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ORIGINAL PAPER

Proton leak induced by reactive oxygen species produced during in vitro anoxia/reoxygenation in rat skeletal muscle mitochondria

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Abstract Superoxide anion generation and the impairment of oxidative phosphorylation yield were studied in rat skeletal muscle mitochondria submitted to anoxia/reoxygenation in vitro. Production of superoxide anion was detected after several cycles of anoxia/reoxygenation. Concomitantly, a decrease of state 3 respiration and phosphorylation yield (ADP/O) were observed. The latter resulted from a proton leak. The presence of palmitic acid during anoxia/reoxygenation cycles led to a dose-dependent inhibition of superoxide anion production together with a partial protection of the ADP/O ratio measured after anoxia/reoxygenation. The ADP/O decrease was shown to be due to a permeability transition pore-sustained proton leak, as it was suppressed by cyclosporine A. The permeability transition pore activation was induced during anoxia/reoxygenation by superoxide anion, as it was cancelled by the spin trap (POBN), which scavenges superoxide anion and by palmitic acid, which induces mitochondrial uncoupling. It can be proposed that the palmitic acid-induced proton leak cancels the production of superoxide anion by mitochondria during anoxia/reoxygenation and therefore prevents the occurrence of the superoxide anion-induced permeability transition pore-mediated proton leak after anoxia/reoxygenation.

Keywords Mitochondria · Anoxia/reoxygenation · H^+ leak · OXPHOS · Uncoupling protein · ROS · Permeability transition pore

Abbreviations

A/R	anoxia/reoxygenation
BSA	bovine serum albumin
CsA	cyclosporine A
EPR	electron paramagnetic resonance
FFA	free fatty acids
*J*o	respiratory rate in phosphorylating state 3
*J*p	rate of ATP synthesis
nBM	*n*-butyl malonate
$O_2^{\bullet -}$	superoxide anion
$^{\bullet}OH$	hydroxyl radical
OXPHOS	oxidative phosphorylation
PA	palmitic acid
POBN	α-(4-pyridyl-1-oxide)-*N*-tert-butylnitrone
PTP	permeability transition pore
ROS	reactive oxygen species
SOD	superoxide dismutase
UCP	uncoupling protein
UQ	ubiquinone
$\Delta\mu H^+$	proton electrochemical gradient

Introduction

In most aerobic cells, mitochondria are the major source of reactive oxygen species (ROS) that are side products of oxidative phosphorylation when ATP synthesis is coupled

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to molecular oxygen reduction. Besides the four-electron reduction of O_2 which occurs at the level of the cytochrome oxidase, a one-electron reduction of molecular oxygen can occur at the level of other respiratory chain complexes, mainly at complex I (NADH–ubiquinone reductase) and complex III (ubiquinol–cytochrome *c* reductase) leading to superoxide anion radical ($O_2^{\bullet -}$) formation (Brand et al., 2004; Brookes, 2005; Turrens and Boveris, 1980). The superoxide anion is the starting point of the production of several ROS, such as hydrogen peroxide (by superoxide dismutase, SOD) and hydroxyl radical ($^{\bullet}OH$). The latter is formed by the Fenton–Haber Weiss reaction when $O_2^{\bullet -}$ and hydrogen peroxide (H_2O_2) coexist in the presence of Fe^{2+}.

The first target of ROS are the mitochondria themselves, where ROS can induce severe damage (protein oxidation, lipid peroxidation, DNA fragmentation) that can finally lead to cell death. Enzymatic detoxifying systems exist both at the level of mitochondria and cytosol to prevent ROS damage. In fact, these detoxification steps represent a second line of cell defense against already produced ROS. Indeed, the first line of defense is the prevention of $O_2^{\bullet -}$ formation at the level of mitochondrial respiratory chain complexes. This $O_2^{\bullet -}$ production is abundant mainly during resting respiration when the proton electrochemical gradient ($\Delta\mu H^+$) is high and when the electron carriers of the respiratory chain are very reduced, i.e., in the absence of ADP (in vitro) or in vivo when the ATP demand is low and the reducing power is high. Thus, systems able to decrease the reduced state of the respiratory chain should decrease $O_2^{\bullet -}$ production at the level of complex III as well as complex I (Brand et al., 2004; Brookes, 2005; Skulachev, 1996).

Uncoupling proteins (UCPs) may be apt candidates to play this preventive role against ROS since their activity increases H^+ leak, accelerates electron flux to oxygen, and consequently decreases the reduced state of mitochondrial electron carriers. UCPs are mitochondrial inner membrane proteins which belong to the mitochondrial anion carrier family that uncouple mitochondrial respiration and ADP phosphorylation by diverting $\Delta\mu H^+$ from ATP synthase (Klingenberg and Echtay, 2001). UCPs are activated by FFA and inhibited by purine nucleotides (Jezek et al., 1998; Klingenberg and Echtay, 2001). Muscle mitochondria contain two UCP isoforms, i.e., a low level of UCP2 (ubiquitous in all tissues) and mainly UCP3 (a muscle specific isoform of UCP). Several lines of indirect observations suggest that UCPs could be implicated in cellular defense against ROS: (i) skeletal muscle mitochondria isolated from UCP3 knockout mice exhibit higher ROS levels and related damage than wild-type mice (Vidal-Puig et al., 2000); (ii) UCP2 knockout mice are more resistant to *Toxoplasma gondii* infection because of the increased ROS production by UCP2 deficient macrophages (Arsenijevic et al., 2000); (iii) over-expressing UCP2 mice show a decrease of cerebral damage after brain trauma and UCP2 in cortical neurone culture decreases apoptosis and inhibits caspase 3 activation induced by oxygen and glucose deprivation (Mattiasson et al., 2003). Moreover, a limitation of ROS production by UCPs has been observed in mammalian, plant, and protist mitochondria (Czarna and Jarmuszkiewicz, 2005; Kowaltowski et al., 1998; Negre-Salvayre et al., 1997; Talbot et al., 2004). Besides the effects of the UCP-mediated H^+ leak on ROS production, the effects of ROS on mitochondrial H^+ leak are also described (for a short critical review, see Brookes, 2005). Furthermore, it has been shown that $O_2^{\bullet -}$ can directly activate H^+ leak mediated by UCPs (Echtay et al., 2002; Talbot et al., 2004).

In normal conditions, enzymatic detoxification systems can cope with ROS production, but in situations, such as in vivo organ ischemia-reperfusion, the defense capacities become overloaded which can lead to mitochondrial damage. In a previous study, we designed an in vitro model of anoxia/reoxygenation (A/R) in order to investigate $O_2^{\bullet -}$ production by isolated liver mitochondria and its effects on mitochondrial function (Du et al., 1998). This experimental model allowed us to show that $O_2^{\bullet -}$ was produced by liver mitochondria after in vitro A/R. Measurements of mitochondrial respiratory parameters showed that this $O_2^{\bullet -}$ production induced mitochondrial damage at the functional level, i.e., a decrease in both oxidative phosphorylation yield (the ADP/O ratio) and phosphorylating respiratory rate (state 3) was observed (Du et al., 1998).

In this study, the in vitro A/R approach has been used to show the production of $O_2^{\bullet -}$ and its damaging effects on rat skeletal muscle mitochondria. Electron paramagnetic resonance (EPR) measurements with POBN/EtOH as a spin trap revealed mitochondrial $O_2^{\bullet -}$ generation. Respiratory measurements performed after A/R cycles showed that a resulting mitochondrial H^+ leak altered the oxidative phosphorylation (OXPHOS) yield. We provided evidence that this extra H^+ leak is mediated by the permeability transition pore (PTP), a cyclosporine (CsA)-sensitive channel of the inner membrane.

Materials and methods

Muscle mitochondria isolation

Hind limb skeletal muscle mitochondria were isolated from male Wistar rats (around 250 g). After fat and conjonctive tissue removal, the muscles were cut into small pieces and homogenized in an ice-cold medium containing 100 mM sucrose, 100 mM KCl, 1 mM K_2HPO_4, 0.1 mM EGTA,

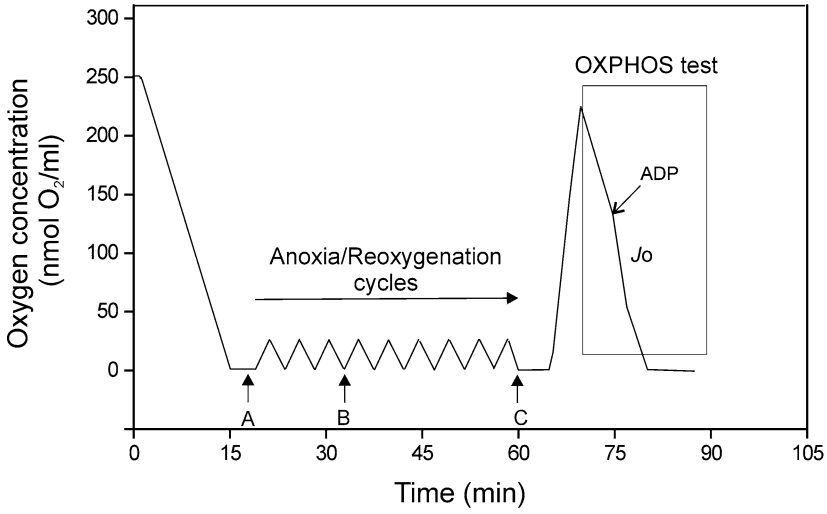


Fig. 1 The experimental setting for the in vitro anoxia/reoxygenation (A/R) treatment of isolated rat skeletal muscle mitochondria. For details, see Anoxia/Reoxygenation (A/R) section. (A–C) Applied A/R cycles: 0, 3, and 9, respectively. OXPHOS test: estimation of state 3 respiratory rate (*J*o) and ADP/O ratio following A/R treatment

0.2% bovine serum albumin (BSA), and 50 mM Tris–HCl, pH 7.4 (Jarmuszkiewicz et al., 2004; Tonkonogi and Salhin, 1997). Fatty-acid-free BSA was used to deplete mitochondria of endogenous FFA. Homogenization was followed by differential centrifugation and the final mitochondrial pellet was resuspended in an ice-cold medium containing 225 mM mannitol, 75 mM sucrose, 0.1 mM EDTA, and 10 mM Tris–HCl, pH 7.4. Protein concentration was determined by the Biuret method.

Mitochondrial respiration

Respiration of skeletal muscle mitochondria (0.5 mg) was measured using a Clark type electrode (Hansatech) with 10 mM succinate (+5 μM rotenone) as an oxidizable substrate in 1.2 mL of an air saturated incubation medium (100 mM KCl, 5 mM K_2HPO_4, 0.5 mM EDTA, 50 mM Tris–HCl, pH 7.4) at 25°C.

Mitochondrial depletion of FFA was checked by measuring respiratory rate in state 4 (in the absence of ADP) in the presence of 10 μM atractyloside and 10 mM glutamate in order to block the protonophoretic activity of the ADP/ATP and glutamate/aspartate carriers, respectively, as described in Jarmuszkiewicz et al. (2004).

The ADP/O ratio was determined with succinate (+rotenone) as a respiratory substrate by an ADP pulse method with 250 nmol of ADP, in the presence of 80 μM ATP (in order to activate the succinate dehydrogenase) and in the presence or absence of 10 μM PA (24 nmol/mg protein). The total amount of oxygen consumed during phosphorylating, state 3 respiration (*J*o) was used for the calculation of the ratio. The ADP/O ratio and *J*o allowed the rate of ADP phosphorylation (ATP synthesis) ($Jp = ADP/O \times Jo$) to be calculated. To establish the *J*p/*J*o relationship, the respiration (*J*o) was titrated with increasing concentrations of *n*-butyl malonate (nBM), a competitive inhibitor of succinate uptake. In the applied *J*o titration range no membrane potential decrease was observed (Jarmuszkiewicz et al., 2004).

Anoxia/reoxygenation (A/R)

Mitochondria reached anoxia by consuming all the oxygen in the 1.2 mL reaction medium in state 4 in the presence of 10 mM succinate (+5 μM rotenone) as oxidizable substrate (Fig. 1). Anoxia was followed by in vitro reoxygenation (up to 10% of oxygen saturation) performed by a brief time opening of the respirometer chamber under stirring. Then, the oxymeter chamber was closed and oxygen was again consumed by mitochondria. The number of A/R cycles tested to check the degree of $O_2^{\bullet -}$ production was 0, 3, and 9. To measure ADP/O and *J*o after A/R treatment, an ADP pulse (250 nmoles) was made after a final reoxygenation step (leading to around 90% of oxygen saturation) performed by a gentle pipetting out and in of the stirred incubation medium.

EPR measurements

The production of $O_2^{\bullet -}$ by isolated skeletal muscle mitochondria was measured by electron paramagnetic resonance (EPR) using 50 mM POBN (α-(4-pyridinyl-1-oxide)-*N*-*tert*-butylnitrone) in the presence of 2% ethanol as a spin trap (Britigan et al., 1996; Morel et al., 1995). The stock solution of POBN was prepared in a phosphate buffer (pH 7.4) and mixed with ethanol before the experiment. Mitochondria (10 mg) were incubated in 1.2 mL of the incubation medium (Mitochondrial Respiration section) in the presence of POBN, with 10 mM succinate as an oxidizable substrate

(+5 μM rotenone, +80 μM ATP). After 0, 3, or 9 A/R cycles (Fig. 1), the mitochondrial suspension was transferred into the quartz flat cell and then into the cavity of the spectrometer. The samples were analyzed with an EPR 300E Spectrometer (Bruker) at operating X-band (9.75 GHz). The instrumental setting was modulation frequency, 100 kHz; amplitude modulation, 1.01 Gauss; receiver gain, 2×10^4; conversion time, 81.92 ms; time constant, 163.84 ms; resulting sweep time, 83.89 s; microwave power, 20 mW; sweep width, 100 Gauss; center field, 3480 Gauss.

To monitor the formation of free radicals ($O_2^{\bullet -}$) in mitochondria, an anoxia/reoxygenation model using EPR methodology was associated with the spin trapping technique. Because of the short half time of free radical species in aqueous medium, hydrosoluble diamagnetic molecules are required. The choice of POBN as a spin trapping agent was justified by its lipophilic properties, very low toxicity, and resistance to cellular reducing agents. POBN was also found to cross the mitochondrial membranes and to interact indirectly with the $O_2^{\bullet -}$ by-products (e.g., hydroxyl radical or lipid radicals) in the presence of ethanol (Pou et al., 1994; Rosen et al., 1990).

The superoxide anion that is produced by mitochondria can cause cell damage (e.g., lipid peroxidation), by reacting with metal–iron complexes within the enzymatic domains. It is well known that mitochondrial aconitase contains a $[4Fe–4S]^{2+}$ cluster that can be attacked by $O_2^{\bullet -}$, thereby generating the inactive $[3Fe–4S]^{+}$ aconitase and the iron II release with the subsequent formation of H_2O_2 (Vasquez-Vivar et al., 2000). Consequently, the inactivation of aconitase by a superoxide may increase the mitochondrial formation of $^{\bullet}OH$ through the Fenton reaction (see Eq. (1)):

$$\begin{aligned}[4Fe–4S]^{2+} + O_2^{\bullet -} &\rightarrow \{[3Fe–4S]^{+} - OO^{2-}\} + Fe^{2+} \\ &\xrightarrow{2H^+} [3Fe–4S]^{+} + Fe^{2+} + H_2O_2 \\ &\rightarrow [3Fe–4S]^{+} + Fe^{3+} + OH^{-} + {}^{\bullet}OH \end{aligned} \quad (1)$$

Trapping of $^{\bullet}OH$ by the POBN/EtOH mixture could occur according to Scheme 1.

Chemicals

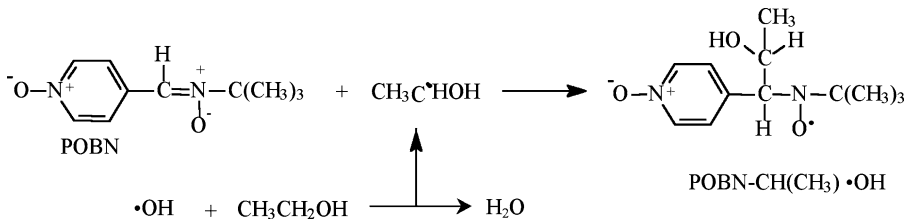
4-POBN (Aldrich) was used without further purification. Iron (II) sulfate, $FeSO_4 \times 7H_2O$, and hydrogen peroxide were purchased from Merck and diethylenetriamine pentaacetic acid (DTPA) was purchased from Aldrich.

Results and discussion

The production of a superoxide anion by isolated rat skeletal muscle mitochondria submitted to anoxia/reoxygenation

It has been previously shown that in rat liver mitochondria, a strong EPR signal was observed after a single A/R cycle (Du et al., 1998). With rat skeletal muscle mitochondria respiring in state 4, no EPR signal was detected after anoxia or after three A/R cycles as shown in Fig. 2. Nine A/R cycles were needed to observe $O_2^{\bullet -}$ production (revealed as a triple doublet of EPR spectrum of POBN/$^{\bullet}$CH(OH)CH_3 adduct) (Fig. 2C). These results strongly suggest that skeletal muscle mitochondria are more resistant to the stress induced by in vitro A/R compared to liver mitochondria. This could be due to a lower $O_2^{\bullet -}$ production with succinate (+rotenone) as a reducing substrate and/or to a higher activity of the enzymatic antioxidant defense in skeletal muscle mitochondria compared to liver mitochondria as shown in Hollander et al. (1999) and Wilson and Johnson (2000). In the present study, the use of succinate (+rotenone) instead of complex I substrates is required in order to perform ADP/O measurements together with *J*o titration (Jarmuszkiewicz et al., 2004).

In our model, $O_2^{\bullet -}$ produced by isolated mitochondria after the A/R cycles is quenched by ethanol, yielding ethoxy radicals which are then trapped by POBN (see Scheme 1) thereby leading to the relatively stable POBN/$^{\bullet}$CH(OH)CH_3 adducts (with the following coupling constants: $a(N) = 15.2$ G and $a(H) = 2.6$ G) (http://EPR.niehs.nih.gov). The efficiency of POBN as a spin trapping agent and the stability of the resulting spin adducts



Scheme 1 Proposed mechanism of hydroxyl radical trapping by the POBN/EtOH mixture. Superoxide anion produced by mitochondria after A/R cycles attacks the cluster $[4Fe–4S]^{2+}$ of aconitase, releasing the iron(II) with the subsequent formation of H_2O_2. Both species form the highly reactive species $^{\bullet}OH$, which may initiate either lipid peroxidation or react with the spin trap. In our in vitro model, the use of the POBN/EtOH mixture allowed the trapping of $^{\bullet}OH$ radicals, leading to POBN–$CH_3{}^{\bullet}$CHOH adducts

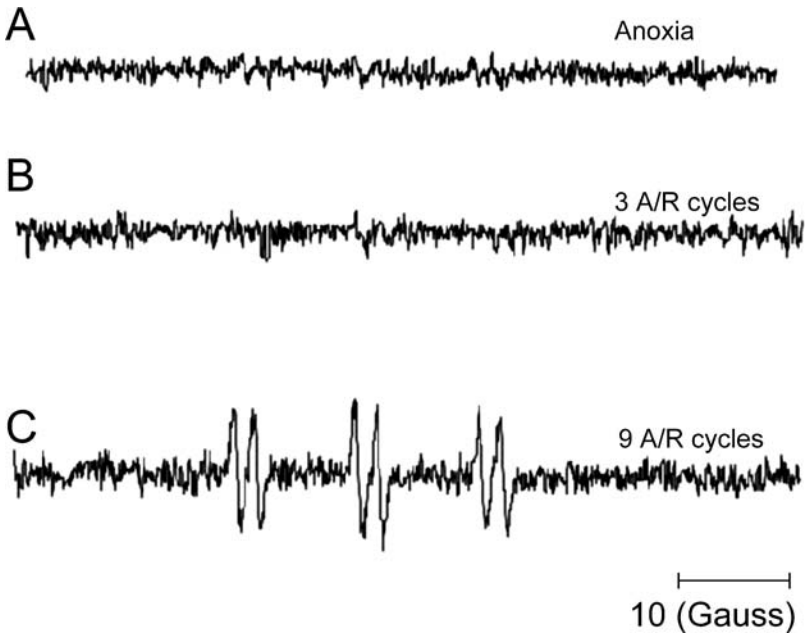


Fig. 2 EPR spectra of rat skeletal muscle mitochondria submitted to anoxia/reoxygenation. As a spin trap 50 mM POBN (in 2% ethanol) was used. Samples were taken from the respirometer chamber when mitochondria reached anoxia as shown in Fig. 1, after 0 (A), 3 (B), and 9 (C) A/R cycles. An example of a representative experiment is shown

confirm the high degree of sensitivity of the POBN/EtOH mixture and demonstrate its potential application to monitor the formation of free radicals in mitochondria by using the EPR technique. On the basis of the EPR results shown in Fig. 2, the effects of nine A/R cycles on the function of isolated rat skeletal muscle mitochondria were then studied.

The effect of anoxia/reoxygenation on skeletal muscle mitochondrial function

Previously, we have shown in vitro with liver mitochondria (Du et al., 1998) as well as in vivo for lung (Willet et al., 2000) and brain (Du et al., 2004) that the most frequent mitochondrial dysfunctions observed after oxygen deprivation (anoxia in vitro or ischemia in vivo) and oxygen refilling (reoxygenation or reperfusion) are a decrease in the ADP/O ratio and in the state 3 respiratory rate (*J*o).

To investigate the effect of A/R on OXPHOS of rat skeletal muscle mitochondrial, we established the *J*p/*J*o relationship both before and after nine A/R cycles for mitochondria respiring with succinate (+rotenone). The phosphorylating respiratory rate was progressively decreased by increasing concentrations of nBM (an inhibitor of succinate uptake).

The *J*p/*J*o relationship obtained without anoxia was linear within the tested range of *J*o and the extrapolation of the regression line crossed the *J*o axis on the right of the origin (Fig. 3) as already shown in (Jarmuszkiewicz et al., 2004). Furthermore, it has been shown that the state 3 membrane potential (158 ± 2 mV) and H^+ leaks (either endogenous or FFA induced) remained stable within the tested range of *J*o titration (Jarmuszkiewicz et al., 2004). The extrapolation of the regression line (Fig. 3, no anoxia, all conditions) crossed the *J*o axis ($Jp = 0$) at about 16 nmol O $\times$ min^{-1} $\times$ mg^{-1} protein which corresponds to an endogenous H^+ leak of around 96 $\times$ nmol H^+ $\times$ min^{-1} $\times$ mg^{-1} protein (taking 6 as the H^+/O ratio of the respiratory chain with succinate as oxidizable substrate). A slope of the straight line (1.589 ± 0.022, SD, $n = 37$) (Fig. 3, no anoxia, all conditions) corresponds to a stoichiometric ADP/O ratio given by the H^+/O ratio of the respiratory chain divided by the H^+/ADP ratio for ADP transport and phosphorylation.

Figure 3 shows that neither 2 μM CsA (an inhibitor of PTP), 50 mM POBN (in 2% ethanol) (a spin trap applied for EPR measurements) nor 2% BSA plus PA (24 nmol/mg protein) (to exclude UCP activity by BSA that chelates PA) have an effect on the control *J*p/*J*o relationship during titration by nBM obtained before anoxia, as all the points obtained in these conditions (■, ▲, ●) were located on the control *J*p/*J*o relationship (◇).

After nine A/R cycles, both *J*o and the measured ADP/O ratio were significantly lowered in the absence of nBM (by 44 and 25%, respectively). These decreases cannot be attributed to mitochondrial disruption by aging in vitro because of the long incubation of mitochondria needed for the nine A/R cycles. Indeed, no significant impairment was observed when O_2 was never depleted in the open respiratory chamber during the same respiratory time course (not shown). The titration with nBM resulted in a *J*p/*J*o straight line (Fig. 3, ×) parallel to the control line and crossing the *J*o axis at about 28 nmol O $\times$ min^{-1} $\times$ mg^{-1} protein. This indicates that nine cycles of A/R induced in respiring muscle

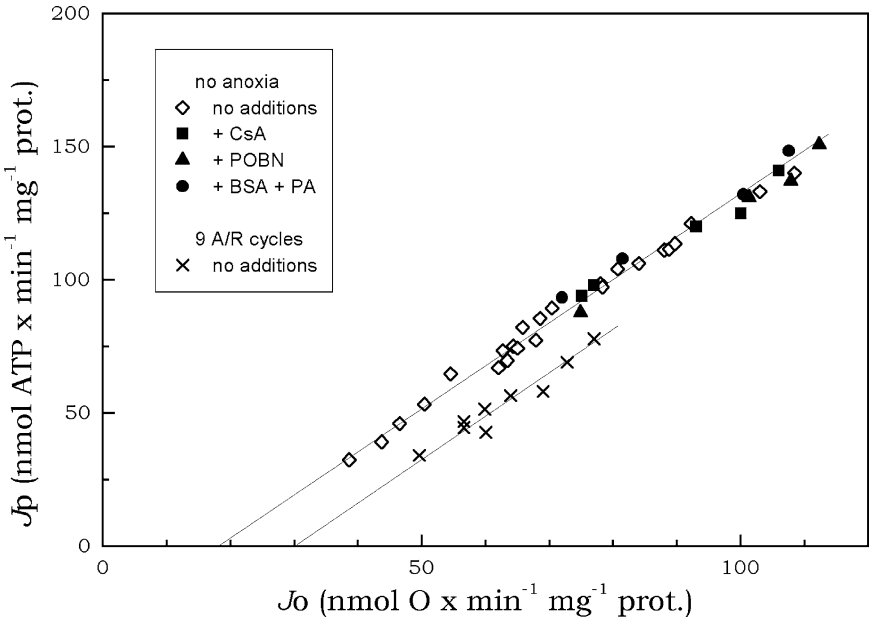


Fig. 3 The effect of anoxia/reoxygenation on the oxidative phosphorylation of rat skeletal muscle mitochondria: the *J*o/*J*p relationship before and after nine cycles of A/R. The respiratory rate was progressively inhibited by nBM (0–0.5 mM). The *J*o/*J*p relationship was defined for no anoxia conditions (◇, control; ■, +2 μM CsA; ▲, +50 mM POBN; ●, +2% BSA and 48 nmol/mg protein PA) and after nine A/R cycles (×). Additions were present in the reaction medium from the beginning of the incubation of mitochondria with oxidizable substrate. Data deal with four mitochondrial preparations. Straight lines are least square regression lines

mitochondria an extra H^+ leak (at around 72 nmol H^+ × min^{-1} × mg^{-1} protein) without modifying the stoichiometric ADP/O ratio (as the slope of the regression line was 1.587 ± 0.167, SD, $n = 9$) which means that the H^+ pumps of the respiratory chain and the ATP synthase were not damaged. From these results one could conclude that in rat skeletal muscle mitochondria, in vitro A/R induced only the extra H^+ leak and inhibition of state 3 respiration.

The effect of PA-induced mitochondrial uncoupling on superoxide anion production during anoxia/reoxygenation

It has been shown on reconstituted systems as well as on isolated mitochondria from organisms of various phyllogenic origins that so far all known UCPs are activated by FFA. For the purpose of this study, a saturated FFA (palmitic acid, C16:0) was chosen to avoid double bound(s), which could act as a spin trap for $O_2^{\bullet -}$. Moreover, PA has been demonstrated to be a good activator of UCPs (Klingenberg and Echtay, 2001). The relationship between $O_2^{\bullet -}$ production and the amount of isolated mitochondria (of rat liver) in the incubation medium has shown that at least 10 mg of mitochondrial protein are necessary for EPR measurements with POBN as a spin trap (Du et al., 1998). Therefore, we determined the optimal amount of PA per mg of mitochondrial protein in order to reach a maximal stimulation of state 4 respiration, and thereby the maximal PA-induced H^+ leak. For that purpose, we measured the PA-induced respiratory rate stimulation in state 4 with various protein concentrations (from 0.5 to 10 mg) at various PA concentrations (not shown). It turned out that 48 nmol PA per mg of mitochondrial protein led to the maximal stimulation of state 4 respiration sustained by H^+ leak which corresponded to more than 300% of the initial state 4 respiratory rate.

The effect of PA on $O_2^{\bullet -}$ production during A/R was assessed by comparing the EPR spectra obtained with mitochondria submitted to nine A/R cycles in the absence or presence of PA (Fig. 4). The negative control was a sample taken out at anoxia without an A/R cycle (Fig. 4A). Then spectrum obtained after nine cycles of A/R in the absence of PA (Fig. 4B) was the positive control (maximal $O_2^{\bullet -}$ production). When A/R cycles were performed in the presence of two concentrations of PA, the intensity of the EPR signals was slightly reduced for 12 nmol/mg^{-1} protein PA (Fig. 4C) and completely abolished for 48 nmol/mg^{-1} protein PA, indicating that no more $O_2^{\bullet -}$ was produced (Fig. 4D). This effect of PA observed on the EPR spectra suggests that the FFA-induced H^+ leak (which could be attributed to the UCP-mediated H^+ leak) (Jarmuszkiewicz et al., 2004) could be very efficient as the first line of defense against ROS production by limiting or canceling the production of $O_2^{\bullet -}$, the precursor of all the other ROS. It must be pointed out that the amount of PA per mg of mitochondrial protein used to abolish $O_2^{\bullet -}$ production is of the same order of magnitude as the amount of linoleic acid that did not modify the function of the H^+ pumps of the respiratory chain and ATP synthase (Jarmuszkiewicz et al., 2004).

To monitor the effect of PA on the production of free radicals in our experimental in vitro model, we made the Fenton reaction in a mitochondria-free system in the presence of POBN/EtOH with or without PA (Fig. 5). Figure 5 shows the EPR spectrum of the POBN/$^{\bullet}$OH adduct resulting from the reaction of a hydroxyl radical with POBN during the Fenton reaction (Fig. 5A). Spectra were also obtained without the addition of Fe(II), or with PA or ethanol

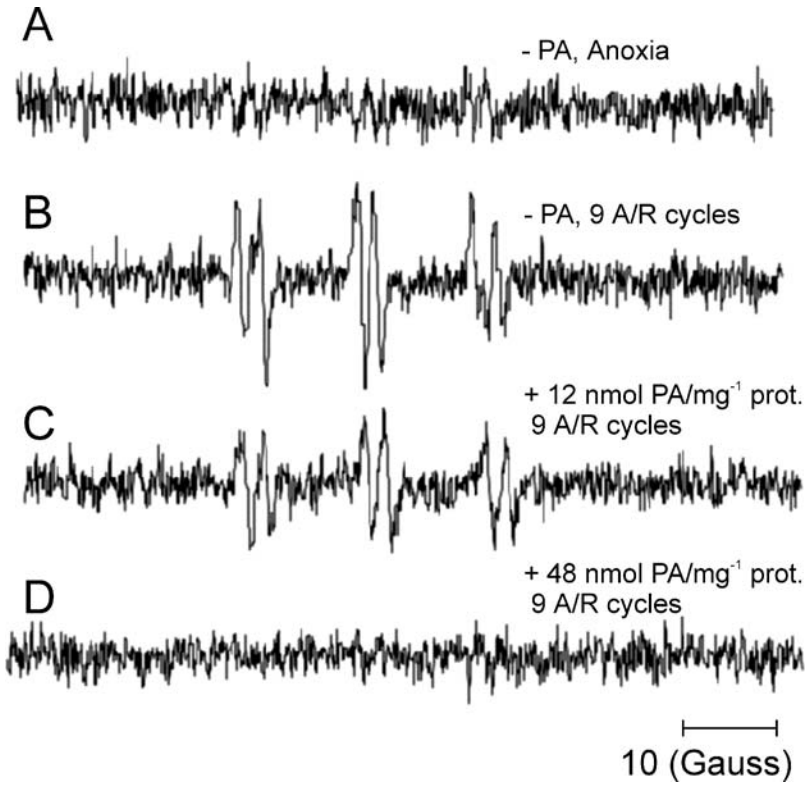


Fig. 4 The effect of PA on the EPR spectra of rat skeletal muscle mitochondria after nine anoxia/reoxygenation cycles. The EPR spectra were obtained in anoxia (A) and after nine A/R cycles in the absence (B) or presence of 12 nmol/mg^{-1} protein PA (C) or 48 nmol/mg^{-1} protein PA (D). PA was present in the reaction medium (C and D) from the beginning of incubation of mitochondria with an oxidizable substrate. An example of a representative experiment is shown

as a vehicle of PA (Fig. 5B, C, and D, respectively). The C and D spectra (Fig. 5) are larger because of the presence of extra EtOH (PA solvent). The results indicate that the presence of PA does not disturb the EPR spectrum of POBN/•OH adduct, hence, the reduction of EPR signals observed for isolated mitochondria (Fig. 4) results only from the PA-induced H^+ leak.

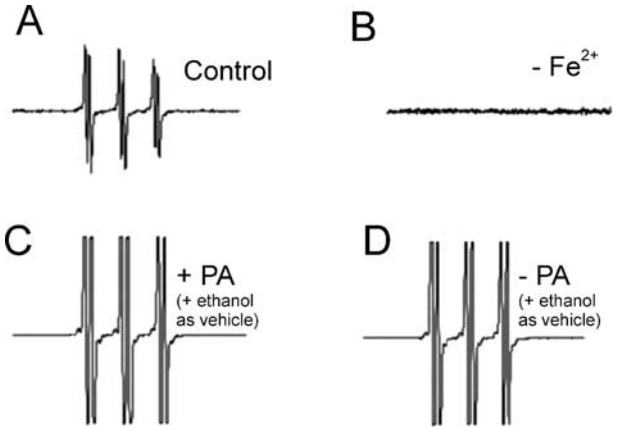


Fig. 5 The effect of PA on the EPR spectrum during the Fenton reaction in a mitochondria-free system. The reaction was performed in a 1.2 mL incubation medium (see Mitochondrial Respiration section) with 1 mM H_2O_2, 0.1 mM $FeSO_4 \times 7H_2O$, and 0.1 mM diethylenetriamine pentaacetic acid (DTPA). Conditions: control (A), −Fe(II) (B), in the presence (C) or absence (D) of 480 nmol of PA. In (D), addition of PA was substituted by the addition of an equal volume of ethanol

The effect of PA-induced uncoupling on the H^+ leak induced by anoxia/reoxygenation

As the H^+ leak induced by nine A/R cycles (Fig. 3) was most likely due to $O_2^{\bullet-}$ production (Fig. 2) and as the H^+ leak induced by PA was able to cancel this $O_2^{\bullet-}$ production (Fig. 4), we studied the effect of PA (removed by 2% BSA before final reoxygenation) during A/R on the A/R-induced mitochondrial H^+ leak (Fig. 6). Mitochondrial parameters (*J*o and ADP/O ratio) allowing the determination of the *J*p/*J*o relationship were measured in no PA conditions (after nine A/R cycles in the absence of PA, Fig. 6, ×) and when PA induced a H^+ leak during A/R cycles. In the latter case, the parameters were measured after nine A/R cycles, which were performed in the presence of PA (48 nmol/mg^{-1} protein) which was chelated by 2% BSA before final reoxygenation (Fig. 6, •). As a result, the *J*p/*J*o points obtained when PA was present during the A/R cycles (Fig. 6, •) were located on the control *J*p/*J*o relationship (Fig. 6, ◇, regression line on the left). This indicates that the PA-induced H^+ leak during the nine A/R cycles cancelled the extra A/R-induced H^+ leak (Fig. 6, ×) as well as its probable source ($O_2^{\bullet-}$ production) (Fig. 4) but did not cancel the *J*o decrease induced by A/R treatment.

Figure 7 shows the typical respiratory traces obtained after nine A/R cycles, when PA was absent or present during A/R. When compare to control mitochondria, nine A/R

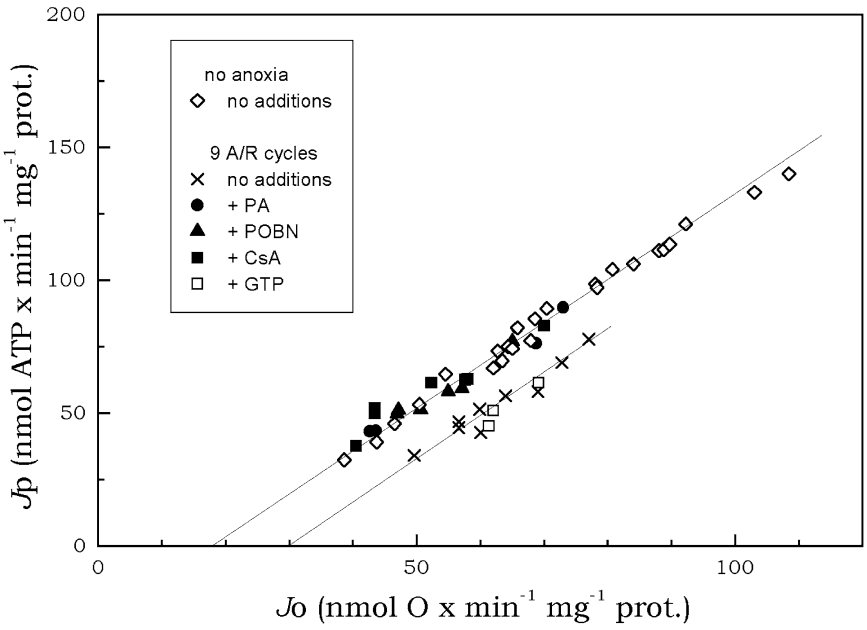


Fig. 6 The effect of PA, POBN, GTP, and CsA on the H^+ leak induced by nine cycles of anoxia/reoxygenation. Mitochondria were submitted to nine A/R cycles in the absence of any additions (×) or in the presence of (from the beginning of incubation with oxidizable substrate) 48 nmol/mg^{-1} protein PA (that was chelated by 2% BSA added before OXPHOS measurements) (●), 50 mM POBN/2% EtOH (▲), 2 μM CsA (■), or 2 mM GTP (□). Control points (no anoxia, no additions, ◇) are those from Fig. 3. Regression lines are those of Fig. 3

cycles caused a decrease in *J*o (state 3) as well as in ADP/O ratio. The presence of PA (48 nmol/mg^{-1} protein) leaded to a recovery of ADP/O ratio to the value observed in control conditions, but did not cancel the *J*o decrease induced by A/R treatment.

Origin of the H^+ leak induced by anoxia/reoxygenation

To check if the extra H^+ leak induced by nine A/R cycles was due to $O_2^{\bullet-}$ production, the effect of the spin trap (50 mM POBN in 2% ethanol) on the ADP/O ratio and *J*o was investigated. As already described, for no anoxia, POBN had no effect on the *J*p/*J*o relationship (Fig. 3, ▲). When nine A/R cycles were applied in the presence of POBN, the points (Fig. 6, ▲) were clearly located on the control *J*p/*J*o relationship (Fig. 6, ◇, regression line on the left), demonstrating that POBN cancelled the extra H^+ leak induced by the nine A/R cycles but, like PA, did not cancel the *J*o decrease. This proved that the used spin trap (POBN/EtOH) has a protective effect against the H^+ leak induced by A/R and that this extra H^+ leak is related and secondary to $O_2^{\bullet-}$ production in muscle mitochondria submitted to A/R in vitro.

If in rat skeletal muscle mitochondria, A/R in vitro can lead to $O_2^{\bullet-}$ production that results in an extra H^+ leak, a question arises as to whether this H^+ leak could be due to the direct activation of muscle UCP isoform by $O_2^{\bullet-}$ itself or by a product of lipid peroxidation. Indeed, it has been shown that $O_2^{\bullet-}$ produced on the matrix side (Talbot et al., 2004) and that the 4-hydroxy-2-nonenal (a product of lipoperoxidation) (Echtay et al., 2003) are able to activate muscle UCPs. GTP inhibits such superoxide-activated UCPs in state 4 respiration (Echtay et al., 2002), contrarily to the FFA-activated muscle

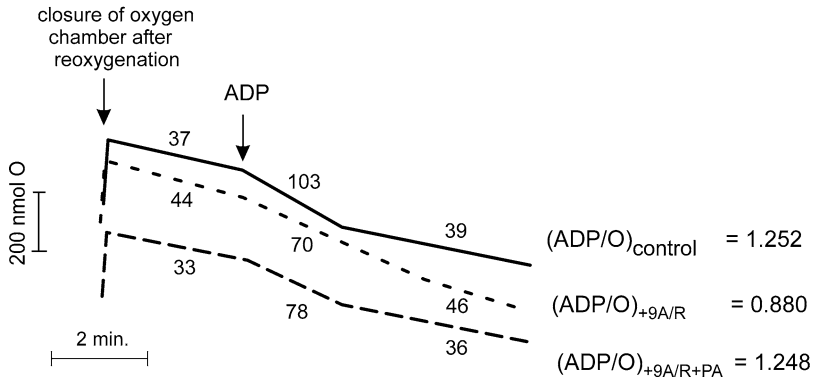


Fig. 7 The effect of PA on coupling of mitochondria submitted to nine cycles of anoxia/reoxygenation. Assay condition as in Fig. 1. OXPHOS test for control mitochondria (no A/R) is shown (*full line*). Mitochondria were submitted to nine A/R cycles in the absence of PA (*dotted line*) or in the presence of (from the beginning of incubation with oxidizable substrate) 48 nmol/mg^{-1} protein PA (that was chelated by 2% BSA added before OXPHOS measurements) (*dashed line*)

UCP (Wilson and Jonson, 2000). The presence of 2 mM GTP had no effect on the *J*p/*J*o relationship in no anoxia condition when FFA were absent (Jarmuszkiewicz et al., 2004).

Nine A/R cycle experiments were made in the presence of 2 mM GTP. The *J*p/*J*o points (Fig. 6, □) did not move from those obtained after A/R treatment (Fig. 6, ×), indicating that the presence of GTP has no effect on the extra H^+ leak induced by A/R. This observation suggests that the H^+ leak induced by A/R was not mediated by ROS-activated UCP. It must be pointed out that in our experimental conditions, $O_2^{\bullet-}$ production occurs mainly at complex III on the external side of the inner mitochondrial membrane, while muscle UCP activation by endogenous $O_2^{\bullet-}$ requires a matrix production (Talbot et al., 2004).

The question which arises is where the $O_2^{\bullet-}$ produced by complex III during A/R could act so as to induce the extra H^+ leak after the A/R cycles. The oxygen deprivation-refilling that occurs during in vitro A/R could be compared to in vivo ischemia-reperfusion. It is well known that during ischemia-reperfusion, high calcium and inorganic phosphate, low ATP content and ROS production induces a mitochondrial permeability transition leading to leaky mitochondria (Di Lisa and Bernardi, 1998). It has been shown that this damage could be reduced by cyclosporine A (CsA) (Halestrap et al., 1997). Moreover, the sequence of events triggered by the opening of the Ca^{2+}-activated CsA-sensitive PTP (dissipation of transmembrane electrochemical gradients, disruption of ionic homeostasis, mitochondrial swelling, and massive ATP hydrolysis) led to assume that the PTP might play an important role in the pathways of cell death (Bernardi et al., 2001; Green and Kroemer, 2005).

After verifying that 2 μM CsA has no effect on the control *J*p/*J*o relationship before anoxia (Fig. 3, ■), we studied the effect of CsA on the H^+ leak induced by nine A/R cycles (Fig. 6). The *J*p/*J*o points obtained in the presence of CsA (Fig. 6, ■) were on the control *J*p/*J*o relationship obtained before A/R (Fig. 6, ◇, regression line on the left) indicating that CsA cancelled the A/R-induced H^+ leak but did not cancel the decrease in *J*o. These results strongly suggest that the extra H^+ leak induced by $O_2^{\bullet-}$ in muscle mitochondria submitted to in vitro A/R treatment could be due to activation of mitochondrial PTP by $O_2^{\bullet-}$ or derived ROS. It must be pointed out that neither PA, nor POBN, nor CsA allowed the recovery of *J*o after A/R. Thus, it can be supposed that the drop in *J*o after anoxia is not related to $O_2^{\bullet-}$ production during A/R.

Conclusions

ROS generation is governed by the redox state of the mitochondrial respiratory chain (more reduced leading to a higher ROS production), which itself is under the control of $\Delta\mu H^+$ that in turn is limited by various H^+ leaks, i.e., the basal leak and several inducible leaks mediated by proteins. The ROS-H^+ leak couple can play an important role in cytoprotection not only against ROS but also against energy wasting (Brookes, 2005). Indeed, in this paper, we have shown that on one hand, ROS generation during A/R treatment of rat skeletal muscle mitochondria leads to the energy wasting H^+ leak through a CsA-sensitive pathway (PTP). On the other hand, the FFA-induced during A/R H^+ leak which could be mediated by muscle UCPs is protective since it cancels A/R-induced $O_2^{\bullet-}$ generation and consequently the A/R-induced detrimental H^+ leak.

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