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Original Contribution

GENERATION OF SUPEROXIDE ANION BY MITOCHONDRIA AND IMPAIRMENT OF THEIR FUNCTIONS DURING ANOXIA AND REOXYGENATION IN VITRO

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Abstract—A small portion of the oxygen consumed by aerobic cells is converted to superoxide anion at the level of the mitochondrial respiratory chain. If produced in excess, this harmful radical is considered to impair cellular structures and functions. Damage at the level of mitochondria have been reported after ischemia and reperfusion of organs. However, the complexity of the in vivo system prevents from understanding and describing precise mechanisms and locations of mitochondrial impairment. An in vitro model of isolated-mitochondria anoxia-reoxygenation is used to investigate superoxide anion generation together with specific damage at the level of mitochondrial oxidative phosphorylation. Superoxide anion is detected by electron paramagnetic resonance spin trapping with POBN-ethanol. Mitochondrial respiratory parameters are calculated from oxygen consumption traces recorded with a Clark electrode. Respiring mitochondria produce superoxide anion in unstressed conditions, however, the production is raised during postanoxic reoxygenation. Several respiratory parameters are impaired after reoxygenation, as shown by decreases of phosphorylating and uncoupled respiration rates and of ADP/O ratio and by increase of resting respiration. Partial protection of mitochondrial function by POBN suggests that functional damage is related and secondary to superoxide anion production by the mitochondria in vitro. © 1998 Elsevier Science Inc.

Keywords—Bioenergetics, Mitochondria, Anoxia-reoxygenation, EPR, Oxidative phosphorylation, Respiration, Superoxide anion, Free radical

INTRODUCTION

Most of the oxygen consumed by aerobic organisms is reduced to water in the terminal reaction of the mitochondrial respiratory chain by the cytochrome *c* oxidase. The reduction of oxygen to water by cytochrome *c* oxidase is believed to occur in four consecutive one-electron steps. Partially reduced oxygen intermediates generated in this process are stable because they are tightly bound to the heme iron. However, a small portion of the oxygen consumed by aerobic cells is converted to superoxide anion radical at the level of other components of the respiratory chain [1–4]. It has been shown that superoxide anion is generated at the level of the three enzymatic complexes interacting with ubiquinone (coenzyme Q) and that this anion is the only reactive oxygen species generated suggesting a monoelectronic donor likely the ubisemiquinone, a quinone-radical intermediate of the redox Q-cycle [5–7].

Mitochondria possess an efficient antioxidant system: enzymes like superoxide dismutase, glutathione peroxidase, NADP transhydrogenase, and compounds such as reduced glutathione, NADPH, vitamins E and C [8–10]. Under physiological conditions, the antioxidant defense system eliminates the superoxide anion produced in mitochondria. But when superoxide anion is generated in excess, or when the oxidant and antioxidant systems are no more in balance, a state of oxidative stress occurs. Under some pathological conditions, especially after ischemia and reperfusion, it has been thought that mitochondria have a substantial capacity to generate reactive oxygen species when the components of the respiratory chain have been reduced and molecular oxygen is refilled [11–13]. In in vivo experiments, reports have shown that the production of hydroxyl increased in rat brain after

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cerebral ischemia. As the inhibitors of mitochondrial complex I, rotenone, and haloperidol, suppressed the production of hydroxyl and as succinic acid, substrate supplying electrons to complex II via FADH, restored the production of hydroxyl, the conclusion has been made that the hydroxyl was produced by mitochondria [11]. Similar results were also obtained from heart, liver, and other organs as well as cultured cells [12–14]. In in vitro experiments, mitochondria isolated from pathological tissues such as tumor, produced more oxygen free radicals than those produced by normal mitochondria [15].

It is well known that the organs are impaired in their functions and structures after ischemia and reperfusion. Many investigations have been reported that mitochondria isolated from ischemia-reperfusion organs are also damaged [16–18]. The major reasons of the damage are considered to be lipid peroxidation and secondary impairment of the mitochondrial functions. However, as the superoxide anion is generated by the respiratory chain in mitochondrial membrane, it should directly and first damage the components in mitochondria (such as respiratory enzymes and surrounding phospholipids).

In the previous reports, almost all results were obtained from mitochondria that were isolated from tissues submitted to ischemia and reperfusion. However, the complexity of the system in such studies prevents from understanding and describing precisely the mechanisms and locations of mitochondrial impairment. Indeed mitochondria are protected in the cytosol by several antioxidant systems that hinder from distinguishing the primary defects occurring directly in mitochondria from secondary damage linked to more general cell impairments.

In order to directly investigate reactive oxygen species generation by mitochondria during anoxia-reoxygenation and to characterize the specific damage at the level of mitochondrial functions, we have established an isolated-mitochondria anoxia-reoxygenation in vitro model. Results reported here show that the mitochondria generate superoxide anion during anoxia-reoxygenation in vitro and that their function is also damaged.

MATERIALS AND METHODS

Chemicals

Adenosin-5′-diphosphate (ADP), α-ketoglutarate (KG), succinate (SUCC), rotenone (ROT), and pyruvate (PYR) were purchased from Boehringer Mannheim GmbH (Germany). Carbonyl cyanide *p*-trifluoro-methoxyphenyl-hydrazone (FCCP), oligomycin (Olig), sucrose, mannitol, phosphoric salts, were obtained from Merck Chemical Company. Ethylene diamine tetraacetic acid (EDTA) and α-(4-Pyridyl 1-oxide)-N-tert-butylnitrone (POBN) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals are of the highest analytical grade available.

Isolation of mitochondria

Male Wistar strain rats of 200–250 g body weight were obtained from the animal house of the Hospital Center of University of Liege. Following decapitation, liver was rapidly dissected out (30 s) and plunged and minced in ice-cold MSET buffer (30 s). The operation from decapitation to homogenization were kept as short as possible and the cooling of minced liver was realized within 1 min. Then the rinsed pieces of the liver were homogenized into large MSET buffer volume during 1 min. The ice-cold MSET buffer contained 210 mM mannitol, 70 mM sucrose, 0.5 mM EDTA, and 10 mM Tris-HCl (pH 7.4). Liver homogenate was centrifuged at $1000 \times g$ for 10 min at 4°C and the subsequent supernatant was centrifuged at $12\,000 \times g$ for 8 min followed by two washings of the mitochondrial pellet in large volume of ice-cold MSET buffer (JA-20 motor and J2-HC centrifuge, Beckman). These conditions were strictly respected to avoid as much as possible warm or cold anoxia during isolation procedure. The final mitochondria pellet was suspended in a sucrose-Tris buffer (280 mM sucrose, 0.5 mM EDTA, and 10 mM Tris-HCl, pH 7.4) and stored on melting ice for measurements. Mitochondrial protein concentration was estimated spectro-photometrically by the method of Peterson [19].

Measurement of mitochondrial respiratory activities

The respiratory parameters of liver mitochondria were measured with α-ketoglutarate and pyruvate or with succinate + rotenone as oxidizable substrates at 25°C in 2 ml of an air saturated reaction medium (0.5 mg of mitochondrial protein/ml). The reaction medium contains 15 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.4), 5 mM α-ketoglutarate plus 5 mM pyruvate (or 5 mM succinate plus 2.5 μg/ml rotenone), 2.5 mM phosphate. The oxygen concentration in medium was followed with a Clark oxygen electrode and its change slope (first derivative = rate of O_2 consumption, e.g., $d(O_2)/dt$) was recorded by Oxygraph (Physica respirometer, Paar Physica, Oroboros, Austria). Respiratory parameters were calculated from oxygen traces.

Anoxia and reoxygenation (A-R)

The anaerobic condition was performed by mitochondria, which consume the oxygen content of the medium

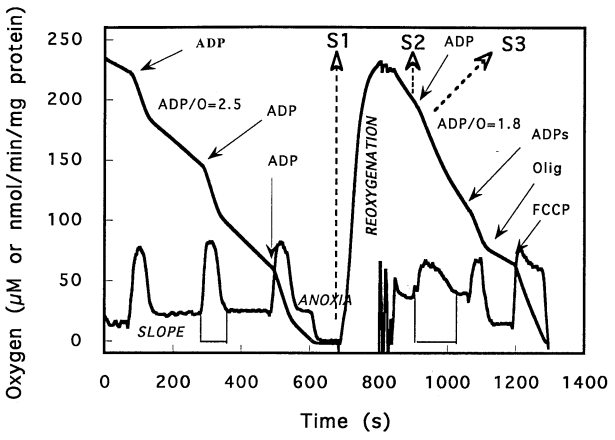

Fig. 1. O_2-consumption trace and its first derivative (respiratory rates) recorded by oxymeter and the time-course of experiments (anoxia, reoxygenation, and EPR experiments samplings). Mitochondria (1 mg protein/ml) were incubated in the medium containing KG 5 mM and PYR 5 mM as oxidizable substrates. ADP pulse: addition of 330 nmol ADP; ADPs: addition of large amount of ADP (2.5 mM); Olig: addition of oligomycin (16 μg/ml); FCCP: addition of FCCP (5 μM); S1, S2, and S3: indicate the moment when samples were taken out for EPR measurement.

after ADP pulses (addition of 330 nmol ADP to 2 ml medium for one pulse) into a closed incubation chamber. Anaerobic conditions after ADP pulses were reached within 10–20 min in state 4 respiration and were kept for 1, 4, or 10 min. Anoxia was followed by 4 $\pm$ 1 min of reoxygenation until 90% of oxygen saturation was reached by exposing the stirred incubation medium to air (open chamber). Then the oxygraph chamber was closed and respiratory activities were measured again as described above (see Fig. 1 for details).

EPR measurements

Superoxide anion production by respiring mitochondrial suspension (4 to 16 mg proteins/ml) was measured by electron paramagnetic resonance (EPR) spin trapping with POBN 50 mM (α-(4-pyridinyl-1-oxide)-N-tert-butylnitrone) in the presence of 2% ethanol [20,21]. Due to its lipophilic properties, POBN was able to cross the mitochondrial membranes and to interact with superoxide anion produced on the matrix side of the membrane. Mitochondria were incubated in the presence of POBN from the beginning of the measurement in order to allow appropriate partitioning of POBN into the matrix. In that way, POBN could reach a protective concentration in this compartment. The samples were taken for EPR measuring at the end of "aerobic" respiration and after reoxygenation during the postanoxic respiration (see Fig. 1 for details). Measurements were performed with EPR 300E Spectometer (Bruker) at operating X-band (9.56 GHz). Instrumental settings were: modulation frequency 100.00 kHz, amplitude modulation 1.01 Gauss, receiver gain 2 $\times$ 104, conversion time 81.92 msec, time constant 163.84 msec, resulting sweep time 83.89 s, number of scans 10, microwave frequency 9.56 GHz, microwave power 20 mW, width sweep 100.00 Gauss, Center field 3480 Gauss.

Calculations and statistics

ADP/O was calculated by the following formula (equation 1):

$$\text{ADP/O} = \text{ADP (nmol)/O (nmol)} \qquad (1)$$

In this formula, ADP is the amount of ADP added in one ADP pulse (330 nmol) and O is the amount of oxygen atoms consumed during phosphorylation of the added ADP. The respiratory activities (V_3, V_4, V_{olig}, and V_{FCCP}) were calculated as formula (equation 2):

$$\text{Respiratory rate} = \text{O(nmol)/t (min)/protein (mg)} \qquad (2)$$

O: amount of oxygen atoms consumed by the mitochondria; t: time (minute), protein: mitochondrial protein. Respiratory parameters data were analyzed with DatGraf Software (Oroboros, Austria). Oxygen concentration in the medium was calculated from pO_2 measurements based on O_2 solubility in the respiration medium at 25°C.

Student *t*-test was used for comparison between groups of data, each indicated as average $\pm$ SD.

RESULTS

Production of superoxide anion by mitochondria during anoxia and reoxygenation

Isolated rat liver mitochondria were incubated as described and submitted to 3 ADP pulses (of 330 nmoles) leading to anoxia in state 4 (Fig. 1 left part). After 1 min (or 4, or 10 min) of anoxia, reoxygenation until 90% was reached in 4 $\pm$ 1 min (see Materials and Methods) and then respiration in state 4 was measured, followed by 330 nmoles ADP pulse. State 4 respiration was recovered when all the ADP is consumed and was equal to the state 4 preceding ADP pulse. This proved that the state 4 respiration after A-R is not a state 3 like respiration. Respiration with saturating amount (2.5 mM) of ADP (V_{3s}) was measured and was equal to the state 3 respiration measured during ADP pulse. Then the respiration with oligomycin (V_{olig}) and the uncoupled respiration (V_{FCCP}) were measured (Fig. 1, right part).

The respiratory time course made in the presence of POBN-ethanol (EPR spin trapping system, see Materials and Methods) allowed to sample mitochondria for EPR

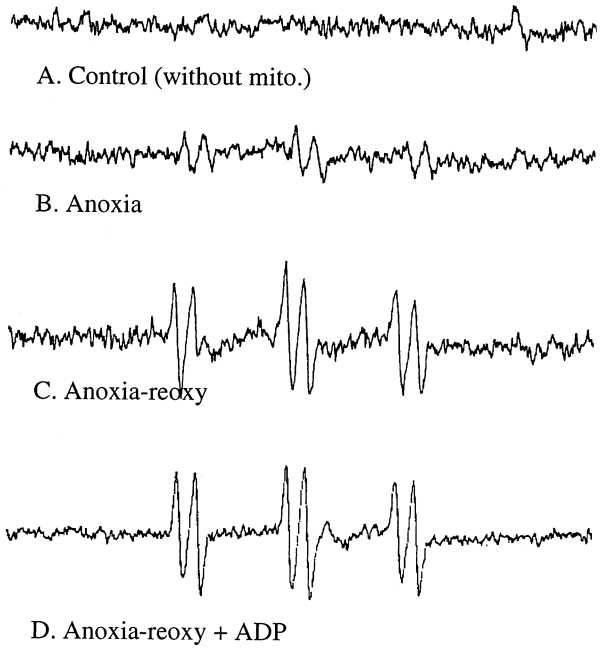

Fig. 2. EPR spectra obtained with POBN/EtOH as spin trap. Mitochondria (10 mg protein/ml) were incubated in the medium containing KG 5 mM and PYR 5 mM as oxidizable substrates. Measuring conditions are described in Materials and Methods. Spectrum A was obtained with the medium containing POBN/EtOH (without mitochondria), spectrum B with the sample taken out after respiration preceding anoxia (S1 in Fig. 1) and spectra C and D correspond to samples taken out after reoxygenation during respiration without or with ADP (S2 and S3 in Fig. 1), respectively. See Materials and Methods for details in procedure.

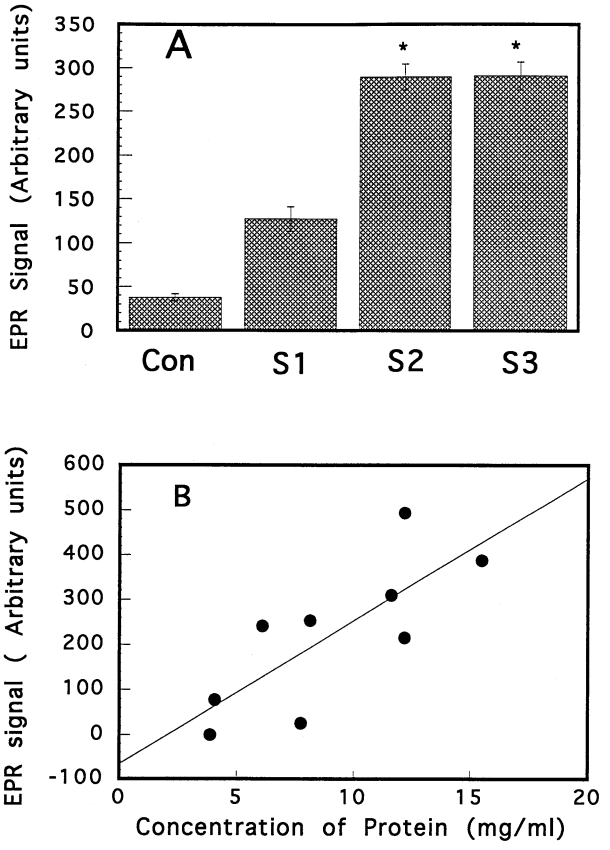

Fig. 3. Production of superoxide anion by mitochondria measured after anoxia-reoxygenation *in vitro*. Mitochondria (10 mg protein/ml) incubated in the medium containing KG 5 mM and PYR 5 mM as oxidative substrates. (A) EPR signal and (B) the relationship between EPR and the amount of mitochondria. EPR signal indicated in arbitrary units (mean ± SD, $n = 6–8$). *$p < .05$ compared with anoxia. Control, incubation medium with POBN/EtOH. S1, S2, and S3 as defined in text.

measurements: when reaching anoxia in state 4 respiration (S1); after 10 min anoxia plus reoxygenation with respiration in state 4 (S2) and in the state 3 (S3) (Fig. 1). Respiratory parameters before anoxia were not modified by the presence of spin trapping system as an example of the ADP/O ratio remains exactly the same (see Fig. 7C, left part and below).

Fig. 2 shows EPR spectra obtained from four different samples: (A) medium contains POBN and EtOH without mitochondria, (B) after the first respiration phase preceding anoxia (Fig. 1, S1), (C) during respiration in state 4 after reoxygenation (without ADP, Fig. 1, S2), and (D) during respiration in state 3 after reoxygenation (Fig. 1, S3, with ADP). It appears (Fig. 2 and Fig. 3A) that a small amount of superoxide anion was produced by mitochondria during ADP pulse respiration preceding anoxia. This confirmed that respiring mitochondria produce superoxide anion in "unstressed" conditions. After reoxygenation, a significant increase in the EPR signal was observed independent from the presence or the absence of ADP phosphorylation. This increase of the signal indicates that superoxide anion production is raised during postanoxic reoxygenation (mitochondria respiring in state 4) and that this overproduction is almost not affected by the respiratory state (± ADP) of the post-reoxygenation respiration period.

Relationship between superoxide production and amount of mitochondria

As superoxide anions are produced at the level of Q redox cycle of the respiratory chain, this production after anoxia/reoxygenation should be related with the amount of mitochondria protein. Indeed, if the amount of mitochondria was increased, more superoxide anions were generated as visualized by the size of EPR signal (Fig. 3B). A rough linear relationship was observed showing that, even if it is not an enzymatic process [22], the level of production of superoxide anion was depending on the amount of respiratory chains and that the trapping system was not saturated.

Influences of anoxia and reoxygenation on the respiratory parameters of mitochondria

A small production of superoxide anion during respiration is believed not to be toxic because their effects are prevented by endogenous antioxidant defenses. It is often proposed that overproduction of superoxide anion can overwhelm the defense processes and cause damage at the level of mitochondria.

In the in vitro experimental model described (Fig. 1) mitochondrial function could be checked before and after a burst of superoxide anion production, i.e., before and after anoxia-reoxygenation. Various respiratory parameters have been measured: V_3 (phosphorylating respiration during ADP pulse), V_4 (resting respiration when ADP is exhausted), V_{olig} (respiration in the presence of oligomycin, the proton translocation inhibitor of F_0 F_1 synthase, not shown in Fig. 1 before A-R), V_{FCCP} (the respiration in the presence of the protonophore FCCP that permeabilizes the membrane to H^+, not shown in Fig. 1 before A-R), V_{3s} (the phosphorylating respiration with saturating 2.5 mM ADP, not shown in Fig. 1 before A-R), and ADP/O the stoechiometric ratio relating the number of ADP phosphorylated per oxygen atom consumed. Fig. 1 illustrates a typical respiratory time course including anoxia-reoxygenation period. It could be seen at a glance by comparing ADP pulse shape on the slope curve (lower curve) that anoxia-reoxygenation impaired oxidative phosphorylation: V_3 was decreased, V_4 was increased, the amount of oxygen consumed to phosphorylate 330 nmol of ADP was increased (peak area before A-R was smaller than after A-R) then ADP/O ratio was decreased. Likewise, V_{3s} and V_{FCCP} decreased but V_{olig} remained unchanged (not shown in Fig. 1 before A-R). Detailed quantitative description is given in Figs. 4–6, for two types of oxidizable substrates, α-ketoglutarate + pyruvate as NADH related substrates and succinate + rotenone as FADH related substrate.

Fig. 4 shows a significant decrease of the ADP/O ratio (21%, from 2.22 ± 0.15 to 1.75 ± 0.08) with α-ketoglutarate + pyruvate as substrates (Fig. 4A) after anoxia-reoxygenation whatever be the duration of anoxia (1 or 10 min). With succinate (Fig. 4B) the same damage was observed: mean value of ADP/O decreases from 1.35 ± 0.16 to 0.92 ± 0.17 (1 min) or to 0.98 ± 0.05 (10 min) (i.e., 27% or 32%). Therefore, the efficiency of the oxidative phosphorylation decreased with both sources of electrons and significantly was not related to the duration of anoxia.

Figs. 5 and 6 show the impairment occurring at the level of various respiratory rates after A-R with NADH-related substrates (Fig. 5) and succinate plus rotenone (Fig. 6). Similar results were obtained with both reducing equivalent sources namely: (i) weak decrease of V_3 during ADP pulse (10–12%), (ii) significant decrease of V_{3s} (20–25%) and V_{FCCP} (30–32%), (iii) important increase in V_4 (30 and 80% for KG and SUCC), and (iv) not significant decrease in V_{olig} (12–16%).

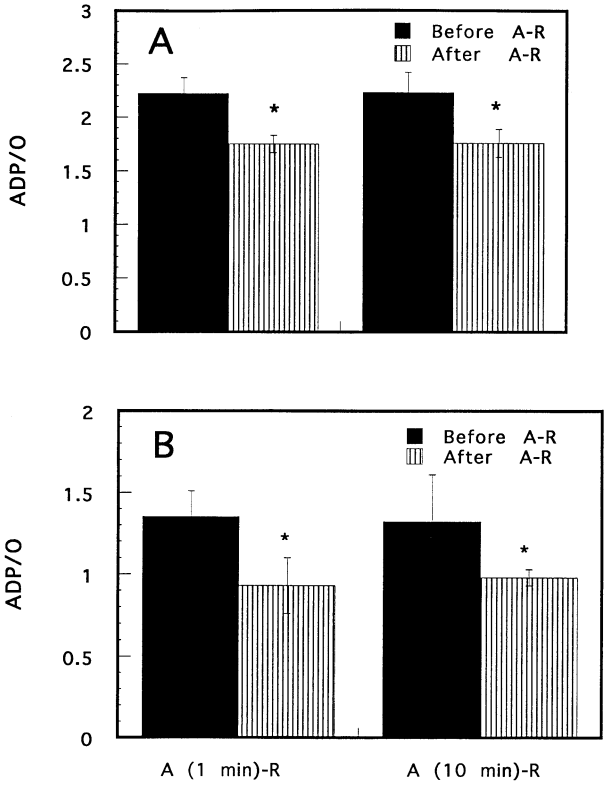

Fig. 4. Damage of mitochondrial oxidative phosporylation efficiency (ADP/O) induced by anoxia and reoxygenation. (A) Mitochondria (1 mg protein/ml) incubated in the medium containing KG 5 mM and PYR 5 mM as oxidizable substrates, and (B) Mitochondria (1 mg protein/ml) incubated in the medium containing SUCC 5 mM as oxidizable substrate and plus ROT (2.5 μg/ml). n = 5–7, mean ± SD, *$p < .01$ compared with ADP/O before A-R. A(1 min)-R: 1 min anoxia followed by reoxygenation and A(10 min)-R: 10 min of anoxia followed by reoxygenation.

Therefore, it seems that a burst of superoxide anion production and the appearance of functional damage were observed simultaneously when isolated mitochondria were submitted to A-R. The functional damage could be due to "in vitro aging" of mitochondria [23]. To check this possibility the same respiratory time course as that one described in Fig. 1 could be carried out in a way that O_2 was never depleted in the open respiratory chamber. Mitochondria received several ADP pulses, then respired in state 4 until the chamber was closed and V_4, V_3, ADP/O, V_{3s}, V_{olig}, and V_{FCCP} were measured (Fig. 7A). No significant impairment was observed in these conditions (Fig. 7A, right part) on the contrary to anoxia-reoxygenation condition (Fig. 7B, right part). Therefore, damage observed after A-R are not due to in vitro aging

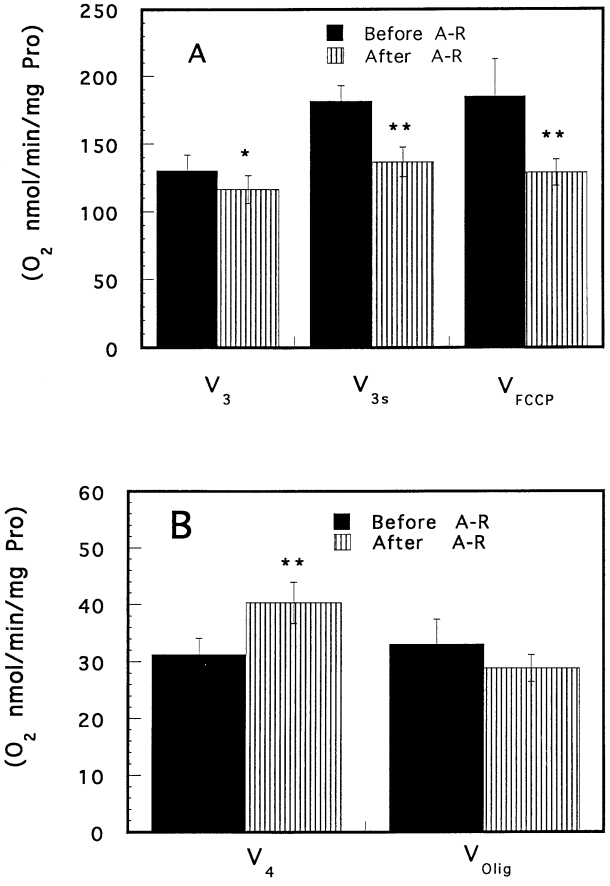

Fig. 5. Influence of anoxia-reoxygenation on NADH related respiratory rate of mitochondria. (A) shows the activities of state 3 respiration and respiration with uncoupler (FCCP). (B) Shows the activities of state 4 respiration and respiration with oligomycin. $n = 5–7$, mean ± SD, $*p < .05$ and $**p < .01$ compared with respiratory parameters before A-R.

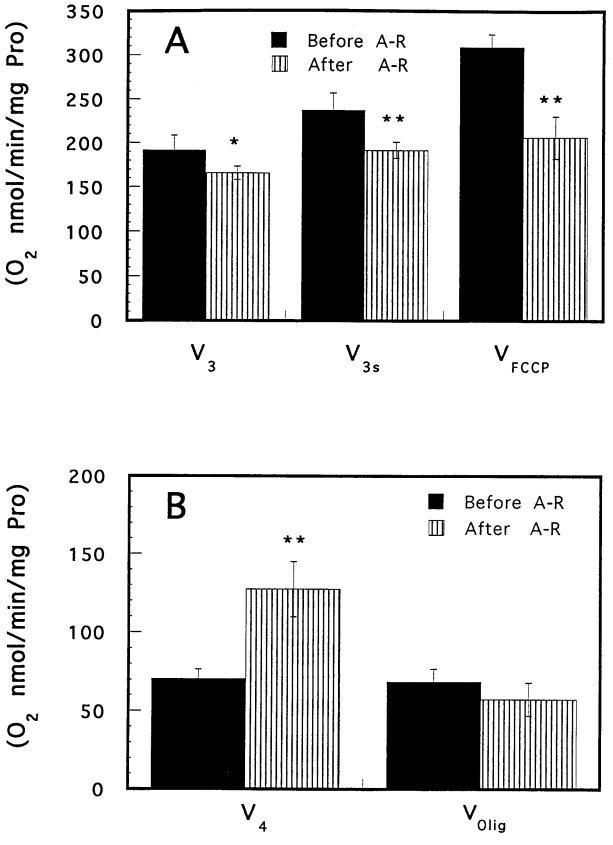

Fig. 6. Influence of anoxia-reoxygenation on the respiratory parameters of mitochondria respiring with succinate plus rotenone. (A) The activities of state 3 respiration and respiration with uncoupler (FCCP). (B) The activities of state 4 respiration and respiration with oligomycin. $n = 5–7$, mean ± SD, $*p < .05$ and $**p < .01$ compared with respiratory parameters before A-R.

of the mitochondria but to oxygen deprivation followed by reoxygenation.

However, it was not because simultaneous production of superoxide anion production and occurrence of functional damage are observed that cause/effect relationship actually exists. If functional damage is secondary to superoxide anion burst production during anoxia/reoxygenation and as POBN ethanol superoxide anion trapping system is not impairing oxidative phosphorylation, then protective effect of POBN-ethanol on functional parameters after anoxia-reoxygenation should be observed. This protection is illustrated in Fig. 7C where ADP pulses before and after anoxia-reoxygenation had almost the same shape (rate curve): V_3 decreased less, V_4 increased less, and ADP/O was the same compared to Fig. 7B. Therefore, a partial protection of mitochondrial function was observed with the trapping system POBN-ethanol. That suggested that functional damage was related and secondary to superoxide anion production by the mitochondria in vitro.

DISCUSSION

According to the chemiosmotic concept the mitochondrial respiration and ATP synthesis are coupled by a proton electrochemical gradient across the mitochondrial membrane. This coupling is built on stoechiometric rules resulting in stoechiometric ratio H^+/O, H^+/ATP, ADP/O, and on the low proton conductivity of the membrane (negligible H^+ leak). The last property has three consequences: (i) the oxygen consumption is tightly controlled by H^+ re-entry into the matrix; (ii) the addition of protonophore (as FCCP) that permeabilizes the membrane to H^+ destroys this control and leads to maximal respiratory rates; (iii) the full inhibition of ATP synthase by oligomycin (H^+-translocation inhibitor in F_0F_1 ATPase) reduces respiration to a residual rate due to H^+ leak (phospholipid membrane H^+ conductivity).

Then, inspection of the respiratory parameters described and of their changes allows to identify and to localize damage induced by A-R. An increase of V_{olig} informs of increase of H^+ leak, i.e., of membrane proton

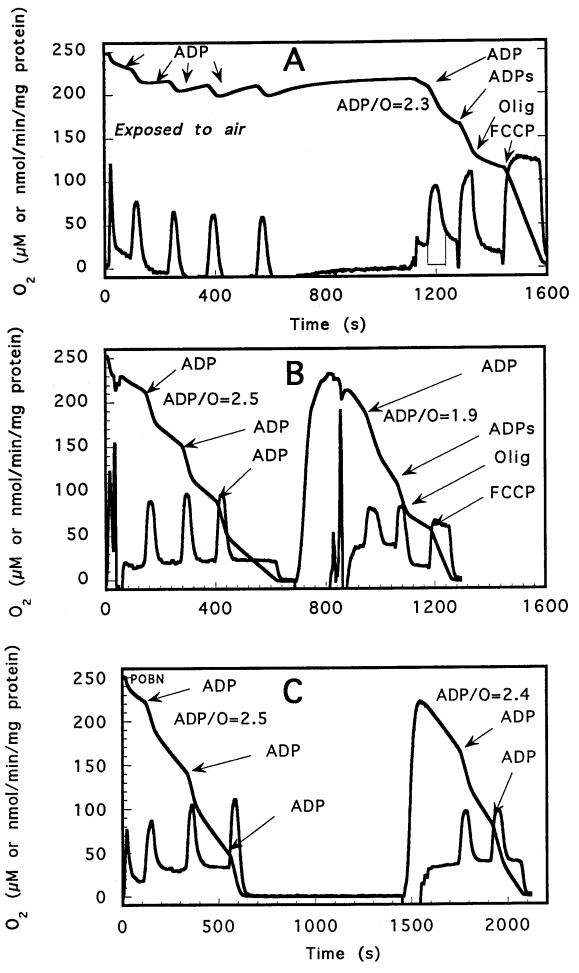

Fig. 7. Oxygen traces and respiratory rates of mitochondria under different conditions. Mitochondria (1 mg protein/ml) incubated in the medium containing KG 5 mM and PYR 5 mM as oxidizable substrates. (A) Time control, mitochondria incubated in the medium exposed to air and the oxygen concentration is kept about 80% of air saturated medium. (B) Mitochondria respiration before and after anoxia-reoxygenation. (C) Effects of POBN-EtOH on the respiration of mitochondria before and after anoxia.

conductivity, therefore, of phospholipid bilayer damage as well as of permeability transition pore opening. If V_4 but not V_{olig} increases, membrane is not damaged but the intrinsic H^+ stoechiometry of the ATP synthase is impaired (H^+ slip, i.e., intrinsic uncoupling of ATP synthase). When V_{3s} and V_{FCCP} decrease similarly either the respiratory chain enzymes are damaged or the upstream feeding with electrons is limiting (oxidizable substrate availability in the matrix). However, if V_{3s} decreases but not V_{FCCP}, there are no oxidative enzyme impairment or substrate shortage but a decrease in ADP availability in the matrix. Proton leak and proton slips (intrinsic uncoupling of respiratory chain redox pump and/or ATP synthase) lead to a decrease of the ADP/O ratio, then to a loss in the efficiency of the oxidative phosphorylation.

Anoxia-reoxygenation resulted in a set of respiratory parameter modifications: decrease of V_3, V_{3s}, V_{FCCP}, ADP/O ratio, increase of V_4 but no change in V_{olig}. According to the analytic scheme presented, these changes mean no damage at the level of the membrane itself (no H^+ leak), impairment of respiratory chain enzymes (or of substrate translocators), intrinsic uncoupling of ATP synthase, and partial loss of the efficiency of the oxidative phosphorylation. Both NADH related and succinate (plus rotenone) oxidative phosphorylation are disturbed in the same way.

This damage is directly related to A-R as shown by the time control experiment that excludes in vitro aging of the mitochondria. The duration of the anoxia (between 1 to 10 min) does not influence the extent of the damage. This indicates that the lesion occurs during the reoxygenation phase of the time course, i.e., at the same time as the superoxide anions are likely to be produced. Indeed, superoxide anion is produced, at a low level during the preanoxic respiration, but at a higher level during postanoxic respiration whether ADP is present (state 3 respiration) or not (state 4 respiration). This last observation could support the possibility that superoxide anion is produced mostly during reoxygenation (burst).

Several reports attribute to Ca^{2+} influx into the matrix some of the damage observed during anoxia or hypoxia-reoxygenation [24], as well as the production of reactive oxygen species itself by the respiratory chain [25]. It must be stressed that our mitochondria were prepared in the presence of 0.5 mM EDTA (see Material and Methods) in order to decrease calcium as much as possible. Nevertheless, endogenous calcium content (Ca^{2+}, which can be released during additional incubations) remains between 7 and 15 nmol Ca^{2+}/mg of protein according to Vercesi [26]. Taking into account the composition of the incubation medium that contained 1 mM EDTA and 5 mM $MgCl_2$, the concentration of free calcium in the system was calculated applying the computer program described by Fabiato et al. [27], making the assumption that all the endogenous calcium is released by the mitochondria at a given moment. The estimated range of free Ca^{2+} concentration in the incubation medium was 0.3 to 0.45 μM, which is far below the free Ca^{2+} concentrations that could be responsible (due to its reuptake) for the damage observed during anoxia-reoxygenation in our experiments. Indeed, due to the K_m of Ca^{2+} uptake and the presence of 5 mM Mg^{2+}, it would take a long time to reach a harmful internal concentration. Therefore, it can be almost excluded that the functional damage observed in our in vitro model (i.e., the increase in state 4 respiration after reoxygenation) is due to Ca^{2+} influx.

Superoxide anion production and functional impairments are observed simultaneously. Moreover, a cause/effect relationship exists as shown by the POBN-ethanol

protection on phosphorylation damage. The latter seems to be secondary to the harmful production of superoxide anion during reoxygenation after the anoxia period that had overreduced the electron carriers of the respiratory chain, particularly ubiquinone. It may be proposed that anoxia leads to a high level of ubiquinol and that under reoxygenation a burst of superoxide anion is produced through ubisemiquinone leading to the mitochondrial function impairment.

As a conclusion, mitochondrial function impairment due to anoxia-reoxygenation in vitro has been described and localized, related to superoxide anion production during reoxygenation, and proposed to be secondary to the superoxide anion production. The dysfunctions look to be exclusively linked to protein damage, probably oxidation of thiol groups producing cross-linkage, which affect their activity as no membrane H^+-conductivity increase has been observed (no increase in H^+ leak). Therefore, the results reported in this article support the view that tissue injuries caused by ischemia-reperfusion could be related to oxygen free radicals generated mainly at the level of the mitochondria and that mitochondrial enzymes are the first targets of these harmful species leading to oxidative phosphorylation deficiency. Then mitochondria would not be able to restore the cellular energetic potential and the cell would die.

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ABBREVIATIONS

ADP—Adenosin-5′-diphosphate
A-R—anoxia-reoxygenation
EPR—electron paramagnetic resonance
FCCP—Carbonylcyanide p-trifluoro-methoxyphenyl-hydrazone
KG—α-ketoglutarate
Olig—Oligomycin
PYR—pyruvate
ROT—Rotenone
SUCC—Succinate
V_3, V_4, $V_{3s,}$ V_{FCCP} and V_{olig} express the states of respiration—state 3, state 4, state 3 with saturating ADP, uncoupled respiration and respiration with oligomycin, respectively.