Arachidonic Acid Causes Cell Death through the Mitochondrial Permeability Transition

IMPLICATIONS FOR TUMOR NECROSIS FACTOR-α APOPTOTIC SIGNALING*

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We have investigated the effects of arachidonic and palmitic acids in isolated rat liver mitochondria and in rat hepatoma MH1C1 cells. We show that both compounds induce the mitochondrial permeability transition (PT). At variance from palmitic acid, however, arachidonic acid causes a PT at concentrations that do not cause PT-independent depolarization or respiratory inhibition, suggesting a specific effect on the PT pore. When added to intact MH1C1 cells, arachidonic acid but not palmitic acid caused a mitochondrial PT in situ that was accompanied by cytochrome c release and rapidly followed by cell death. All these effects of arachidonic acid could be prevented by cyclosporin A but not by the phospholipase A2 inhibitor aristolochic acid. In contrast, tumor necrosis factor α caused phospholipid hydrolysis, induction of the PT, cytochrome c release, and cell death that could be inhibited by both cyclosporin A and aristolochic acid. These findings suggest that arachidonic acid produced by cytosolic phospholipase A2 may be a mediator of tumor necrosis factor α cytotoxicity in situ through induction of the mitochondrial PT.

The mitochondrial PT† is a regulated permeability increase to solutes with molecular masses up to 1,500 Da mediated by opening of a high conductance channel, the PTP, whose molecular nature remains debated (1). The PT is modulated by a variety of effectors of cell death, including reactive oxygen species that are produced early by mitochondria after stimulation of the TNFα receptor (2). Early evidence indicated that PTP opening can mediate cell death caused by oxidative stress and anoxia through ATP depletion and dysregulation of Ca2+ homeostasis (3–5). The PT could also be instrumental in the release of intermembrane proteins such as cytochrome c (6, 7), which plays a key role in caspase 9 activation (8), and apoptosis-inducing factor (9), which causes nuclear degradation independent of caspase activation (10).

Mitochondria play a role in apoptotic signaling in the TNFα pathway, which is activated by ligand binding to the TNFα receptor. Several cytosolic proteins are recruited to the receptor death domain, which in turn leads to the production of a variety of second messengers. As a result, multiple protein-mediated signaling cascades are activated, including caspase-dependent and caspase-independent pathways, phospholipases, protein kinases, and protein phosphatases (see Ref. 11 for a critical and comprehensive review). Lipid mediators may be involved in both in signaling and in the late cellular responses by receptors of the TNFα superfamily, and solid evidence indicates that mitochondria can be the target of lipid mediators of pathophysiological relevance. Ceramide produced by acidic sphingomyelinase is essential for production of GD3 ganglioside, a cell death inducer (12) that is able to cause PT-dependent apoptosis (13) with cytochrome c release and caspase activation (14). Ceramide itself has prominent effects on mitochondria (14–19) and may turn physiological Ca2+ signals acting through the inositol 1,4,5-trisphosphate receptor into initiators of cell death (20).

TNFα receptor engagement causes caspase-dependent degradation of cytosolic PLA2, followed by membrane translocation of a 70-kDa active fragment and production of AA (21). AA can in turn activate the ceramide pathway by stimulating neutral sphingomyelinase (22, 23), thereby creating a feed-forward mechanism in the propagation of the apoptotic signal by TNFα. Prompted by these observations we have investigated the effects of AA in isolated mitochondria and intact cells. We show that AA is a powerful PT inducer that mimics the mitochondrial effects of TNFα in MH1C1 cells and that AA generated by PLA2 activation plays a role in apoptotic signaling through the mitochondrial pathway.

MATERIALS AND METHODS

Liver mitochondria were prepared by standard centrifugation techniques from albino Wistar rats weighing about 300 g (24). Mitochondrial volume changes were monitored as the absorbance changes at 540 nm with a PerkinElmer Life Sciences Lambda-10 or with an Aminco DW2000 spectrophotometer (25). Mitochondrial membrane potential was calculated based on the equilibrium distribution of rhodamine 123 (26), assuming a matrix volume of 1 μl per mg protein 1. Rhodamine 123 uptake was determined under the specified conditions from the fluorescence changes of mitochondrial suspensions containing 0.1 μM rhodamine 123 at the wavelength pair 503–525 nm, with excitation and emission slits set at 2 and 5 nm, respectively. Oxygen consumption was
determined polarographically using a Clark-type oxygen electrode. All assays were performed at 25 °C in instruments equipped with thermostatic control and magnetic stirring.

Measurement of PLAr activation was based on the cleavage of bis-BODIPY®-FL-C11-PC (Molecular Probes, Eugene, OR), a synthetic phospholipid containing fluorescein that is dequenched upon cleavage of PC. For loading, MH1C1 cells seeded on uncoated 22-mm round glass coverslips were incubated for 4 h at 37 °C with a 2% (v/v) suspension of liposomes prepared in Dulbecco’s phosphate-buffered saline (Sigma) containing dipalmitylophosphatidylserine (Sigma), cholesterol (Sigma), and bis-BODIPY®-FL-C11-PC in a molar ratio of 271:74:1.8. After washing twice with Dulbecco’s phosphate-buffered saline, cells were incubated for 30 min at 37 °C with vehicle, 50 μM aristolochic acid, or 2 μM CsA and then imaged as described below. Fluorescence images were acquired with an Olympus IMT-2 inverted microscope, equipped with a xenon light source (75 watts) for epifluorescence illumination and with a 12-bit digital cooled CCD camera (Micromax, Princeton Instruments). Filter settings were 480 ± 25 nm excitation and 525 nm long pass emission, and images were collected with an exposure time of 100 ms using a × 40, 1.3 NA oil immersion objective (Nikon). Data were acquired and analyzed using Metamorph software (Universal Imaging). Sequential digital images were acquired at the times indicated in Fig. 6A, and the whole cellular fluorescence of 10 cells in each field was recorded and stored. Control experiments showed that bis-BODIPY®-FL-C11-PC was properly inserted in the bilayer, since no fluorescence increase was elicited by added PLAr (not shown). The data in Fig. 6A were corrected for background fluorescence and are normalized for comparative purposes. Culture conditions for MH1C1 rat hepatoma cells, staining and imaging with calcein, TMRM, annexin-V, and propidium iodide, and immunodetection of the bc1 complex and cytochrome c were carried out exactly as described in detail in the companion paper (7).

Calcine, bis-BODIPY®-FL-C11-PC, TMRM, and rhodamine 123 were purchased from Molecular Probes (Eugene, OR); TNFα, aristolochic acid, and AA were purchased from Alexis Biochemicals (Laufelfingen, Switzerland); and all other chemicals were purchased from Sigma and were of the highest available grade. CsA was a generous gift of Novartis (Basel, Switzerland).

RESULTS

Effects of AA and PA on Isolated Mitochondria—The experiments of Fig. 1 compare the effects of PA and AA on the volume maintained by energized rat liver mitochondria, measured on the basis of the absorbance changes at 540 nm (25). Mitochondria were first loaded with a small Ca2+ pulse, followed by EGTA to prevent Ca2+ release via the uniporter (27). The subsequent addition of increasing concentrations of PA (Fig. 1A) or of AA (Fig. 1B) caused the expected (28) absorbance decrease that accompanies the PT, which is documented by its inhibition by CsA (traces d in both panels). Importantly, the PT was completely insensitive to aristolochic acid (traces e in both panels), which inhibits PLAr (29); and AA was a more potent inducer of mitochondrial swelling than PA (compare A and B).

We next assessed whether PT induction by PA and AA was due to a direct effect on the PTP or rather to an indirect effect, an issue that has been the subject of some debate (28).

The experiments of Fig. 2 illustrate the concentration dependence of the effects of PA (Fig. 2A) and AA (Fig. 2B) on the membrane potential (closed circles), on uncoupled respiration (closed triangles), and on PT-dependent swelling (open circles). From these titrations it is apparent that the PT-inducing effects of PA occurred in the same range of concentrations causing respiratory inhibition and depolarization. This is consistent with the finding that the PT-inducing effects of nonesterified, long chain fatty acids correlate with their effects on the membrane potential (30) and are only partly due to a direct effect on the PTP (25, 31). On the other hand, AA induced a relevant effect on the PTP (70% of maximal) at concentrations (10 μM) that had relatively little effect on respiration and membrane potential (Fig. 2B), suggesting a direct effect of the PTP.

We further characterized the mechanism of PT induction by AA by testing its sensitivity to a set of inhibitors. The experiments of Fig. 3 demonstrate that PT induction by AA was insensitive to NEM and MBM, which are able to block the effects of reagents acting on thiol nucleotides and thiol oxidation (32, 33). On the other hand, the inducing effects of AA could be fully blocked by sphingosine, which inhibits PTP induction by a variety of fatty acids (34), and by PGO, an arginine reagent that is able to prevent PTP opening by the protonophore FCCP (35).

Effects of AA, PA, and TNFα on Mitochondria in Situ—The
PTP is affected by a very large number of individual effectors (36). Despite recent progress in understanding the basis for its modulation in vitro, it remains extremely difficult to predict whether a given set of conditions can promote the PT in situ (see Refs. 1 and 37 for reviews). We therefore tested whether AA and PA were able to cause a PT in situ. The experiments of Fig. 4A document that the addition of AA caused a time-dependent decrease of mitochondrial TMRM fluorescence (closed squares) due to a PT, as shown by the inhibitory effects of CsA (closed circles). On the other hand, aristolochic acid was without effect (open squares), suggesting that AA is acting on mitochondria directly. As could be predicted on the basis of its effects in isolated mitochondria, Fig. 4B shows that PA caused a very small decrease of TMRM fluorescence (closed squares) that was sensitive to CsA (closed circles) but not to aristolochic acid (open squares). It is noteworthy that PA had much smaller effects than AA even at a concentration of 0.6 mM (results not shown).

We next assessed whether PT opening by AA was causally related to cell death. In MH1C1 cells AA-induced PT was followed by release of cytochrome c, as assessed with the in situ double labeling technique, which is described in detail in the companion paper (7) (Fig. 5A), and by cell death, documented by the appearance of a large number of cells positive for surface phosphatidylserine with annexin V staining (Fig. 5B). Both events were largely inhibited by CsA (Fig. 5), whereas neither cytochrome c release nor cell commitment to apoptosis by AA was affected by aristolochic acid (results not shown). On the other hand, in good agreement with the results obtained in isolated mitochondria and in situ, PA had a very small effect on cytochrome c release and cell survival (Fig. 5).

TFNα inhibits respiration and increases the mitochondrial production of reactive oxygen species, an early event in the apoptotic cascade (2) that could be due to cytochrome c release following a mitochondrial PT (15, 38, 39). Since TNFα causes activation of cytosolic PLA₂ (21), we investigated whether the PT could be triggered by TNFα-dependent production of AA. The experiments depicted in Fig. 6A show that the addition of TNFα initiated the cleavage of the synthetic phospholipid bis-BODIPY® FL C11-PC (closed squares). Consistent with a role of PLA₂ upstream of mitochondria, probe cleavage was prevented by aristolochic acid (open squares) but not by CsA (closed circles), whereas PT opening was completely prevented by both aristolochic acid and CsA (Fig. 6B). In our model system treatment with TNFα was followed by cytochrome c release (Fig. 7A) and by the appearance of annexin V-positive cells (Fig. 7B), and both events were inhibited by CsA and aristolochic acid (Fig. 7).

**DISCUSSION**

In this paper we have shown that AA is an effective inducer of the PT in isolated mitochondria and intact cells. At variance from the case of PA, low concentrations of AA affect the PTP directly rather than through a decrease of the mitochondrial membrane potential. Since PT induction by TNFα is inhibited by the PLA₂ inhibitor aristolochic acid, which does not inhibit the PTP per se, we suggest that AA released by PLA₂ may participate in early apoptotic signaling to mitochondria in the TNFα pathway.

**Fatty Acids and the PT**—Fatty acids exert prominent effects
through which fatty acids exert this multiplicity of effects is at the level of Complex I (46). A discussion of the mechanisms involving transport of the deprotonated form through the adenine nucleotide translocase (45). Fatty acids also cause re-involvement of Complex I (46). A discussion of the mechanisms involving transport of the deprotonated form through the adenine nucleotide translocase (45). Fatty acids also cause re-involvement of Complex I (46).

Effects of arachidonic and palmitic acids on mitochondrial TMRM fluorescence in MH1C1 cells. MH1C1 cells were loaded with 10 nM TMRM, and changes of fluorescence intensity over mitochondrial regions of interest were determined as described in the companion paper (7). Where indicated 0.2 mM AA (A) or 0.2 mM PA (B) was added (closed squares). In both panels, open squares and closed circles denote cells pre-treated with 50 μM aristolochic acid or 2 mM CsA, respectively, and open circles report the fluorescence intensities after the addition of vehicle (0.02% ethanol v/v). Where indicated (arrows) 1 μM FCCP was added. The values are mean of 6 experiments ± S.D. Note that the standard deviations were omitted from selected traces in B for the sake of clarity.

Effects of arachidonic and palmitic acids on cytochrome c release and apoptosis in MH1C1 cells. MH1C1 cells grown on coverslips were treated for 30 (A) or 45 min (B) with 0.2 mM AA, 0.2 mM PA, or vehicle (ethanol 0.02% v/v). When indicated cells were pre-treated with 2 μM CsA for 15 min. A, cells were fixed and treated with antibodies against the bc1 complex and cytochrome c, and the cytochrome c localization index was determined exactly as described in the companion paper (7). B, cells were stained with annexin-V-FLUOS and propidium iodide exactly as described (13). The fraction of annexin-V positive cells (gray bars) and double positive cells (black bars) was calculated from 20 randomly chosen fields from four independent experiments, and values are mean ± S.D. Data are from three different experiments ± S.D., and the localization index was determined exactly as described (7). Downward and upward error bars refer to annexin-V positive and double positives, respectively.

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Effects of TNFα, aristolochic acid, and CsA on phospholipid hydrolysis and mitochondrial calcein fluorescence. A, MH1C1 cells were loaded with bis-BODIPY®-FL-C11-PC as described under “Materials and Methods.” Whole cellular fluorescence intensities minus background (fields not containing cells) are reported after normalization of the initial fluorescence, and they represent the mean of 10 cells from three different experiments ± S.D. B, MH1C1 cells were co-loaded with calcein-AM and CoCl2, and changes of calcein fluorescence intensity over mitochondrial regions of interest were determined as described in the companion paper (7). Reported values are mean ± S.D. of four different experiments. In both panels, in the experiments denoted by closed circles cells were treated with 2 μM CsA, whereas in those denoted by open squares cells were treated with 50 μM AA. The experiments were carried at 37 °C by using a water-jacketed coverslip holder. Where indicated (arrow), 10,000 IU TNFα were added.
variety of inducers are able to shift the apparent threshold voltage for opening closer to the resting value thereby favoring onset of a PT \((25, 31)\). A first important result of the present work is the demonstration that, at variance from PA, AA is able to cause PTP opening at concentrations that do not cause respiratory inhibition and membrane depolarization (Fig. 2). We conclude that low concentrations of AA \((\text{below 10 } \mu\text{M})\) selectively interact with the PTP, possibly by modifying its putative voltage sensor. Consistent with this view is our finding that pore opening by AA can be fully inhibited by the arginine reagent PGO (Fig. 3), which is also able to block PTP opening by the proponentophore FCCP (35). It is important to stress that the PT-inducing effects of AA were insensitive to inhibition by NEM (Fig. 3), which is able to block PTP opening caused by a variety of oxidants (like organic hydroperoxides, diamide, menadione, and paraquat) as well as diithiol reagents (like arsenite and phenylarsine oxide) \((32, 33, 48)\). It therefore appears extremely unlikely that the inducing effects of AA are mediated by oxidative processes triggered by AA metabolism.

**AA and Apoptosis**—AA is emerging as a very important player in the pathways to cell death. Nonsteroidal anti-inflammatory drugs, which are among the few agents that can effectively prevent neoplasias \((49)\), induce apoptosis through elevation of the intracellular levels of AA, and their effects can be mimicked by the addition of exogenous AA \((50)\). The role of AA has been recently addressed in an elegant model where the levels of intracellular AA could be modulated by overexpression of cyclooxygenase-2 and fatty acyl-CoA ligase, two of the major regulators of cyclooxygenase and arachidonic acid. The extracellular levels of AA can be modulated by overexpression of cyclooxygenase and arachidonic acid.

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