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Interrelated influence of superoxides and free fatty acids over mitochondrial uncoupling in skeletal muscle

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ABSTRACT

Mitochondrial uncoupling protein 3 (UCP_3)-mediated uncoupling has been postulated to depend on several factors, including superoxides, free fatty acids (FFAs), and fatty acid hydroperoxides and/or their derivatives. We investigated whether there is an interrelation between endogenous mitochondrial superoxides and fatty acids in inducing skeletal muscle mitochondrial uncoupling, and we speculated on the possible involvement of UCP_3 in this process. In the absence of FFAs, no differences in proton-leak kinetic were detected between succinate-energized mitochondria respiring in the absence or presence of rotenone, despite a large difference in complex I superoxide production. The addition of either arachidic acid or arachidonic acid induced an increase in proton-leak kinetic, with arachidonic acid having the more marked effect. The uncoupling effect of arachidic acid was independent of the presence of GDP, rotenone and vitamin E, while that of arachidonic acid was dependent on these factors. These data demonstrate that FFA and O^{2-} play interrelated roles in inducing mitochondrial uncoupling, and we hypothesize that a likely formation of mitochondrial fatty acid hydroperoxides is a key event in the arachidonic acid-induced GDP-dependent inhibition of mitochondrial uncoupling.

1. Introduction

The flux of substrate through the tricarboxylic acid cycle and the flux of electrons through the respiratory chain are coupled to ATP synthesis via the formation of a proton gradient across the mitochondrial inner membrane. ATP is generated from ADP and Pi as protons pass down their electrochemical gradient through the action of ATPsynthase. However, in some conditions a dissipation of the electrochemical gradient may take place [in the absence of ADP or in the presence of inhibitors of the proton flux through the Fo portion of ATPsynthase, for instance, the protons pumped out of the matrix pass back into the mitochondria through other proton-conductance routes (proton leak)].The proportion of respiration that is used to drive the energy-dissipating futile proton cycle across the mitochondrial inner membrane is high [1,2], and up to 20% of the basal metabolic rate may be used to drive this process [3].

Although the proton leak has been related to the phospholipid composition of the inner membrane [4,5], studies on liposomes constituted by inner membrane phospholipids have revealed that they are several-fold less proton-permeable than native mitochondria [6]. Moreover, the proton conductance is the same in such liposomes prepared from mitochondria with very different endogenous proton conductances [7]. Thus, simple diffusion through bulk regions of the membrane bilayer does not explain the proton conductance, and protein components present in the mitochondrial inner membrane must be involved. Besides the basal proton conductance, mitochondria also have an inducible proton conductance that requires the presence of cofactors for its activation.

Among the proteins involved in this inducible proton conductance are adenine nucleotide translocase (ANT) [8] and the uncoupling proteins (UCPs) [9,10].

In brown adipose tissue, uncoupling protein 1 (UCP_1) catalyzes the inducible proton conductance, generating physiologically important heat production in response to physiological stimuli such as cold and the animal's diet. Within the last decade, several genes have been discovered that encode proteins closely related to UCP_1, including uncoupling protein 2 (UCP_2) and uncoupling protein 3 (UCP_3). Despite the ubiquitous presence of UCP_2 mRNA, UCP_2 protein has been detected in only a few tissues [10], and a causal role in pancreatic beta cell dysfunction and type 2 diabetes has been attributed to it [10]. UCP_3 – because of its preferential expression in skeletal muscle (a metabolically very active tissue endowed with a high mitochondrial activity) – has attracted the interest of several researchers. Many studies have been carried out to try to clarify the role played by this protein, but its physiological function is still in doubt. It seems likely to

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contribute to inducible proton leak [9] as it does not uncouple unless it is first activated. Indeed, growing evidence suggests that the presence of cofactors is crucial for UCP_3-mediated uncoupling [11–13], and that the absence of one of them can adversely affect UCP_3 uncoupling activity [13,14]. While studying the changes in UCP_3 expression/activity occurring during the transition hypo-hyperthyroidism, we first demonstrated that UCP_3-mediated uncoupling can be observed only in the presence of free fatty acids (FFAs) [14]. Moreover, it could be argued that an absence of mitochondrial uncoupling, despite an upregulation of UCP_3 protein, does not necessarily mean that UCP_3 is devoid of the capacity to uncouple. The above situations might be the result of changes in the levels/production of one or more putative cofactors. Indeed, a requirement for additional activators has been indicated by studies on reconstituted systems in isolated mitochondria and on animal tissues [11–13], and it seems likely that UCP_3 is not functional in the absence of activators. However, it is not clear if and in what way these cofactors are related to each other.

The identities of the activators for UCP_3 remain unknown, but possible activators include free fatty acids [9], CoQ [11,15,16], superoxides [12,17,18], AAPH (a carbon-centered radical generator) [19], reactive alkenals such as 4-hydroxy-2-nonenal (HNE) [20], and alkenal analogs such as cinnamate and retinal [20].

It has been shown that UCP_3 is a highly active H+ transporter when activated by CoQ [16], and that CoQ, superoxide, and AAPH work indirectly, by generating carbon-centered radicals on the polyunsaturated fatty acid chains of the phospholipids located in the inner mitochondrial membrane. The idea that the action of CoQ on UCP_3-mediated uncoupling occurs via O^{2-} seems to be confirmed by studies in which the activation of UCP_3 induced by adding CoQ to mitochondria disappeared when superoxide dismutase was also added [15]. Superoxides, therefore, seem to play a pivotal role in activating UCPs [12,17,18], even though compounds formed downstream of superoxide formation have also been proposed to be involved in the activation of UCPs. These downstream compounds include hydroperoxy fatty acids [21,22] and lipid peroxidation products such as HNE [20,23] and/or 4-hydroxinoneic acid [24].

Since: i) the proton leak contributes significantly to the setting of the level of the metabolic rate in animals [2,25] (ii) among all tissues skeletal muscle mitochondria exhibit the highest proton leak [2], iii) a change in the skeletal muscle mitochondrial proton conductance could significantly affect the energy expenditure of the animal, and iv) activation of proton-leak processes is a potential target for the treatment of pathologies such as obesity via a modulation of metabolic rate, we thought it both interesting and important to try to gain a deeper insight into the mechanism by which the skeletal muscle proton leak may be induced.

In the present study, we therefore investigated the interrelated influences of superoxides and fatty acids over the mitochondria proton-leak kinetic in skeletal muscle (and we discuss the putative involvement of UCP_3 in this process). To this end, we evaluated proton-leak kinetics while modulating both mitochondrial endogenous superoxide production and levels of FFAs. In addition, we tested the effects of rotenone, vitamin E, and GDP on FFA-induced uncoupling.

2. Material and methods

2.1. Animals

Male Wistar rats (250–300 g) (Charles River) were used throughout. They were kept one per cage in a temperature-controlled room at a thermoneutral temperature of 28 °C under a 12-h light, 12-h dark cycle. A commercial mash and water were available ad libitum. Rats were anesthetized by means of an i.p. injection of chloral hydrate (40 mg/100 g BW), then killed by decapitation.

2.2. Isolation of mitochondria and evaluation of respiratory parameters

Subsarcolemmal skeletal muscle mitochondria were isolated by differential centrifugation at 8000 ×g, as previously described [14]. They were then immediately used for measurement either of respiratory rate or membrane potential. We chose to perform our experiments on subsarcolemmal mitochondria because the mitochondrial H_2O_2 release per unit of O2 consumed is reportedly 2-fold higher in subsarcolemmal mitochondria than in intermyofibrillar ones [26].

Mitochondrial oxygen consumption was measured polarographically using a Clark-type electrode, the measurements being carried out in duplicate using succinate (6 mM) as substrate. The analyses were performed at 37 °C in a final volume of 0.5 ml of standard incubation medium [consisting of 80 mM KCl, 50 mM HEPES (pH 7.2–7.4), 1 mM EGTA, 5 mM K_2HPO_4, 5 mM $MgCl_2$, 4 μM rotenone, and 0.3% bovine serum albumin (BSA) (w/v)], either in the absence or presence of ADP (300 μM).

The respiratory control ratio (RCR) was calculated as the ratio of respiration measured in the presence of ADP to that measured in its absence.

2.3. Proton-leak kinetic assay

To evaluate proton conductance we detected the kinetic response of proton leak to a change in membrane potential. Respiration rate and membrane potential ($\Delta\Psi$) were measured simultaneously, the latter being detected using a triphenylmethylphosphonium ($TPMP^+$)-sensitive electrode. For these measurements, 0.5 mg mitochondrial proteins was incubated in 1 ml standard incubation medium from which rotenone was omitted (unless otherwise specified) and to which 1 μg/ml oligomycin, 80 ng/ml nigericin, and 0.3% BSA were added. At the concentration of BSA used, BSA is the predominant protein, and endogenous FFAs can therefore be considered to be buffered by the albumin.

First, mitochondria were incubated in the respiratory medium for 2 min to calibrate the $TPMP^+$-sensitive electrode, calibration being achieved by means of sequential additions of up to 2 μM $TPMP^+$. Then, mitochondria were energized using 6 mM succinate, and respiration was titrated with increasing amounts of malonate (up to 2 mM).

Next, we tested the effect of FFAs on mitochondrial proton-leak kinetics. Since: i) among FFAs, polyunsaturated fatty acids (PUFAs) display the highest sensitivity to oxidative damage, and ii) many studies have shown that free-radical damage and lipid peroxidation each increase as a function of the degree of unsaturation of the fatty acids [27], we decided to evaluate the effect of two different FFAs with the same chain length, but a different saturation index, on mitochondrial uncoupling. These FFAs were arachidonic acid and arachidic acid. Arachidonic acid, being a PUFA, easily enters peroxidative processes. Moreover, Beck et al. [28] reported that among the free fatty acids tested, arachidonic acid had the highest capacity to induce proton conductance in planar lipid membranes reconstituted with purified recombinant human uncoupling proteins.

To test the effect of the above FFAs on mitochondrial uncoupling, malonate titrations of respiration rate were performed in the presence of either arachidonic acid (30 μM) or arachidic acid (30 μM). In the case of these two FFAs, the binding affinity constant of BSA is known only for arachidonic acid [29], and so we will refer in this study to the nominal amount of each fatty acid added.

To test the involvement of O^{2-} production via electron reverse from complex II to complex I on both the mitochondrial basal and FFA-inducible proton leaks, in some experiments rotenone was present in the respiration medium (at a concentration of 4 μM). To test the effect of vitamin E on both the basal and FFA-inducible proton leaks, in some experiments it was present in the respiration medium at a concentration of 1 mM. The effect of GDP on both the basal and FFA-induced proton leaks was tested by adding it to the incubation medium at a concentration of 0.5 mM. Each of the above compounds was added to the incubation medium before the mitochondria were added.

In some experiments, to exclude the involvement of adenine nucleotide translocase (ANT) in fatty acid-induced uncoupling, carboxyatractiloside (CAT; 10 μM) was added to the incubation medium. When CAT was present, to test the effect of GDP on fatty acid-induced uncoupling, malonate titrations of respiration rate were performed in the presence of either arachidonic acid (60 μM) or arachidic acid (60 μM), in each case with or without GDP (0.5 mM).

The concentrations of arachidonic acid used in the basal condition and in the presence of CAT were chosen after carrying out arachidonic-acid titration of the oligomycin-inhibited respiration rate. From the arachidonic-acid titration data, we chose the concentrations of arachidonic acid that were able to induce the same mild uncoupling in the two different experimental conditions.

Baseline correction was obtained by the addition of 0.2 μM FCCP, which induces complete release of $TPMP^+$. A $TPMP^+$-binding correction of 0.4/(μl/mg protein) was applied, as reported by Rolfe et al. [2].

In all the experimental conditions, EGTA was present in the incubation medium to prevent the opening of transition pores [30].

2.4. Mitochondrial H_2O_2 release

The rate of mitochondrial H_2O_2 release was measured at 37 °C the following, using a fluorimeter, the linear increase in fluorescence (ex 312 nm and em 420 nm) that occurred due to the oxidation of homovanillic acid (HVA) by H_2O_2 in the presence of horseradish peroxidase (HRP) [31]. Reaction conditions were 0.2 mg of mitochondrial protein/ml, 6 U/ml HRP, 0.1 mM HVA, 5 mM succinate, and 75 U/ml superoxide dismutase, dissolved in the same incubation buffer as that used for the oxygen-consumption measurements.

The effects of arachidonic acid, arachidic acid,and other compounds (rotenone, vitamin E, GDP, CAT) on mitochondrial H_2O_2 release was detected by adding them (individually or in combination) to the incubation medium. Mitochondria were