

# Arachidonic Acid Causes Cytochrome c Release from Heart Mitochondria

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**Arachidonic acid interaction with heart mitochondria is known to cause uncoupling as well as inhibition of pyruvate + malate and succinate-supported respiration. Here we present experiments showing that arachidonic acid causes cytochrome c release from Ca<sup>2+</sup>-loaded heart mitochondria. We have also measured mitochondrial matrix swelling and found a fairly good correlation between the two processes, as revealed by the same arachidonic acid concentration dependence and by the same susceptibility toward different free fatty acid species. The effects produced by arachidonic acid are not related to its protonophoric activity since, under the experimental conditions used, saturating concentrations of FCCP did not cause any effect.** © 2000 Academic Press

**Key Words:** arachidonic acid; cytochrome c release; heart mitochondria; mitochondrial swelling; permeability transition; cell death.

The role and the effects of long chain free fatty acids (FFA) are actively studied in connection to several physiopathological conditions. FFA concentration was indeed found to increase greatly in ischemic and post-ischemic brain (1) and heart (2) as well as in blood of patients following acute myocardial infarction (3). Perturbations in the control of cellular FFA level, and of arachidonic acid (AA) in particular, appear to affect cell proliferation and survival (4), owing to the involvement

of AA in the induction of necrosis (5) as well as apoptosis (4, 6, 7).

Mitochondria play a critical role in cell death through several different mechanisms including (i) energy failure following inhibition of oxidative phosphorylation, (ii) altered inner membrane permeability with consequent loss of membrane potential ( $\Delta\Psi$ ), (iii) generation of reactive oxygen species (ROS), and (iv) release of apoptogenic activators of caspase cascade, such as cytochrome c and the so-called apoptosis-inducing factor (AIF) (see 8–13 for reviews).

The ability of FFA to uncouple oxidative phosphorylation has been known for a long time and a mechanism was put forward by Skulachev, according to which  $\Delta pH$  driven movement to the mitochondrial matrix of protonated FFA is coupled to electrophoretic export of FFA anions, mediated by several anion carriers, namely the ADP/ATP antiporter, the aspartate/glutamate antiporter, the dicarboxylate carrier and uncoupling proteins (see 14, 15 for reviews). We have recently found that unsaturated FFA strongly inhibit respiratory activity in heart mitochondria oxidizing pyruvate + malate or succinate and that the inhibition is accompanied by a substantial enhancement of ROS production at the steady-state respiration (16).

It was furthermore reported that FFA promote membrane permeability transition causing opening of the permeability transition pore (PTP) in Ca<sup>2+</sup>-loaded mitochondria. Whether this effect may (17–19) or may not (20–22) be entirely related to their protonophoric activity is still unclear.

Here we present experiments showing that the interaction of arachidonic acid with heart mitochondria causes a large release of cytochrome c together with a concomitant permeability transition of the inner membrane. Under the experimental conditions used, these effects appear definitely unrelated to the protonophoric action of the fatty acids.

Abbreviations used: FFA, free fatty acids; AA, arachidonic acid; ROS, reactive oxygen species; PTP, permeability transition pore; MPT, mitochondrial permeability transition;  $\Delta\Psi$ , transmembrane electrical potential gradient;  $\Delta pH$ , transmembrane pH gradient; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HRP, horseradish peroxidase; CsA, cyclosporin A; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; NEM, *N*-ethylmaleimide.

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## MATERIALS AND METHODS

**Preparation of mitochondria.** Heart mitochondria from adult male Wistar rats were isolated by differential centrifugation, as previously described in details (23). The final pellet was resuspended in 0.25 M sucrose, 10 mM Tris-Cl, pH 7.4, 0.25 mM phenylmethylsulfonyl fluoride (PMSF), 10  $\mu$ M EGTA, at a protein concentration of 50–60 mg/ml, as determined by the Biuret method. Estimation of mitochondrial cytochrome content was carried out as reported in (24). Typically, a content of  $0.57 \pm 0.05$  nmol of cytochrome *c* per mg prot ( $n = 12$ ) were found in our preparations.

**Measurement of oxygen consumption.** The respiratory activity of rat heart mitochondria was measured polarographically in a Rank Brothers oxygraph by suspending mitochondria at 0.1 mg/ml in a basic reaction mixture containing 75 mM sucrose, 50 mM KCl, 30 mM Tris-Cl, pH 7.4, 2 mM  $\text{KH}_2\text{PO}_4$ , 10  $\mu$ M EGTA, 10 mM succinate, 1  $\mu$ g/ml rotenone, at 25°C. After 1 min loading with 30  $\mu$ M  $\text{Ca}^{2+}$ , 0.5 mM EGTA was added, followed by the addition of 20  $\mu$ M AA (vehicle in the control). After a further 5-min incubation, succinate-supported respiration was stopped with 1.2  $\mu$ M antimycin A and cytochrome *c* oxidase activity was measured by adding 1.4 mM ascorbate/0.4 mM TMPD.

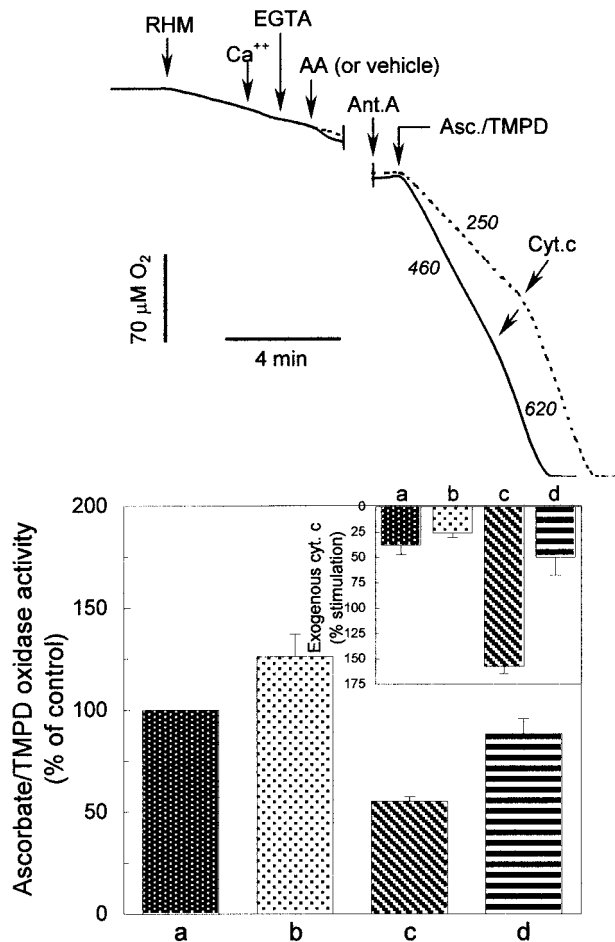
**Mitochondrial swelling.** Changes in absorbance of rat heart mitochondria were monitored at 540 nm in a Beckmann DU 7400 spectrophotometer, equipped with a thermostatted and magnetically stirred automatic sampling unit. Mitochondrial suspension medium and the experimental procedure were those described for oxygen consumption experiments. Swelling was triggered after 1 min of  $\text{Ca}^{2+}$  loading, followed by the addition of EGTA as described in (25).

**Detection of cytochrome *c* release.** Mitochondria were incubated at 25°C under the same conditions described for swelling and oxygen consumption experiments. Unless otherwise specified,  $\text{Ca}^{2+}$ -loaded mitochondria were supplemented with 20  $\mu$ M FFA and, after 5 min incubation, spun down at 14,000g for 10 min at 4°C. The pellets were solubilized at 1 mg/ml in 5% SDS, 15% glycerol, 50 mM Tris-Cl, pH 6.8, 2% mercaptoethanol and subjected to SDS-PAGE according to (26), while the resulting supernatants were centrifuged at 100,000g for 15 min, at 4°C. The supernatants of the second centrifugation were concentrated 200 times using Millipore ultrafree-4 centrifugal filter and subjected to electrophoresis. After electrophoresis, cytochrome *c* detection was performed by either staining with tetramethylbenzidine, to detect covalently bound heme polypeptides (27), or immunoblotting with a mouse anti-cytochrome *c* antibody according to (28). Immunoblot analysis was performed with HRP-conjugated anti-mouse antibody using enhanced chemiluminescence Western blotting reagents (NEN). Relative optical densities and areas of bands were quantified using a Camag TLC scanner II densitometer equipped with a D-2000 Chromato-integrator (Merck-Hitachi). Conversion of areas of bands in cytochrome *c* concentration was performed using linear concentrations of cytochrome *c* as standard.

**Chemicals.** Free fatty acids, antimycin A, rotenone, PMSF, cyclosporin A and horse-heart cytochrome *c* (type VI) were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-cytochrome *c* antibody was a gift of Prof. R. Scarpulla (Northwestern Medical School, Chicago, IL). All other reagents were of high purity grade.

## RESULTS

We have previously shown that AA, in the concentration range of 10–20  $\mu$ M, while exhibiting the well known uncoupling effect on state 4 respiration of heart mitochondria, did also cause inhibition of pyruvate + malate and succinate (+rotenone) respiration in the presence of uncoupler (16). This inhibition was due to a



**FIG. 1.** Effect of arachidonic acid on mitochondrial respiration. Rat heart mitochondria were reacted with AA and ascorbate/TMPD activity measured as described under Materials and Methods. Where indicated, 0.2  $\mu$ M cytochrome *c* was added. Solid line, control; dotted line, in the presence of arachidonic acid. Lower panel: ascorbate/TMPD oxidase activity of mitochondria supplemented with 20  $\mu$ M AA without (b) and with (c, d)  $\text{Ca}^{2+}$  loading. In d 1  $\mu$ M CsA plus 0.2 mM ADP were also present in the reaction medium. Specific activity in the control (column a) was  $460 \pm 26.5$  nmol  $\text{O}_2 \cdot \text{min}^{-1} \cdot \text{mg prot}^{-1}$ , and was almost unaffected by 30  $\mu$ M  $\text{Ca}^{2+}$ . The inset shows the percentage stimulation of the oxidase activity caused by the addition of cytochrome *c* under the corresponding conditions described above. The values reported are the means  $\pm$  SD of six determinations from three different experiments.

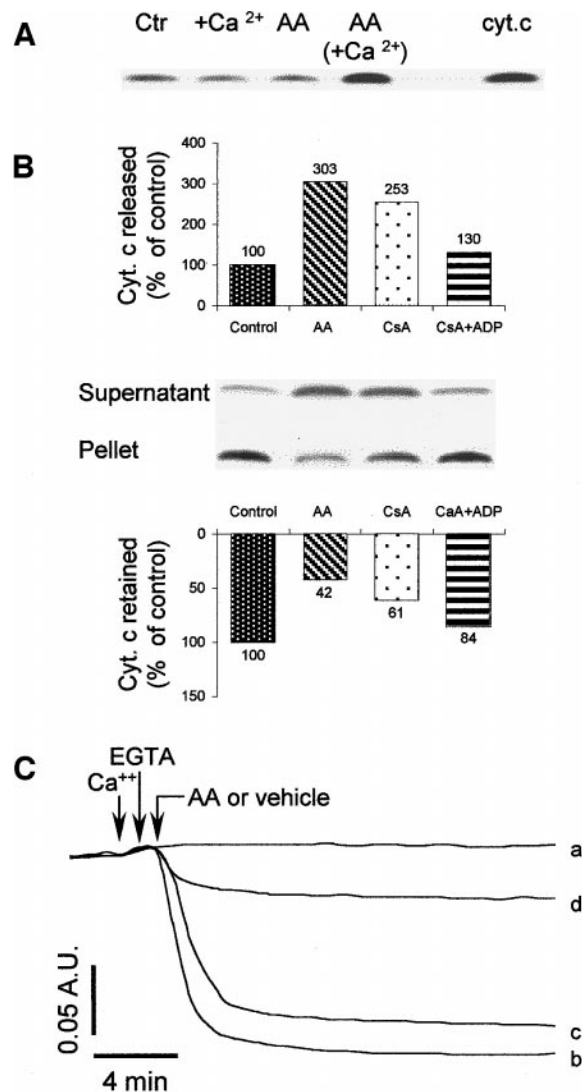
direct interaction of AA with complexes I and III of the respiratory chain (16). On the contrary, AA caused a small stimulation of ascorbate/TMPD oxidase activity (see ref. 16 and column b in Fig. 1). The latter is, basically, almost the same either in mitochondrial state 4 or in uncoupled state, since of the very poor respiratory control ratio (23). Thus ascorbate/TMPD-supported respiration represents an excellent system to ascertain whether AA may cause effects other than uncoupling and those consequent to its interaction with complexes I and III.

Tracing of oxygen consumption reported in Fig. 1 shows that when AA (dotted trace) was added to  $\text{Ca}^{2+}$ -loaded mitochondria, ascorbate/TMPD oxidase activity, measured after 5 min incubation and in the presence of antimycin, was markedly inhibited (see also column c). The inhibitory effect of AA was largely prevented by supplementing the incubation mixture with cyclosporin A (CsA) and ADP (column d). Noteworthy the addition of cytochrome *c* at the steady-state respiration allowed a complete recovery of AA-inhibited oxidase activity (dotted line) to the value of control (solid line). The inset shows the per cent stimulation of the oxidase activity by the addition of cytochrome *c* to control (a), to AA-supplemented mitochondria (b), to AA-supplemented  $\text{Ca}^{2+}$ -loaded mitochondria in the absence (c) and in the presence (d) of CsA and ADP.

The above results are suggestive of AA-dependent induction of permeability transition (PT) of  $\text{Ca}^{2+}$ -loaded mitochondria with outer membrane rupture and consequent depletion of cytochrome *c*. In the experiment of Fig. 2 we measured directly the release of cytochrome *c* from mitochondria. It is shown in Fig. 2A that neither  $\text{Ca}^{2+}$  nor AA *per se* caused cytochrome *c* to be released, but there was a large release when AA was added to  $\text{Ca}^{2+}$ -loaded mitochondria. Scanning densitometry determinations of the blots from supernatants of AA-treated mitochondria revealed that cytochrome *c* released under these conditions amounted to  $0.25 \pm 0.048$  ( $n = 8$ )  $\text{nmol} \cdot \text{mgprot}^{-1}$ , that is around 45% of its total mitochondrial content, and that its concentration increment in the supernatant was more than three times (mean of  $335 \pm 30\%$ ,  $n = 8$ ) with respect to the control (Fig. 2B). It has to be noted that mitochondria subjected to hypotonic rupture in saline medium (29) released almost the same amount of cytochrome *c* (not shown). Quite consistently with the release values in the supernatant, it was found that around 46% ( $\pm 4\%$ ,  $n = 8$ ) of the total cytochrome *c* content remained in the pellet (42% in the experiment of Fig. 2B). The release of cytochrome *c* was only partially sensitive to CsA, but almost completely prevented by CsA and ADP in the incubation medium (Fig. 2B). Staining of gels with tetramethylbenzidine gave the same results (not shown).

Optical absorption measurements at 540 nm (Fig. 2C) showed that addition of AA to  $\text{Ca}^{2+}$ -loaded mitochondria ( $\text{Ca}^{2+}$ , at concentrations used, and AA did not cause any effect *per se*) brought about swelling of mitochondria, which was again scanty CsA sensitive, but largely suppressed by CsA plus ADP. ADP alone caused a little lower effect (not shown). It is worth mentioning that swelling was abolished by  $\text{Mg}^{2+}$  (5 mM) and by *N*-ethylmaleimide (NEM, 20  $\mu\text{M}$ ) (results not shown).

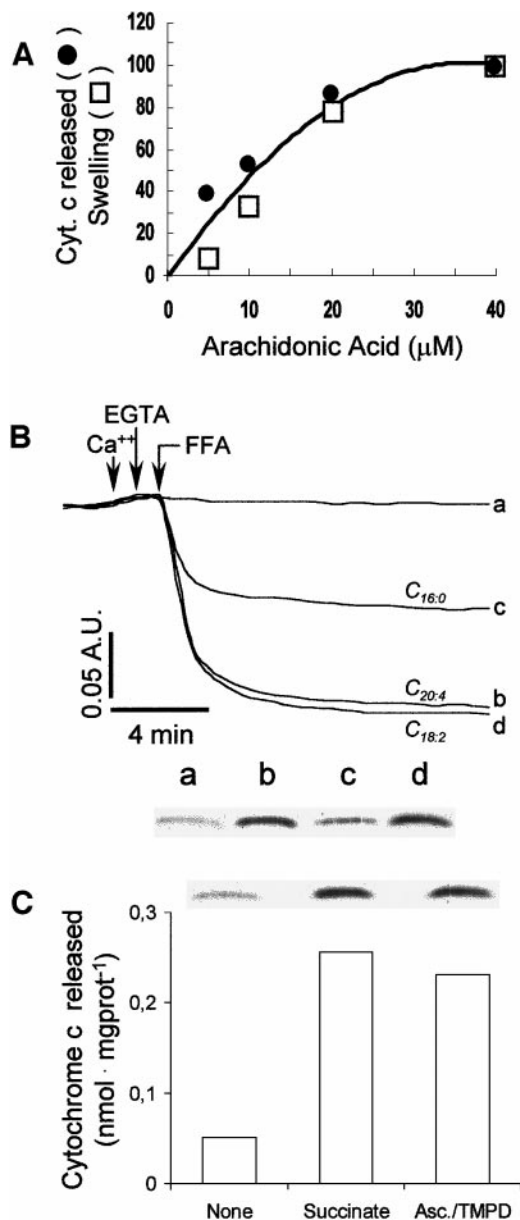
The concentration dependence of AA effect on both cytochrome *c* release and mitochondrial swelling is shown in Fig. 3A. The two processes appear fairly well



**FIG. 2.** Effect of arachidonic acid on cytochrome *c* release and mitochondrial swelling. Freshly isolated mitochondria were incubated at 25°C in the presence of 10 mM succinate and 1  $\mu\text{g/ml}$  rotenone, as described under Materials and Methods. (A) Western blot analysis of cytochrome *c* released into the supernatant. Mitochondria were incubated with  $\text{Ca}^{2+}$  or AA alone, and with  $\text{Ca}^{2+}$  followed by AA, as indicated. Purified horse-heart cytochrome *c* (0.14 nmol) was used as internal standard. (B) Cytochrome *c* release in  $\text{Ca}^{2+}$ -loaded mitochondria caused by 20  $\mu\text{M}$  AA, in the absence and in the presence of 1  $\mu\text{M}$  CsA, and CsA plus 0.2 mM ADP. Values on the columns represent percentage variations of cytochrome *c* content measured by scanning densitometry of the blots from pellet and supernatant fractions. Representative results are shown from a typical experiment. (C) Mitochondrial swelling was measured as reported under Materials and Methods. Where indicated, 30  $\mu\text{M}$   $\text{Ca}^{2+}$ , 0.5 mM EGTA, and 20  $\mu\text{M}$  AA (vehicle in the control) were added. (a) Control, (b) AA, (c) AA in the presence of 1  $\mu\text{M}$  CsA, and (d) in the presence of CsA plus 0.2 mM ADP.

correlated, with an  $\text{ED}_{50}$  value (the concentration of AA giving half-maximal effect) of around 10  $\mu\text{M}$ . A close correlation is also emerging from the experiment of Fig. 3B, where it is shown that palmitic acid is much





**FIG. 3.** Correlation between AA-dependent cytochrome *c* release and mitochondrial swelling. Measurement of mitochondrial swelling and detection of cytochrome *c* release by Western blot analysis of supernatant fractions were performed as described under Materials and Methods. (A) Concentration dependence of AA effects. The values reported are expressed as percentage of the maximal effect measured at  $40 \mu\text{M}$  AA. (B) Effect of different FFA on mitochondrial swelling and cytochrome *c* release. FFA were added at a final concentration of  $20 \mu\text{M}$ . (a) Control, (b) arachidonic acid, (c) palmitic acid, and (d) linoleic acid. (C) substrate dependence of AA-induced cytochrome *c* release. Mitochondria were incubated in the absence or in the presence of the indicated respiratory substrates. Estimation of cytochrome *c* release was carried out as reported under Materials and Methods.

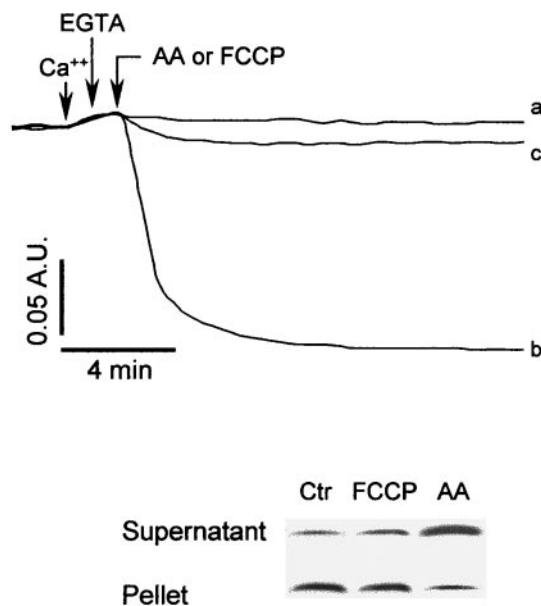
less effective than, and linoleic acid is as effective as AA towards both cytochrome *c* release and mitochondrial swelling. Figure 3C shows that AA, in order to

cause cytochrome *c* release, as well as swelling (not shown), requires steady-state respiring mitochondria, with its effect independent of the substrate being used. This experiment further suggests that cytochrome *c* is released under these conditions independently of its redox state. Separate controls have indeed shown that cytochrome *c* is almost completely oxidized during succinate respiration, while fully reduced when ascorbate/TMPD was used as substrate, in the presence of antimycin.

The question then arises whether the effects described above for AA and other FFA are related to their protonophoric activity, considering that (i) the concentrations used here are fully uncoupling, as revealed by their effect on respiration and membrane potential (data not shown, see also ref. 16) and (ii) as uncouplers, FFA may cause MPT to initiate by decreasing  $\Delta\Psi$  below the gating potential (30). The experiment reported in Fig. 4 shows that under the conditions where AA causes mitochondrial swelling and cytochrome *c* release, saturating concentrations of FCCP did not have appreciable effects. Under these conditions, FCCP could still cause swelling provided that phosphate concentration was increased to  $10 \text{ mM}$  in the incubation mixture (not shown).

## DISCUSSION

In this study we have shown that the interaction of long chain free fatty acids, and AA in particular, with



**FIG. 4.** Comparison of the effect of AA and FCCP on mitochondrial swelling and cytochrome *c* release. The experimental conditions for swelling measurements and analysis of cytochrome *c* release by Western blot of supernatant and pellet fractions were those described under Materials and Methods. AA (b) and FCCP (c) were added to a final concentration of  $20$  and  $0.1 \mu\text{M}$ , respectively. Vehicle in the control (a).

Ca<sup>2+</sup>-loaded respiring heart mitochondria causes cytochrome *c* release. Importantly, this process appears closely related with mitochondrial swelling, as revealed by the same AA concentration dependence and by the same susceptibility towards different free fatty acid species (Fig. 3).

Although the FFA concentrations used here cause a drop of membrane potential, we have shown that the effects produced cannot be accounted for by their protonophoric-dependent membrane depolarization. In fact, under the experimental condition used, saturating concentrations of FCCP did not cause similar effects (Fig. 4).

The swelling of mitochondria ensuing upon addition of arachidonic acid is indicative of the opening of PTP, followed by outer membrane rupture and release in the external medium of a large proportion of cytochrome *c*. Under the experimental conditions we selected, both mitochondrial swelling and cytochrome *c* release were, however, only marginally sensitive to cyclosporin A, which is generally taken as a diagnostic tool for the involvement of PTP in isolated mitochondria as well as in cell and organs (31). Involvement of PTP opening in the AA-induced effects, is supported by the following observations: (i) CsA-insensitive reversible PTP induction has already been reported previously (32); (ii) in heart mitochondria CsA is scantily effective, while ADP and CsA are synergistically regulating the permeability transition (33); (iii) Mg ions and NEM definitely prevented swelling; (iv) FFA themselves were reported to be a factor limiting pore inhibition by CsA (22); (v) finally, and more importantly, the processes elicited by AA require Ca<sup>2+</sup>-loaded mitochondria (Fig. 2A) and the presence of phosphate in the reaction medium. Thus FFA behave as inducers, increasing the susceptibility of mitochondria towards strong PTP opening agents such as phosphate.

In conclusion we can outline different consequences ensuing upon interaction of FFA with heart mitochondria. Few micromolar FFA concentrations may give rise to a so-called mild uncoupling effect, thus preventing generation of ROS by mitochondria in the resting state (34). Following a given stimulus, such as TNF- $\alpha$  which causes activation of cytosolic PLA<sub>2</sub> (35), cellular concentration of AA may rise substantially. Under these conditions there occur both a larger uncoupling and inhibition of respiratory enzymes. ROS will be now extensively produced (16). This condition, which is typical of postischemic heart, is likely accompanied by opening of PTP and release of cytochrome *c*.

FFA have been described to cause either apoptosis or necrosis in living cells. In cultured cardiomyocytes palmitate has been reported to cause both necrosis and apoptosis (36), with cytochrome *c* release, largely CsA insensitive. As discussed by Lemasters *et al.* (37), progression to necrotic or apoptotic cell death depends upon ATP level. This will be, in turn, regulated by the

extent of the uncoupling produced by FFA and by their effect on the membrane permeability.

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