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Original Contribution

ARACHIDONIC ACID INTERACTION WITH THE MITOCHONDRIAL ELECTRON TRANSPORT CHAIN PROMOTES REACTIVE OXYGEN SPECIES GENERATION

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Abstract—A study has been carried out on the interaction of arachidonic acid and other long chain free fatty acids with bovine heart mitochondria. It is shown that arachidonic acid causes an uncoupling effect under state 4 respiration of intact mitochondria as well as a marked inhibition of uncoupled respiration. While, under our conditions, the uncoupling effect is independent of the fatty acid species considered, the inhibition is stronger for unsaturated acids. Experiments carried out with mitochondrial particles indicated that the arachidonic acid dependent decrease of the respiratory activity is caused by a selective inhibition of Complex I and III. It is also shown that arachidonic acid causes a remarkable increase of hydrogen peroxide production when added to mitochondria respiring with either pyruvate+malate or succinate as substrate. The production of reactive oxygen species (ROS) at the coupling site II was almost double than that at site I. The results obtained are discussed with regard to the impairment of the mitochondrial respiratory activity as occurring during the heart ischemia/reperfusion process. © 1999 Elsevier Science Inc.

Keywords—Mitochondria, Respiratory chain, Uncoupling, Site specific inhibition, Arachidonic acid, Free fatty acids, Hydrogen peroxide, ROS, Free radicals

INTRODUCTION

Much attention has recently been addressed to the study of the role and the effects of long chain free fatty acids (FFA) on mitochondrial oxidative phosphorylation. Although the major part of these molecules is associated with fatty acid binding proteins, a small proportion occurs freely in the cell, so that, according to their actual concentration, FFA can exert a series of effects on energy conserving respiratory system. We have recently found that submicromolar concentrations of arachidonic acid give rise to a substantial reactivation of (ΔpH-depressed proton pumping activity in liposome reconstituted bovine complex III (the bc_1 complex) [1]. These experiments, besides indicating the involvement of intrinsic acidic residue(s) in the pump mechanism of the bc_1 complex, also suggest a supportive role of endogenous fatty acids in the proton translocation process. Similar conclusions were also attained by Sharpe et al. [2], who showed that the cytochrome *c* oxidase proton pumping activity was lost after extensive incubation of proteoliposomes with bovine serum albumin (BSA), but was restored upon incubation of BSA-depleted proteoliposomes with fatty acids.

The interaction of micromolar concentrations of fatty acid with mitochondrial membranes, as revealed mainly by experiments on brain mitochondria, consists of an increase of state 4 respiration, decrease of state 3, or uncoupler stimulated respiration (state 3u) and decrease of the respiratory control ratio (RCR) [3]. While the stimulation of state 4 respiration by FFA (the uncoupling effect) appears to be due to their protonophoric activity, with the involvement in the process of the ATP/ADP antiporter [4] as well as the aspartate/glutamate antiporter [5], the mechanism by which they inhibit state 3 respiration still remains an open question.

This effect is of particular interest when studied in connection to tissue ischemia. It was in fact found that

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accumulation of unesterified arachidonic acid takes place in ischemic myocardium [6,7] and brain [8]. Importantly, restoration of blood flow results in further accumulation of arachidonic acid in the heart [6]. A significant increase of long chain FFA was measured in mitochondrial preparation from rat brains subjected to ischemia and this was correlated with an increase of state 4 and marked inhibition of state 3 respiration [9,10]. Furthermore, arachidonic acid, at concentrations relevant to celebral ischemia, caused a strong inhibition of ADP- and uncoupler-supported respiration in brain mitochondria [11,12].

Because a large body of evidence has shown that an excess of reactive oxygen species (ROS), generated within mitochondria, are released during ischemia/reperfusion of various tissues [13–19], the question then arises whether unesterified fatty acid, and arachidonic acid in particular, may mediate ROS production in mitochondria. This is the subject of the present work.

Here we report that arachidonic acid interaction with intact bovine heart mitochondria, while causing uncoupling as well as inhibition of uncoupled respiration, does indeed promote hydrogen peroxide production at the steady-state respiration, with either pyruvate+malate or succinate as substrate. Thus, a causal relationship appears to be envisaged between tissue ischemic injury and arachidonic acid accumulation that causes, in turn, ROS production with consequent mitochondrial dysfunction.

MATERIALS AND METHODS

Chemicals

2′7′-dichlorofluorescin-diacetate was obtained from Estman Kodak (Rochester, NY). Catalase, phenylmethanesulfonylfluoride (PMSF), bovine serum albumin (BSA), antimycin A, rotenone, 2,6-dichlorophenolindophenol (DCPIP), decyl-ubiquinone, and horse-heart cytochrome *c* (type VI) were obtained from Sigma Chemical Co. (St. Louis, MO). Myxothiazol and horseradish peroxidase (POD) were from Boehringer, Mannheim (Germany). All other reagents were of the highest purity grade commercially available.

Isolation of mitochondria

Mitochondria were isolated from bovine heart following the basic procedure by Burnette and Batra [20] with some modification. The modifications to the basic procedure for the preparation of beef heart mitochondria were performed by A. M. Sardanelli (unpublished work). The ventricles, quickly excised, were placed in ice-cold 0.25 M sucrose, pH 7.4. The tissue was then finely minced and homogenized in 10 volumes of isolation medium containing 0.25 M sucrose, 10 mM TRIS-Cl, pH 7.4, 1 mM EGTA, and 0.25 mM PMSF. The homogenate was centrifuged at 1200 $\times$ *g* for 10 min. The resulting supernatant was centrifuged at 9500 $\times$ *g* and the pellet, resuspended in the same buffer, was centrifuged again at 14,000 $\times$ *g* for 10 min. The final pellet was washed gently to remove any light or loosely packed damaged mitochondria and resuspended in the buffer described above.

Measurement of oxygen consumption rate

The respiratory activity of freshly prepared bovine heart mitochondria was measured polarographycally with a Clark-type electrode, in an all-glass reaction chamber, at 25°C. Mitochondrial proteins (0.1 mg) were suspended in a medium containing 50 mM KCl, 75 mM sucrose, 30 mM TRIS-Cl, pH 7.4, 1 mM K-phosphate, 0.5 mM EDTA, and 1 mM $MgCl_2$ (final volume 1.6 ml). State 4 respiration was started by the addition of pyruvate (3.5 mM)/malate (1.7 mM) or succinate (7 mM) in the presence of 1 μg/ml rotenone. Uncoupled state respiration was obtained by adding 1.5 μM CCCP. When BSA was used, the concentration of the uncoupler CCCP was doubled.

Measurement of redox activities of the respiratory enzymes

All enzyme activities were measured spectrophotometrically with a double-beam, dual-wavelength Johnson Research Foundation spectrophotometer by suspending 0.1 mg protein of mitochondrial particles, prepared by freezing and thawing (three times) of isolated mitochondria, in a medium containing 50 mM K-phosphate buffer, pH 7.4, and 25 μM EDTA (final volume 1.6 ml), at 25°C.

NADH-cytochrome *c* oxidoreductase (complex I+III) activity was assayed by measuring the rotenone-sensitive initial rate of cytochrome *c* reduction at 550–540 nm ($\Delta\varepsilon = 19.1\ \mathrm{mM}^{-1} \cdot \mathrm{cm}^{-1}$). The basic reaction mixture was supplemented with 30 μM NADH and 1.5 mM KCN. The reaction was started by the addition of 8 μM ferricytochrome *c*.

Succinate-cytochrome *c* oxidoreductase (complex II+III) activity was measured at 550–540 nm as succinate-supported, antimycin-sensitive, initial rate of cytochrome *c* reduction. The basic reaction mixture also contained 7 mM succinate, 1.5 mM KCN, and 1 μg/ml rotenone. The reaction was started by the addition of 8 μM ferricytochrome *c*.

NADH-CoQ oxidoreductase (complex I) was assayed by following the rotenone-sensitive initial rate of NADH oxidation at 360–374 nm ($\Delta\varepsilon = 2.3\ \mathrm{mM}^{-1} \cdot \mathrm{cm}^{-1}$). The

basic reaction medium also contained 30 μM NADH, 1.2 μM antimycin A, 1.5 mM KCN. The reaction was initiated by adding 56 μM decyl-ubiquinone.

Succinate-CoQ oxidoreductase (complex II) activity was measured by following the reduction of 2,6-dichlorophenolindophenol (DCPIP) at 600 nm ($\Delta\varepsilon$ = 19.1 $mM^{-1} \cdot cm^{-1}$). The basic reaction medium also contained 1 μg/ml rotenone, 1.2 μM antimycin A, 1.5 mM KCN, 50 μM DCPIP, and 10 mM succinate. The reaction was initiated by adding 56 μM decyl-ubiquinone.

Ubiquinol-cytochrome *c* oxidoreductase (complex III) activity was determined following at 550–540 nm the initial rate of antimycin-sensitive cytochrome *c* reduction upon addition of 10 μM reduced decyl-ubiquinone. The reduced quinone was obtained from the oxidized form following the procedure described by Trumpower and Edwards [21]. The reaction medium also contained 1 μg/ml rotenone, 1.5 mM KCN, and 8 μM ferricytochrome *c*.

Cytochrome *c* oxidase (complex IV) activity was estimated from the initial rate of ferrocytochrome *c* oxidation at 550–540 nm. The reaction mixture was supplemented with 9 μM ferrocytocrome *c* and 1.2 μM antimycin A. The reaction was started by the addition of mitochondrial particles.

Measurement of H_2O_2 production

The rate of mitochondrial hydrogen peroxide production was estimated by measuring the linear fluorescence increase (excitation at 475 nm, emission at 525 nm) caused by the H_2O_2-dependent oxidation of dichlorofluorescin (DCFH), to the fluorescent compound dichlorofluorescein in the presence of horseradish peroxidase (POD) [22]. Immediately prior to determinations DCFH was obtained from the stable reagent dichorofluorescin-diacetate (DCFH-DA) by alkaline treatment [22].

Mitochondrial proteins (0.1 mg) were suspended in a medium containing 225 mM mannitol, 75 mM sucrose, 30 mM TRIS-Cl, pH 7.4, 1.5 μM CCCP, 0.4 μM POD, and 5 μM DCFH (final volume 2.5 ml). Pyruvate/malate or succinate were used as substrates at the same concentrations used in oxygen consumption assays.

Conversion of fluorescence units to nmol of H_2O_2 produced was performed by measuring the fluorescence changes upon addition of known amounts of H_2O_2.

RESULTS

The effect of FFA on oxygen consumption by bovine heart mitochondria was examined using pyruvate+malate or succinate as substrates. As shown in Fig. 1, addition of low concentrations of arachidonic acid (up to 2.5 μM) to mitochondrial suspension under substrate supported respiration, caused, in state 4, a substantial increase of the respiratory rate with both pyruvate+malate (Fig.1A) and succinate (Fig. 1B). By increasing the arachidonic acid concentration further, the respiration rate started to decrease to values well below the controls. Oxygen consumption measured in uncoupled state (lower panels of Fig. 1) was progressively inhibited by arachidonic acid. The I_{50} value, the concentration of arachidonic acid causing 50% inhibition, was around 3 and 9 μM with pyruvate+malate and with succinate as substrate, respectively (Table 1). As a result of these effects, arachidonic acid caused a drop of the RCR for both substrates to a value of 1 (complete uncoupling). Several other FFA were tested on the oxygen consumption activity. For comparison the effect of palmitic acid is reported here (Fig. 1, Table 1). Palmitic acid was a weaker inhibitor of the uncoupled rate of oxygen consumption. However, the apparent ED_{50} value for palmitic acid (the concentration giving half-maximal respiration increase) was comparable to that for arachidonic acid. Oleic and linoleic acids had an effect on mitochondrial respiration very similar to that produced by arachidonic acid (not shown).

It was of interest to test the effect of BSA, for which contrasting results have been reported [10,12,23]. Under our experimental conditions, the effect of BSA was dependent on both the respiratory substrate used and the arachidonic acid concentration. With succinate as substrate the effect of any concentration used of arachidonic acid on either state 4 or uncoupled state respiration was readily reversed by 15 μM BSA added to the mitochondrial suspension 2–4 min after the arachidonic acid addition. With pyruvate+malate as substrate, BSA reversed up to a concentration of 20 μM arachidonic acid. Beyond these concentrations, the inhibition by arachidonic acid could not be removed.

Because several mechanisms, including substrate transport, are possibly involved in the inhibition of respiratory activity by FFA [3], attempts were made to measure in mitochondrial particles the individual redox system activities. The effect of 40 μM arachidonic acid on individual redox reactions of mitochondria is reported in Fig. 2. While succinate-Q reductase (complex II) and cytochrome *c* oxidase (complex IV) activities remained unaffected, all of the others were inhibited by arachidonic acid. In particular, the rotenone-sensitive NADH- Q reductase (complex I) and NADH-cytochrome *c* reductase (complex I+III) activities were inhibited to roughly the same extent, consistently with the decrease of pyruvate+malate supported respiration. Similarly, the succinate-cytochrome *c* reductase (complex II+III) activity de-

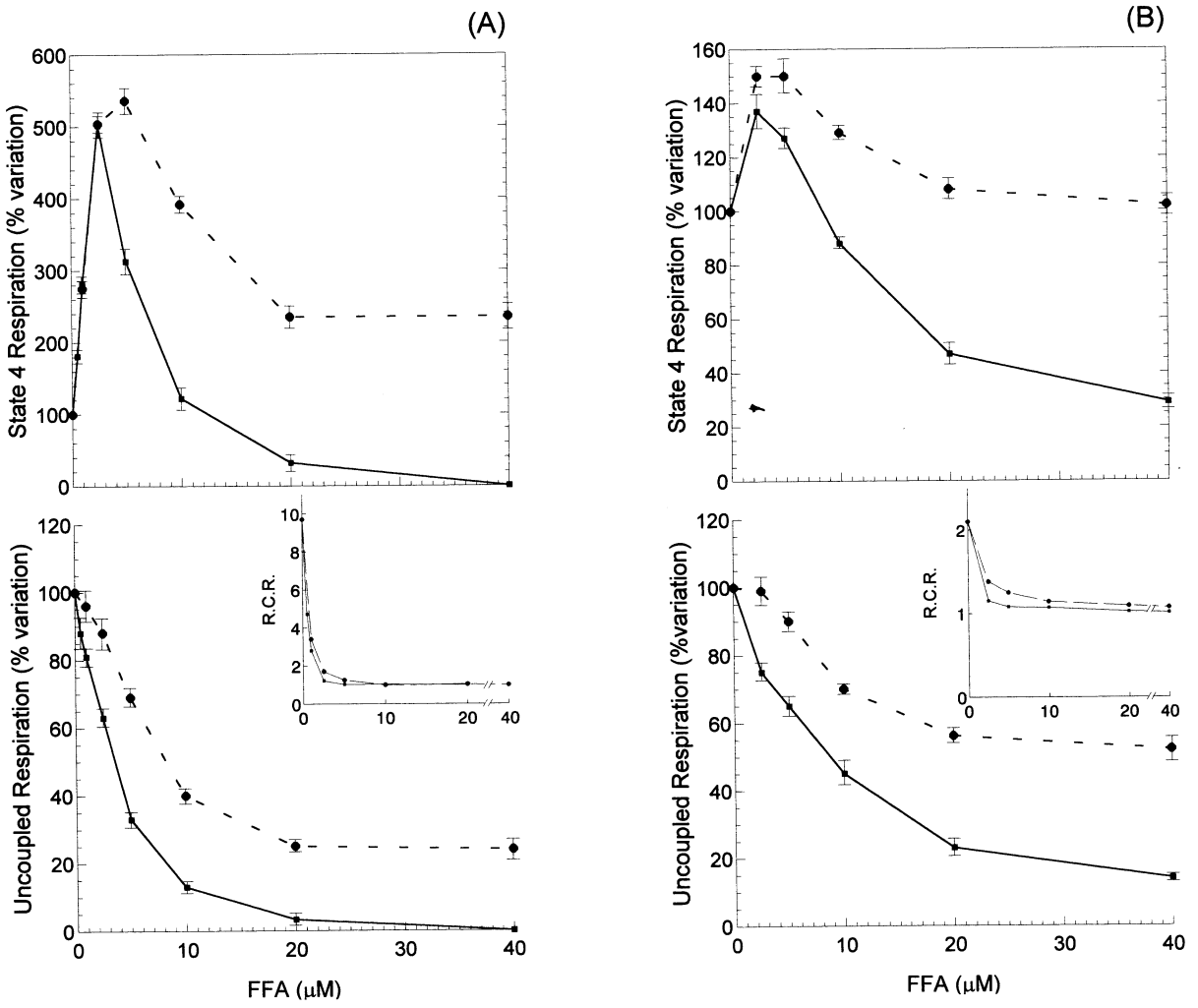

Fig. 1. Effect of arachidonic and palmitic acid on mitochondrial respiration. Freshly prepared mitochondria were suspended in the reaction medium described in the Materials and Methods section. State 4 respiration was started by the addition of the substrate pyruvate + malate (A) or succinate (in the presence of rotenone) (B), followed by 1.5 μM CCCP (uncoupled respiration). RCR value represents the ratio of uncoupled versus state 4 respiration rate. Fatty acids were added as ethanolic solutions. Control values for state 4 and uncoupled respiration were 10.4 $\pm$ 2.2 and 101 $\pm$ 12.7 nmol $O_2 \cdot min^{-1} \cdot$ mg $prot^{-1}$, respectively, with pyruvate/malate as substrate, and 50.2 $\pm$ 3.5 and 105.4 $\pm$ 4.1 nmol $O_2 \cdot min^{-1} \cdot$ mg $prot^{-1}$ with succinate. The values reported represent the mean $\pm$ SD of measurements from three to six different experiments. Arachidonic acid (solid line); palmitic acid (dashed line). For other experimental conditions see the Materials and Methods section.

creased to the same extent as did the succinate oxidase activity of intact mitochondria (Fig. 1). The activity of QH_2-cytochrome *c* reductase decreased less than others, being inhibited by around 35%. It has to be noted that, contrary to what was found here in the heart, 50 μM arachidonic acid inhibited among all others only NADH-Q reductase activity in brain mitochondria [23]. Again, palmitic acid exerted a much weaker effect on the redox reactions considered here (Fig. 2). It appears, however, that pyruvate+malate supported respiration was inhibited more than NADH-Q or NADH-cytochrome *c* reductase activities. This suggests that FFA might also inhibit substrate transport systems as well as the primary dehydrogenases [3].

FFA-promoted H_2O_2 production

In order to approach this study properly, it was necessary to settle preliminarily the experimental conditions for substrate dependence and site specificity of FFA-promoted hydrogen peroxide production in intact bovine heart mitochondria. As shown in Fig. 3, addition of antimycin to succinate respiring mitochondria (trace a) promoted hydrogen peroxide production [24–28]. When rotenone was added before antimycin to prevent backflow of electrons to complex I site, the mixothiazol sensitive H_2O_2 production rate decreased to 65% (trace b). This indicated, as suggested previously [27,28], that H_2O_2 production in succinate re-

Table 1. Uncoupling and Inhibition by Fatty Acids of Heart Mitochondrial Respiration

	ED_{50} (μM)		I_{50} (μM)	
	$C_{20:4}$	$C_{16:0}$	$C_{20:4}$	$C_{16:0}$
Pyruvate+malate	1.3 ± 0.1	1.4 ± 0.1	3.5 ± 0.3	8.5 ± 0.5
Succinate	1.3 ± 0.1	1.3 ± 0.1	9.0 ± 0.5	>40

ED_{50} and I_{50} represent the fatty acid concentration giving 50% increase of state 4 respiration and 50% inhibition of uncoupled respiration, respectively. The data are taken from the experiments reported in Fig. 1. $C_{20:4}$ = arachidonic acid; $C_{16:0}$ = palmitic acid.

spiring mitochondria occurs at both coupling site I and II. The rate of H_2O_2 production measured under the latter condition was thus taken as the maximal potential rate of H_2O_2 generation at site II.

Addition of 30 μM arachidonic acid to succinate respiring mitochondria, in the presence of rotenone, gave rise to H_2O_2 production, whose rate amounted to around 50% of that measured in the presence of antimycin. Interestingly, subsequent addition of antimycin did not cause hydrogen peroxide to be generated at the rate measured in the absence of arachidonic acid, neither did myxothiazol suppress completely the arachidonic acid promoted H_2O_2 production. However, in all the conditions used, the fluorescence increase signal was over 90% sensitive to catalase. Importantly the addition of arachidonic acid before succinate did not cause per se ROS to be produced at an appreciable rate (trace d).

With pyruvate+malate as substrate, the maximal potential rate of H_2O_2 production was taken as that measured after the addition of rotenone to respiring intact mitochondria (trace a'). The addition of arachidonic acid

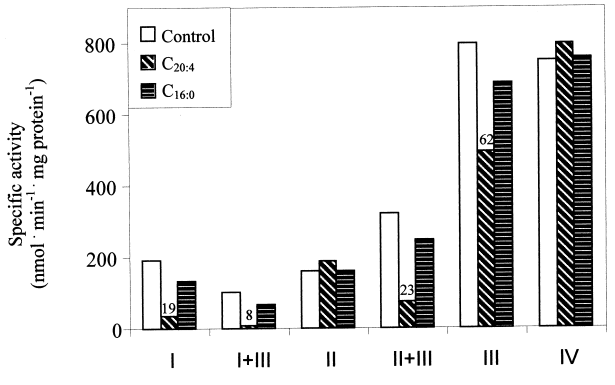

Fig. 2. Effect of FFA on the activity of respiratory enzymes. The determinations of enzyme activities were performed as reported in the Materials and Methods section, using 0.1 mg of bovine heart mitochondrial particles. Where indicated, 40 μM arachidonic acid (20:4) and palmitic acid (16:0) were present. The data are the mean of three different experiments. Figures on the columns refer to the remaining activities, as percent of the control.

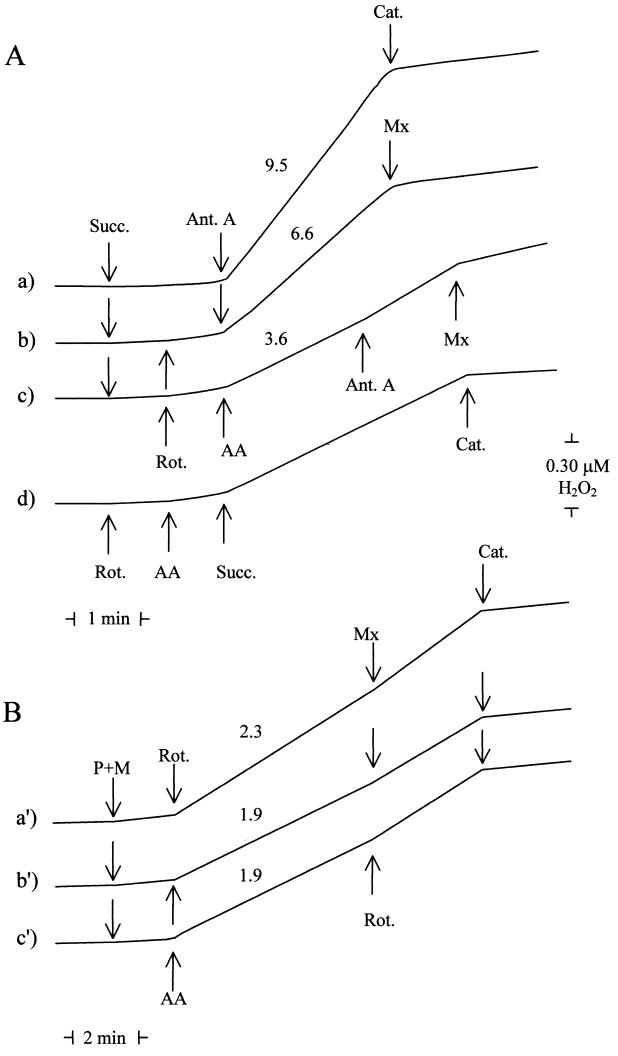

Fig. 3. H_2O_2 production in substrate supplemented bovine heart mitochondria. Freshly prepared mitochondria were suspended in the medium described in the Materials and Methods section. Succinate (Succ) (A) and pyruvate+malate (P+M) (B) were the substrates at the same concentrations used for the determination of oxygen consumption. Where indicated, 0.3 μM rotenone (Rot), 0.2 μM antimycin (Ant.A), 0.5 μM myxothiazol (Mx), 30 μM arachidonic acid (AA), and 0.5 μM Catalase (Cat.) were added. Figures on the traces refer to the rate of H_2O_2 production as nmol · min^{-1} · mg $protein^{-1}$.

(traces b' and c') promoted hydrogen peroxide production at a rate of 80–90% of that measured with rotenone. The arachidonic acid dependent H_2O_2 generation was further potentiated by either myxothiazol (trace b') and rotenone (trace c').

The dependence of hydrogen peroxide generation on arachidonic acid concentration is shown in Fig. 4. With both pyruvate+malate (Fig. 4A) and succinate (Fig. 4B), the curves have a characteristic sigmoidal shape and approach saturation at apparent maximal rate values of 3.5 (at site II) and 1.8 nmol H_2O_2 · min^{-1} · mg $protein^{-1}$ (at site I), thus approximately at a ratio 2:1 for H_2O_2

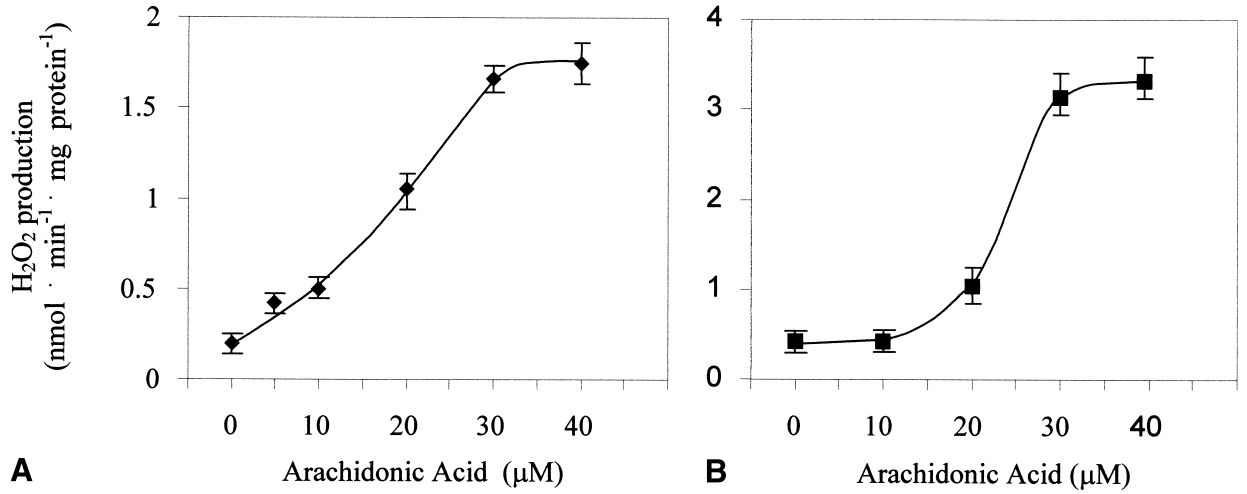

Fig. 4. Effect of arachidonic acid on H_2O_2 production in respiring mitochondria. Measurement of H_2O_2 production by mitochondria at the steady-state respiration was performed as described in the Materials and Methods section. Pyruvate+malate (A) and succinate (B) were used as substrate. The assay was performed as described for the experiments shown in traces c and c' of Fig. 3. The values reported are the means ± SD of measurements from three to six different mitochondriae preparations.

production at site II and I, respectively (see also Turrens and Boveris [29], Hansford et al. [27]). The half-maximal effective concentration of arachidonic acid for H_2O_2 production was higher at site II than at site I, consistently with the I_{50} values for the inhibition of uncoupled respiration with either substrates.

A comparison of the efficacy of various fatty acids on hydrogen peroxide generation in respiring mitochondria is shown in Fig. 5. Oleic and linoleic acids had an effect similar to that elicited by arachidonic acid with both succinate and pyruvate+malate as substrate. Palmitic acid was much less effective. It caused, in fact, H_2O_2 generation at a rate that was 60% of the maximal potential rate at site I and only 17% of that at site II.

DISCUSSION

The results we have obtained show that arachidonic acid and other long chain fatty acids affect heart mitochondrial redox activity and greatly enhance ROS production at the steady state respiration. The effects described here and by others in different tissues are likely negligible in vivo, under normal physiological conditions, where the concentration of free fatty acids is kept low, but may become relevant under various conditions including fasting, exhaustive exercise [30], ethanol abuse [31,32], cold stress [33], and in several hereditary disorders [34,35], diabetes [36], and tissue ischemia.

As far as the heart ischemia/reperfusion process is concerned, a dramatic increase of FFA concentration from 250 to 4000 nmol/g dry weight tissue takes place, with the AA concentration being increased up to ten times [37,38]. This effect has been attributed mainly to an imbalance in the deacylation-reacylation cycle of membrane phospholipid [7,37], in addition to ischemia-related increased synthesis of long chain fatty acids [39] and decrease of their mitochondrial oxidation. Phospholipase A_2 activation appears, anyhow, to contribute greatly to the accumulation of FFA in the ischemic heart [37]. Different types of phospholipase A_2 have been described in the heart, either membrane-bound or cytosolic, the latter being specific for arachidonyl residue at the sn-2 position of the phospholipid molecules [40]. However, because various species of long chain fatty acids accumulate during ischemia/reperfusion process [41], then the membrane bound phospholipase A_2 appears to be certainly involved. Relevant to this regard is the finding by Smith et al. [42], who showed that an activation of mitochondrial phospholipase A_2 occurs during rat kidney ischemia, which is responsible for a decrease of phospholipid fatty acids and a distinct increase of the mitochondrial unsaturated free fatty acids. A significant increase of FFA was also measured in mitochondria isolated from rat brains subjected to ischemia [10].

In this work, our attention was mainly addressed to arachidonic acid as (i) together with linoleic acid it is the main fatty acyl component of myocardium phospholipid and cholesterol esters [37]; (ii) among all others it is the fatty acid species that accumulates during the ischemia/reperfusion process; (iii) it is the precursor of those biologically active compounds commonly referred to as eicosanoids; and (iv) may be the signal molecule of the intracellular transduction pathway leading to activation of phospholipase A_2 in the heart [43].

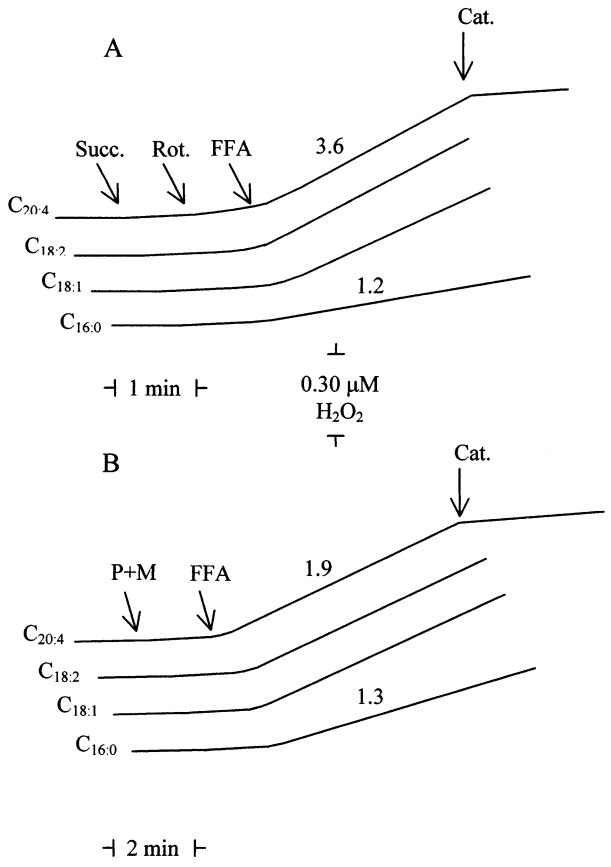

Fig. 5. Effect of different FFA on the H_2O_2 production in respiring mitochondria. Measurement of H_2O_2 production by mitochondria was performed as described in the Materials and Methods section, and in the legends to Figs. 3 and 4. Succinate (A) and pyruvate+malate (B) were used as substrate. FFA were added to a final concentration of 40 μM. $C_{20:4}$ = arachidonic acid; $C_{18:2}$ = linoleic acid; $C_{18:1}$ = oleic acid; $C_{16:0}$ = palmitic acid. Figures on the traces refer to the rate of H_2O_2 production as nmol $\cdot$ min^{-1} $\cdot$ mg $protein^{-1}$.

When tested on the respiration rate of intact heart mitochondria, fatty acids showed a dual effect, as already reported for brain mitochondria [12]: a decoupling effect and inhibition of uncoupler stimulated respiration. It has to be noted that under our conditions the uncoupling effect was independent of the respiratory substrate used (site I o site II substrate) as well as of the fatty acid species examined. On the other hand, arachidonic acid was a stronger inhibitor as compared to palmitic acid on both the respiratory sites; furthermore NADH oxidase activity appeared to be more susceptible to inhibition by fatty acids than succinate oxidase activity (Table 1).

The inhibition of the respiratory activity by arachidonic acid is accompanied by a substantial increase of ROS production rate. A slight arachidonic acid dependent ROS production increase has been reported in a crude synaptosomal-mitochondrial fraction [44]. These authors did not show whether ROS production was coupled to inhibition of respiratory activity. Our results show that ROS production is a consequence of the selective inhibition by arachidonic acid of respiratory chain complex I and III. In particular, it can be noted that (i) the stronger is the inhibitory effect, the higher is the increase of ROS production rate (compare in Figs. 1 and 5 arachidonic and palmitic acid effects); (ii) arachidonic acid seems to be more effective at site I than at site II on both inhibition of the respiratory activity and ROS production, as revealed by the effective half-maximal concentrations (Figs. 1 and 4). Nevertheless, there does not appear to exist a simple direct relationship between ROS production and inhibition of the respiratory activity (see also Turrens et al. [25]). In fact the arachidonic acid concentration-dependent curves for inhibition of the respiration and ROS production are clearly different (Figs. 1 and 4). In addition, arachidonic acid does not behave as the classical site II inhibitor antimycin in the generation of ROS: the effect of arachidonic acid and antimycin are not adding to each other; furthermore, the arachidonic acid effect is not completely suppressed by myxothiazol (Fig. 3, upper traces).

The inhibition of respiratory activity by arachidonic acid appears anyhow to precede ROS production. In fact, the reversibility by BSA of the arachidonic acid induced effects on the respiration, found in most the conditions used, suggests that the inhibition of the respiration comes first, followed by ROS production, which can, in turn, lead to subsequent effects like lipid peroxidation and oxidation of proteins, with further impairment of the respiratory activity.

The question arises whether AA can accumulate to reach the concentration used here and by others [11,12, 23] and ranging from 10 to 50 μM. Although it appears difficult to determine the actual concentration of AA to which mitochondria might be exposed in the cell under pathologic conditions, concentrations of 80, 120, 40, and 80 μM were calculated for $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, and $C_{20:4}$ during cerebral ischemia [12]. Similarly, concentrations of fatty acid ethyl esters as high as 115 μM were measured in human myocardium after ethanol consumption [31]. Furthermore, the activation of mitochondrial phospholipase A_2 ensuing from the ischemic process might cause an increase of the local concentration of FFA [10,42]. Finally, it is worth noting that the AA effect observed here, on both respiratory activity and ROS production, is shared by other long chain unsaturated fatty acids like linoleic acid, which represents, together with AA, the bulk of mitochondrial and sarcoplasmic reticulum phospholipid components. Thus, for the effects described to be realized, the overall concentration of the effective FFA has to be considered.

In conclusion, our findings together with the data reported by others on this subject, allow us to suggest a

time course of the ischemia/reperfusion process with regard to metabolic modifications, progressive energy failure and cell damage. As the ischemic process starts, the initial accumulation of arachidonic acid mainly causes uncoupling of the respiration. This can indeed be considered a beneficial effect as it could prevent a potential harmful increase of the reduction level of the electron transport carriers and of the semiquinone radical lifetime [45]. On increasing the arachidonic acid concentration, as it occurs upon prolongation of ischemia [6], the electron transport at site I is inhibited with minor effect on succinate oxidase activity [12,26,46]. Under these conditions the mitochondrial ROS production is restricted at the level of site I and represents, in our conditions, one-third or less of the maximal potential value (see also Turrens and Boveris [29], Hansford et al. [27]; see Fig. 4). If the AA concentration increases further, as it ensues from the reperfusion process [6], then the succinate oxidase activity will also be markedly inhibited. Under these conditions the rate of ROS production increases up to maximal potential values (see also Gonzalez-Flecha and Boveris [26]) and can lead to mitochondrial dysfunction and a definite cell damage.

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ABBREVIATIONS

BSA—bovine serum albumin
CCCP—carbonyl cyanide m-chlorophenylhydrazone
DCFH-DA—dichlorofluorescin-diacetate
DCFH—dichlorofluorescin
DCPIP—2,6-dichlorophenolindophenol
ΔpH—transmembrane pH difference
EDTA—ethylene diamine tetra-acetic acid
EGTA—ethylene glycol-bis (-aminoethyl ether) tetra-acetic acid
FFA—free fatty-acid
POD—peroxidase
RCR—respiratory control ratio
ROS—reactive oxygen species