, 50 mM Tris, pH 7.5, 0.1% (w/v) fatty acid free BSA. The homogenate precipitate was resuspended in a small amount of isolation medium and treated with protease Nagarse (9 U/g tissue) for 5 min at a temperature of 4 °C. The suspension was then homogenised, filtered through sterile gauze, and centrifuged at 3000 g_{av} for 10 min. The resulting supernatant was rapidly discarded and the pellet was resuspended and centrifuged at 500 g_{av} for 10 min. The supernatant containing the IMF mitochondria was centrifuged at 3000 g_{av} for 10 min, the pellet was washed once and resuspended in the suspension medium. In control experiments, we assured that the differences in functionality of IMF and SS mitochondria were not due to differences in isolation procedures.

Measurements of mitochondrial respiration, thermodynamic coupling and efficiency. Oxygen consumption was measured polarographically with a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, Ohio) in a 3-ml glass cell, at a temperature of 30 °C. Isolated SS or IMF mitochondria (0.1 mg protein/ml) were incubated in a medium containing 30 mM KCl, 6 mM $MgCl_2$, 75 mM sucrose, 1 mM EDTA, 20 mM KH_2PO_4 pH 7.0, and 0.1% (w/v) fatty acid-free BSA. In the presence of 10 mM succinate, 3.75 µM rotenone and 0.6 mM ADP, state 3 oxygen consumption was measured. State 4 was obtained in the absence of ADP. The respiratory control ratio (RCR) was calculated as the ratio between states 3 and 4 [13].

The degree of thermodynamic coupling, q, was determined by applying equation 11 from Cairns et al. [14]: $q = \sqrt{1 - (J_o)_{sh}/(J_o)_{unc}}$. $(J_o)_{sh}$ was measured as the oxygen consumption rate in the presence of oligomycin (2 µg/ml), which inhibits ATP synthase, and $(J_o)_{unc}$ was measured as the uncoupled rate of oxygen consumption induced by FCCP (1 µM), which dissipates the transmitochondrial proton gradient.

The optimal thermodynamic efficiency of oxidative phosphorylation, η, was calculated using equation 13 from Cairns et al. [14]: $\eta = \frac{q^2}{\left(1 + \sqrt{1 - q^2}\right)^2}$.

Measurements of basal proton leak kinetics. If the activity of the respiratory chain is titrated with inhibitors in the presence of oligomycin to prevent ATP synthesis, the resulting titration curve of membrane potential against respiration rate represents the kinetic response of the proton leak to changes in membrane potential. Mitochondrial oxygen consumption was measured polarographically with a Clark-type electrode, whereas mitochondrial membrane potential recordings were performed in parallel with safranin O using a JASCO dual-wavelength spectrophotometer (511–533 nm) [15]. The absorbance readings were transformed into mV membrane potential using the Nernst equation: $\Delta\psi = 61\ \text{mV} \cdot \log([K^+]_{in}/[K^+]_{out})$. Calibration curves made for each preparation were obtained from traces in which the extramitochondrial K^+ level ($[K^+]_{out}$) was altered in the 0.1–20 mM range. The change in absorbance caused by the addition of 3 µM valinomycin was plotted against $[K^+]_{out}$. Then, $[K^+]_{in}$ was estimated by extrapolation of the line to the zero uptake point. Titration of mitochondrial oxygen consumption and membrane potential were carried out at 30 °C by sequential additions of increasing malonate concentrations, in a medium containing 30 mM LiCl, 6 mM $MgCl_2$, 75 mM sucrose, 1 mM EDTA, 20 mM Tris-PO_4 pH 7.0, succinate (10 mM), rotenone (3.75 µM), oligomycin (2 µg/ml), safranin O (83.3 nmol/mg), nigericin (80 ng/ml) and 0.1% (w/v) fatty acid-free BSA.

Measurement of palmitate-induced uncoupling. To determine the effect of palmitate on proton leak kinetics and parameters of thermodynamic coupling and effi-

ciency, mitochondrial membrane potential and oxygen consumption titration as well as q and η determinations were carried out as above in the presence of palmitate at a concentration of 45 µM for SS mitochondria or 75 µM for IMF mitochondria. Due to the presence of 0.1% BSA in the incubation medium, the above concentrations of palmitate correspond to 17 (for SS mitochondria) and 62 (for IMF mitochondria) nM free (not bound) fatty acid, calculated using the equation of Richieri et al. [16].

Measurement of ANT content. The ANT content of SS and IMF mitochondria was determined by titrating state 3 respiration with increasing concentrations of carboxyatractyloside (CAT) [17] in a medium containing 30 mM KCl, 6 mM $MgCl_2$, 75 mM sucrose, 1 mM EDTA, 20 mM KH_2PO_4 pH 7.0, succinate 10 mM and rotenone 3.75 µM, and 0.1% (w/v) fatty acid-free BSA. Mitochondria were preincubated with CAT in the respiratory medium for 1 min before ADP (0.6 mM) was added to initiate state 3 respiration. The mitochondrial content of ANT was determined by the extrapolation of the linear part of the titration curve to obtain the amount of CAT required to completely inhibit state 3 respiration.

Measurement of UCP3 protein content. Mitochondrial protein content of UCP3 was estimated by Western blot analysis, as previously reported [18], by using UCP3 polyclonal antibodies (Chemicon).

Determination of mitochondrial aconitase and superoxide dismutase specific activity. Aliquots of isolated SS and IMF mitochondria were immediately frozen in liquid nitrogen and stored at –80 °C. On the day of assay, mitochondria were incubated in 1% Triton X-100, 20–30 µg protein/ml for aconitase and 2–3 µg protein/ml for superoxide dismutase (SOD). Determination of aconitase specific activity was carried out in a medium containing 30 mM sodium citrate, 0.6 mM $MnCl_2$, 0.2 mM NADP, 50 mM TRIS-HCl pH 7.4, and 2 units of isocitrate dehydrogenase. The formation of NADPH was followed spectrophotometrically (340 nm) at 25 °C [19]. The level of aconitase activity measured equals active aconitase (basal level). Aconitase inhibited by ROS *in vivo* was reactivated so that total activity could be measured by incubating mitochondrial extracts in a medium containing 50 mM dithiothreitol, 0.2 mM Na_2S, and 0.2 mM ferrous ammonium sulphate [20]. SOD specific activity was measured in a medium containing 0.1 mM EDTA, 2 mM KCN, 50 mM KH_2PO_4 pH 7.8, 20 mM cythocrome c, 0.1 mM xanthyne, and 0.01 units of xanthyne oxidase. Determinations were carried out spectrophotometrically (550 nm) at 25 °C, by monitoring the decrease in the reduction rate of cythocrome c by superoxide radicals, generated by the xanthine-xanthine oxidase system. One unit of SOD activity is defined as the concentration of enzyme that inhibits cythocrome c reduction by 50% in the presence of xanthine+xanthine oxidase [21].

Table 1. Respiratory parameters in SS and IMF skeletal muscle mitochondria from fed and fasted rats.

	Fed	Fasted
SS		
State 3 (ngatoms O/min ×mg protein)	383 ± 20	344 ± 30
State 4 (ngatoms O/min ×mg protein)	82 ± 8	82 ± 6
RCR	4.7 ± 0.2	4.2 ± 0.2
q	0.902 ± 0.002	0.873 ± 0.002*
η	0.396 ± 0.003	0.345 ± 0.005*
IMF		
State 3 [ngatoms O/(min ×mg protein)]	670 ± 15#	627 ± 15#
State 4 [ngatoms O/(min ×mg protein)]	126 ± 3#	114 ± 10#
RCR	5.3 ± 0.1	5.2 ± 0.2#
q	0.913 ± 0.001#	0.911 ± 0.002#
η	0.421 ± 0.002#	0.418 ± 0.001#

Values are the means ± SE of eight different experiments. State 3 and state 4 were measured in the presence of 10 mM succinate + 3.75 µM rotenone. RCR, Respiratory control ratio; q, thermodynamic degree of coupling; η, optimal efficiency. *$p < 0.05$ main effect of fasting; #$p < 0.05$ main effect of mitochondria type (two-way ANOVA with Bonferroni's post hoc test).

Statistical analysis. Data are expressed as means ± SE. Statistical analyses were performed using two-way analysis of variance (ANOVA) for main effects (mitochondrial type and fasting) and interactions, followed by Bonferroni's post hoc test to determine individual differences. Two-tailed unpaired Student's t test and non-linear regression curve fit were also performed. Statistical differences were considered significant if $p < 0.05$. All analyses were performed using GraphPad Prism 4 (GraphPad Software Inc., San Diego, Calif.).

Materials. All reagents were purchased from Sigma (St. Louis, Mo.) except for CAT, which was purchased from Calbiochem (San Diego, Calif.).

Results

Skeletal muscle mitochondrial respiration using succinate plus rotenone as substrate, during maximal ADP-stimulated (state 3) and resting (state 4) states together

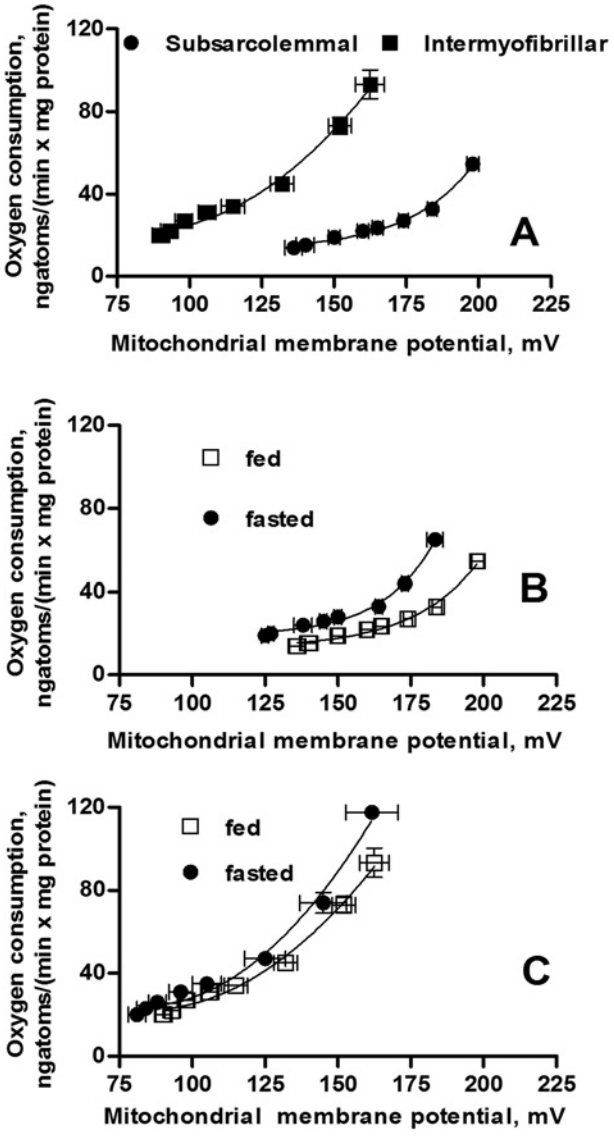

Figure 1. Kinetics of basal proton leak in subsarcolemmal and intermyofibrillar mitochondria from fed rats (*A*), in subsarcolemmal (*B*) and intermyofibrillar mitochondria (*C*) from fed and fasted rats. Values are the means ± SE of 8 different experiments. Basal proton leak was significantly lower in subsarcolemmal mitochondria compared to intermyofibrillar mitochondria from fed rats (*A*), basal proton leak was significantly higher in subsarcolemmal mitochondria from fasted rats compared to fed rats (*B*) and there was no significant difference in intermyofibrillar mitochondria from fasted rats compared to fed rats (*C*), as shown by non-linear regression curve fits.

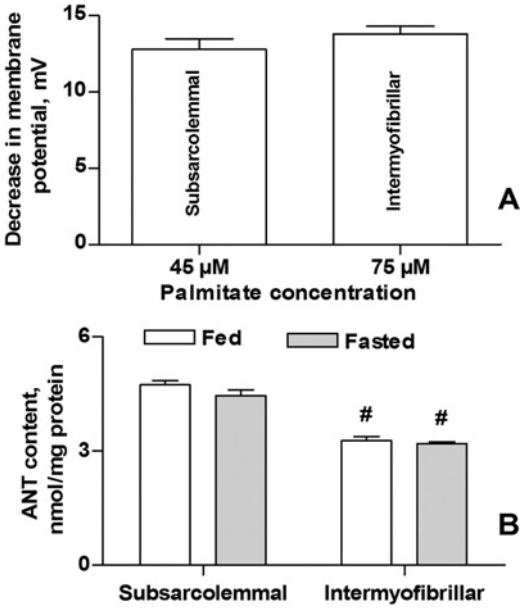

Figure 2. Palmitate-induced uncoupling measured as a decrease in mitochondrial membrane potential in subsarcolemmal and intermyofibrillar mitochondria from fed rats (*A*), and adenine nucleotide translocator (ANT) content in subsarcolemmal and intermyofibrillar mitochondria from fed and fasted rats (*B*). Values are the means ± SE of eight different experiments. #p<0.05 main effect of mitochondrial type (two-way ANOVA with Bonferroni's post hoc test).

with RCR values are shown in table 1. In the fed state, state 3 and 4 respiratory rates of IMF mitochondria were significantly higher than those of SS mitochondria. On the other hand, no variation in state 3 and 4 respiration was found in the fed-fasting transition in SS and IMF mitochondria. RCR values were consistent with those of intact, functional isolated mitochondria.

Table 1 also shows calculated q and η values, parameters of oxidative phosphorylation coupling, which were determined to allow evaluation of energetic efficiency in skeletal muscle mitochondria. These parameters were different in fed rats, with IMF mitochondria showing significantly higher q and η values than SS ones. In addition, the results show that only SS mitochondria from fasted rats exhibited significantly lower q and η values compared to mitochondria from fed rats (table 1).

Figure 1 shows basal proton leak kinetics measured in SS and IMF skeletal muscle mitochondria isolated from fed and fasted rats. It should be noted that the presence of fatty acid-free BSA both during the preparation of isolated mitochondria and basal proton leak measurements is necessary to avoid contaminating free fatty acids, which are known mitochondrial uncouplers [9]. BSA chelates the free fatty acids and therefore abolishes their effect. The titration curves reported in figure 1 are an indirect measurement of the dependence of mitochondrial proton leak on membrane potential, since steady-state oxygen consumption (i.e. proton efflux rate) in non-phosphorylating mitochondria is equivalent to proton influx rate due to proton leak [22]. The results show that in the fed condition, SS mitochondria had lower basal proton leak kinetics compared to IMF mitochondria (fig. 1A). In addition, basal proton leak kinetics significantly increased in SS mitochondria after fasting (fig. 1B), but

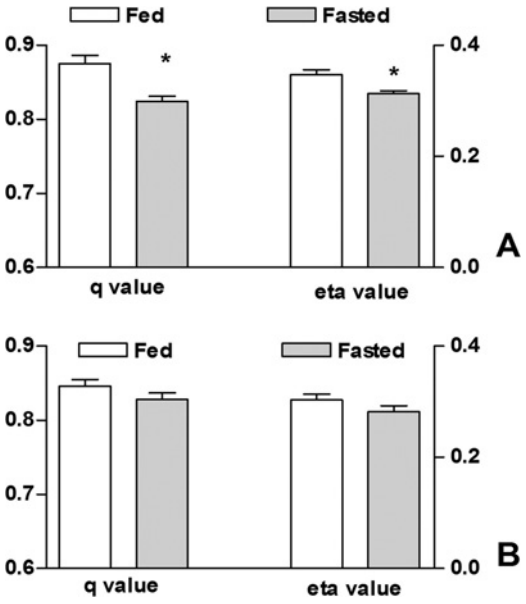

Figure 3. Calculated thermodynamic coupling (q) values and optimal thermodynamic efficiency (η) of oxidative phosphorylation in subsarcolemmal (*A*) and intermyofibrillar (*B*) skeletal muscle mitochondria from fed and fasted rats measured in the presence of palmitate at a concentration of 45 µM for subsarcolemmal mitochondria or 75 µM for intermyofibrillar mitochondria. Values are the means ± SE of eight different experiments. *p < 0.05 compared to fed rats (two-tailed unpaired Student's t test).

Table 2. Aconitase and SOD activity in SS and IMF skeletal muscle mitochondria from fed and fasted rats.

	Fed	Fasted
SS		
Basal aconitase specific activity (mU/mg protein)	46 ± 4	47 ± 2
Total aconitase specific activity (mU/mg protein)	157 ± 8	126 ± 6*
Basal aconitase/total aconitase	0.30 ± 0.02	0.37 ± 0.02*
SOD specific activity (U/mg protein)	15 ± 1	16 ± 1
IMF		
Basal aconitase specific activity (mU/mg protein)	17 ± 2#	16 ± 1#
Total aconitase specific activity (mU/mg protein)	106 ± 6#	103 ± 2#
Basal aconitase/total aconitase	0.16 ± 0.01#	0.16 ± 0.01#
SOD specific activity (U/mg protein)	13 ± 2	12 ± 2

Values are the means ± SE of eight different experiments. SOD; superoxide dismutase. *p < 0.05 main effect of fasting; #p < 0.05 main effect of mitochondria type (two-way ANOVA with Bonferroni's post hoc test)

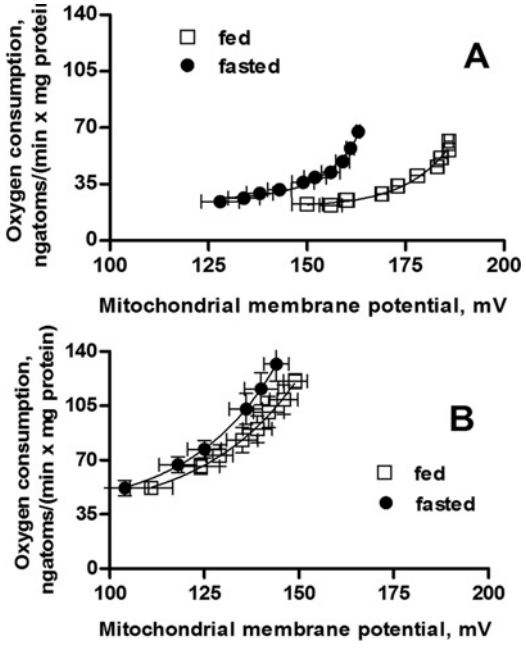

Figure 4. Kinetics of palmitate-induced proton leak in subsarcolemmal (*A*) and intermyofibrillar (*B*) mitochondria from fed and fasted rats. Values are the means ± SE of eight different experiments. Palmitate-induced proton leak was significantly higher in subsarcolemmal mitochondria from fasted rats compared to fed rats (*A*), while there was no significant difference in intermyofibrillar mitochondria from fasted rats compared to fed rats (*B*), as shown by non linear regression curve fits.

were unchanged between IMF mitochondria from fed and fasted rats (fig. 1C).

The sensitivity of SS and IMF mitochondria to the uncoupling effect of palmitate was evaluated by comparing the decrease in mitochondrial membrane potential induced by palmitate addition (fig. 2A). The results show that in fed rats, SS mitochondria were more sensitive to the fatty acid uncoupling effect. In fact, a similar decrease in membrane potential was obtained using 45 µM of palmitate in SS mitochondria and 75 µM in IMF mitochondria. For ANT, a protein involved in fatty acid-dependent uncoupling pathways [10], the results of content determination showed that in fed rats, SS mitochondria had a higher amount of ANT/mg protein than IMF ones, while no change was found in the fed-fasting transition (fig. 2B).

Figure 3 shows the values of q and η (eta), parameters of oxidative phosphorylation coupling, obtained in the presence of palmitate. These parameters significantly decreased in fasted rats compared to fed rats only in SS mitochondria (fig. 3A). In agreement with this result, increased palmitate-induced proton leak kinetics were found only in SS mitochondria isolated from fasted rats (fig. 4A).

To obtain some information about ROS damage and antioxidant defence in SS and IMF mitochondria, skeletal muscle mitochondrial aconitase and SOD specific activ-

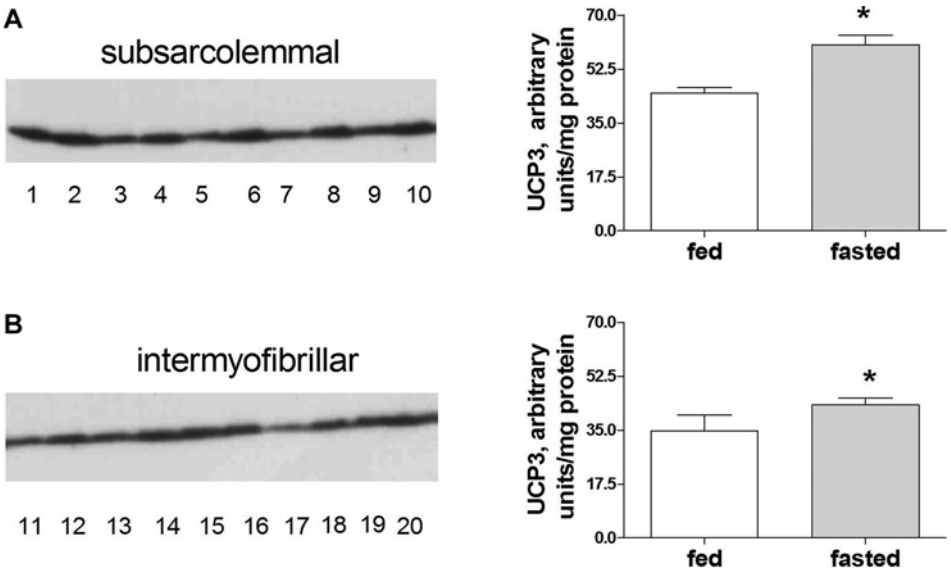

Figure 5. Uncoupling protein 3 (UCP3) contents in subsarcolemmal (*A*) and intermyofibrillar (*B*) skeletal muscle mitochondria from fed and fasted rats. Results are given as arbitrary units/mg protein. Values are the means ± SE of five different experiments. Odd numbers, fed rats; even numbers, fasted rats. *$p < 0.05$ compared to fed rats (two-tailed unpaired Student's t test).

ity were tested. Since aconitase activity is very sensitive to superoxide exposure, this enzyme can be used to estimate indirectly the superoxide concentration. We, therefore, measured mitochondrial aconitase activity in isolated mitochondria as an *in vivo* indicator of ROS damage. To avoid mistakes due to a different amount in total aconitase activity, we measured active aconitase (basal level) and total aconitase, obtained after reactivation of the inactive fraction of aconitase, and the results are expressed as the basal/total aconitase activity ratio. In the fed condition, this ratio was significantly higher in SS mitochondria compared to IMF mitochondria (table 2). This result indicates that ROS damage is lower in SS mitochondria. For the fed-fasting transition, the basal/total aconitase activity ratio was significantly higher in SS mitochondria isolated from fasted rats (table 2). Therefore, these mitochondria had less oxidative damage compared to SS mitochondria from fed rats. On the other hand, no difference in basal/total aconitase activity ratio was observed between IMF mitochondria isolated from fed and fasted rats (table 2). Finally, no variation was found in the SOD specific activity in SS and IMF mitochondria and no change was found in the fed-fasting transition (table 2). Both SS and IMF mitochondria from fasted rats showed significantly higher contents of uncoupling protein 3 (UCP3) than those from fed rats (fig. 5).

Discussion

Skeletal muscle is characterised by the presence of the functionally and biochemically distinct IMF and SS mitochondria, which are localised to discrete cellular compartments. The results of this study have furthered our insight into the energetic features of these mitochondrial populations. Interestingly, the results reveal that, in skeletal muscle, SS and IMF mitochondria exhibit profound differences in their efficiency at producing ATP. Since SS and IMF mitochondria generate ATP for different energy-requiring processes, this finding is of fundamental importance to understanding the role played by these mitochondrial populations in cellular energetic handling, and hence in skeletal muscle functionality.

Previous studies on the behaviour of skeletal muscle mitochondria showed that SS mitochondria, located beneath the plasma membrane, have a lower respiratory capacity than IMF ones, located between the myofibrils [1, 2]. Here we show that not only respiratory capacity but also the degree of coupling and hence the efficiency of oxidative phosphorylation differ in mitochondria, according to their cellular localisation. In fact, SS mitochondria exhibited lower q and η values than IMF ones, and can therefore be considered less efficient at producing ATP. The lower efficiency of SS mitochondria is not due to increased basal proton leak, since we have found, in agreement with previous results [6], that this parameter is lower in SS than in IMF mitochondria. Thus, the regulation of efficiency is achieved through differences in state 3 respiration, which is substantially lower in SS than in IMF mitochondria. Consequently, in SS mitochondria the basal leak contribution to total respiration is higher [23] and hence the q value is lower. The different degree of coupling of SS and IMF mitochondria is in agreement with their functional role in the muscle cell. In fact, con-

sidering Stucki's [24] definition of specific thermodynamic degrees of coupling to obtain maximal output power or output flow, the value of q here found for IMF mitochondria (0.913) is close to that reported by Stucki (0.910), which allows maximal ATP production to support the actomyosin cycle during muscle contraction. On the other hand, the lower q value found here for SS mitochondria could allow higher rates of ATP production to support energy-requiring processes at the cell surface, including ion pump activity, substrate transport, cellular signaling, and protein synthesis [25].

While q and η values measured in isolated mitochondria give an estimate of innate coupling, one should consider that mitochondrial coupling is also under the control of external factors, such as unbound cellular fatty acids. In fact, fatty acids are responsible for the well-known phenomenon of 'mild uncoupling' [9–11], which adds its contribution in setting the final degree of coupling of oxidative phosphorylation. Again, testing the behaviour of SS and IMF mitochondria showed significant differences. SS mitochondria exhibited a higher sensitivity to the uncoupling effect of fatty acid palmitate, compared to IMF ones. It should be noted that our measurements were made in the presence of fatty acid-free BSA, so that unbound palmitate concentrations were in the order of nanomolar, similar to those probably present in the intact cell, where fatty acid-binding proteins have a Kd lower than 1 µM [26]. The higher sensitivity of SS mitochondria to 'mild uncoupling' is in line with their higher content of ANT, since ANT has been established as an important contributor to the above phenomenon [10], and is also in agreement with the decreased ROS damage found in SS mitochondria compared to IMF ones. In fact, one of the postulated physiological roles for the uncoupling is known to be maintenance of mitochondrial membrane potential below the critical threshold for ROS production [12]. The protective role of mild fatty acid uncoupling in SS mitochondria is confirmed by the results of Servais et al. [27]. They found that in the presence of BSA (and therefore in conditions in which fatty acid uncoupling is absent), SS mitochondria exhibited twofold higher H_2O_2 production than IMF ones. In conclusion, it seems that there is a different susceptibility to ROS damage for SS mitochondria and IMF mitochondria. In agreement, very recently Adhihetti et al. [28] found that SS mitochondria have a minor susceptibility to apoptosis evoked by ROS compared to IMF mitochondria.

Taking into account all the above data, we can deduce that SS mitochondria are intrinsically less coupled compared to IMF ones and this feature becomes more relevant in the presence of unbound fatty acids in the cell. This mitochondrial population could therefore make a primary contribution to the regulation of skeletal muscle energy efficiency in response to changes in the metabolic status of the organism. Interestingly, obesity and type 2 diabetes have recently been found to be associated with a deficiency of SS mitochondria in human skeletal muscle [29]. Fasting is a physiological condition, in which skeletal muscle energy metabolism needs to be modulated to face changes in nutrient supply. Therefore, we investigated the regulation of mitochondrial efficiency during the fed-fasting transition. Energetic efficiency parameters of IMF mitochondria were not affected by 24 h fasting, whereas SS mitochondria from fasted rats exhibited a higher basal, in agreement with a previous result [6], and palmitate-induced uncoupling. Consequently, a decrease in the degree of coupling (q), and hence efficiency (η), was found both in the absence and in the presence of palmitate in SS mitochondria.

Compared with fed rats, fasted animals showed increased serum non-esterified fatty acid (NEFA) levels [30], and decreased muscle carbohydrate supply, which was compensated by enhanced fatty acid oxidation [31, 32]. Therefore, one can suggest that, during fasting, fatty acids are the principal fuels oxidised by SS mitochondria, which, being localised beneath the sarcolemmal membrane, experience a higher NEFA amount than IMF mitochondria. In fact, NEFA concentration then dilutes in the sarcoplasm. In SS mitochondria, the higher NEFA availability associated with the increased palmitate-induced uncoupling may contribute to lower efficiency. Superoxide production from the electron transport chain has been reported to be high during fatty acid oxidation in isolated muscle mitochondria [33]. In addition, ROS production is more pronounced if ATP demand is lower in the presence of a great availability of energy substrates [34, 35]. As for ATP demand, SS mitochondria supply ATP to energy-requiring reactions, such as protein synthesis and ATPase pumps that are lowered during fasting [36, 37], while IMF mitochondria produce ATP for contractile elements [1, 4] that are not influenced by fasting [38]. It follows that SS mitochondria during fasting could produce a large amount of ROS. On the other hand, we found in SS mitochondria from fasted rats a reduction in oxidative damage as indicated by determination of the basal/total aconitase activity ratio. Since no increase in SOD specific activity was found in the fed-fasting transition, the above reduction could be due to enhanced palmitate-induced uncoupling found in SS mitochondria from fasted rats, which is one way to mitigate ROS damage [12].

The determination of ANT content in the two mitochondrial populations allows us to exclude the possibility that the above increase in palmitate-induced uncoupling found in SS mitochondria isolated from fasted rats is due to ANT, which was found unchanged in SS and IMF mitochondria. To establish a relationship between UCP3 expression and palmitate-induced uncoupling, UCP3 protein content was measured. The results obtained confirm previous ones [6]. In fact, UCP3 protein content was significantly higher in SS and IMF mitochondria isolated

from fasted rats. This result is partially in agreement with the work by Jimenez et al. [39], who found an increase only in skeletal muscle SS mitochondria, isolated from fasted mouse. On the other hand, our result is consistent with upregulation of UCP3 mRNA expression found in fasted rat skeletal muscle [40]. The discrepancy between the increased UCP3 levels found in both the mitochondrial populations and the increased fatty acid-induced uncoupling found only in SS mitochondria can be explained by a higher activation of UCP3 that may occur in SS mitochondria because of a greater mitochondrial superoxide production. In fact, uncoupling proteins activated by mitochondrial matrix superoxide has been proposed to cause mild uncoupling and so diminish mitochondrial superoxide production, hence protecting against oxidative damage, at the expense of a small loss of energy [41]. Therefore, in our experimental condition, it seems that the physiological role of UCP3 could be to minimise ROS production by a counter-regulatory mechanism.

In conclusion, taking together the above results, it appears clear that energetic efficiency in the two skeletal muscle populations is heterogeneous and differently regulated by physiological stimuli. In fact, SS mitochondria seem to be the population more prone to palmitate-induced uncoupling in fed and fasted rats, probably to reduce oxidative damage in this mitochondrial population, which plays an important role in bioenergetic support of important phenomena, such as signal transduction, fat oxidation and substrate transport. In addition, since uncoupling of oxidative phosphorylation results in the release of energy in the form of heat, SS mitochondria could also represent a physiological therapeutic target for some pathological conditions, where it is necessary to increase thermogenesis, such as obesity.

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