Role of phospholipase A₂ activation and calcium in CYP2E1-dependent toxicity in HepG2 cells.

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Running title: Role of phospholipase A₂ in CYP2E1 toxicity
SUMMARY

Previous studies suggested a role for calcium in CYP2E1-dependent toxicity. The possible role of phospholipase A2 (PLA2) activation in this toxicity was investigated. HepG2 cells that overexpress CYP2E1 (E47 cells) exposed to arachidonic acid (AA) +Fe-NTA showed higher toxicity than control HepG2 cells not expressing CYP2E1 (C34 cells). This toxicity was inhibited by the PLA2 inhibitors aristolochic acid, quinacrine, and PTK. PLA2 activity assessed by release of preloaded $^3$H-AA after treatment with AA+Fe was higher in the CYP2E1 expressing HepG2 cells. This $^3$H-AA release was inhibited by PLA2 inhibitors, $\alpha$-tocopherol, and by depleting $Ca^{2+}$ from the cells (intracellular + extracellular sources), but not by removal of extracellular calcium alone. Toxicity was preceded by an increase in intracellular calcium caused by influx from the extracellular space and this was prevented by PLA2 inhibitors. PLA2 inhibitors also blocked mitochondrial damage in the CYP2E1 expressing HepG2 cells exposed to AA+Fe. $Ca^{2+}$ depletion and removal of extracellular calcium inhibited toxicity at early time periods, although a delayed toxicity was evident at later times in $Ca^{2+}$-free media. This later toxicity was also inhibited by PLA2 inhibitors. Analogous to PLA2 activity, $Ca^{2+}$ depletion but not removal of extracellular calcium alone prevented the activation of calpain activity by AA+Fe. These results suggest that release of stored calcium by AA+Fe, induced by lipid peroxidation, can initially activate calpain and PLA2 activity, that PLA2 activation is critical for a subsequent increased influx of extracellular $Ca^{2+}$, and that the combination of increased PLA2 and calpain activity, increased calcium and oxidative stress cause mitochondrial damage, that ultimately produces the rapid toxicity of AA+Fe in CYP2E1-expressing HepG2 cells.
INTRODUCTION

Phospholipase A\textsubscript{2} (PLA\textsubscript{2}) comprises a set of extracellular and intracellular enzymes that catalyze the hydrolysis of the sn-2 fatty acyl bond of phospholipids to yield free fatty acids and lysophospholipids. The extracellular (secreted) PLA\textsubscript{2}s (sPLA\textsubscript{2}) require millimolar calcium concentrations for catalytic activity, and do not manifest significant fatty acid selectivity \textit{in vitro}. In mammalian cells, as many as five different sPLA\textsubscript{2} exist: groups I, IIA, IIC, V and X. The intracellular PLA\textsubscript{2}s are further divided into groups IV (cytosolic Ca\textsuperscript{2+} dependent PLA\textsubscript{2} or cPLA\textsubscript{2}) and VI (intracellular Ca\textsuperscript{2+} independent PLA\textsubscript{2} or iPLA\textsubscript{2}) based on the Ca\textsuperscript{2+} requirements needed for basal activity. cPLA\textsubscript{2} requires \textmu{}M Ca\textsuperscript{2+} for membrane translocation but not for catalysis, and possesses a preference for phospholipids containing AA. iPLA\textsubscript{2} exhibits no substrate specificity for AA-containing phospholipids and no Ca\textsuperscript{2+} requirement for activity. Coexpression of different forms of PLA\textsubscript{2} has been found within the same cell or tissue (1,2). The physiological functions of individual PLA\textsubscript{2}s in the hepatocyte are currently unknown (3).

Several studies have shown that reactive oxygen species in cellular systems can activate PLA\textsubscript{2} activity, measured as increased release of radioactive AA in prelabeled cells (4-11). Liver mitochondria isolated from vitamin E-deficient rats (12), rats fed fish oil (13), or rats treated with bacterial endotoxin (14), all conditions associated with increased oxidant production, showed an increase in PLA\textsubscript{2} activity. Phospholipase A\textsubscript{2} activities of the mitochondrial and cytosolic fraction in the kidney were significantly enhanced after 1h ischemia followed by 1h reperfusion (15). Liver microsomes isolated from rats chronically treated with ethanol showed increased PLA\textsubscript{2} and CYP2E1 activity, decreased content of AA and increased content of conjugated dienes (16).

Oxidative stress-associated activation of PLA\textsubscript{2} has been proposed to be a critical factor in cytotoxicity. PLA\textsubscript{2} activation results not only in the degradation of membrane phospholipids but also in the accumulation of unsaturated free fatty acids and lysophospholipids which by themselves can be injurious (17). On the contrary, PLA\textsubscript{2} has been shown to be protective in some settings of lipid peroxidation and hypoxia/reoxygenation (18-21). There is mounting evidence that membrane lipid peroxidation stimulates
phospholipid hydrolysis via Ca\(^{2+}\) dependent PLA2 activity. It is well known that Ca\(^{2+}\) mobilization and phospholipid degradation are closely interrelated. Membrane lipid peroxidation alters Ca\(^{2+}\) homeostasis, and this appears to be an important initial signal for PLA2 activity. Peroxides are known to increase intracellular free Ca\(^{2+}\) concentrations through either membrane disruption and physical disruption of ionic homeostasis, or by signaling release of intracellular calcium (22). In other cell systems, the mechanism of oxidant-induced AA release appears to be independent of Ca\(^{2+}\), resulting either from the activation of an iPLA2 (8,23,24), or the inhibition of AA esterification into phospholipids (25,26).

Many cytochrome P450s especially CYP2E1 can produce superoxide anion and hydrogen peroxide during their catalytic cycle (27,28). CYP2E1 is of interest because of its ability to metabolize and activate many substrates to reactive intermediates and to its role in alcohol liver injury. CYP2E1 overexpression in HepG2 cells (E47 cell line), in the absence of added toxin, is associated with increased cellular production of ROS and lipid peroxidation (29,30) and increased cytotoxicity of arachidonic acid (31), GSH depletion (29,30), iron (32) and ethanol (33). These cells when exposed to Fe-NTA showed an elevation of intracellular calcium and reactive oxygen species that occurs before the onset of cellular toxicity, events blocked by antioxidants. Control HepG2 cells not expressing P450 (C34 cells) showed very low toxicity and a small increase in intracellular calcium under the same conditions (34). Activation of calpain was observed together with cell death, both processes blocked by calpain inhibitors (34). An increase in intracellular calcium seems to be a very early and critical event in the toxicity caused by CYP2E1-dependent oxidative stress, leading to the activation of Ca\(^{2+}\)-dependent processes such as calpain activity.

The objectives of this work were: i) to investigate the possible activation of PLA2 activity in HepG2 cells that overexpress CYP2E1, and exposed to a CYP2E1-dependent oxidative stress (Fe+AA); ii) to evaluate the possible relationship between an increased PLA2 activity and cytotoxicity, and iii) to study the Ca\(^{2+}\)-dependence of the induction of PLA2 activity by CYP2E1-dependent oxidative stress.
EXPERIMENTAL PROCEDURES

Chemicals: PBS and fatty acid free BSA were from Roche (Newark, NJ). G418 was from Invitrogen (Carlsbad, CA). Ethanol 95% was from Pharmaco Products (Brookfield, CT). Fluo3-AM and pluronic acid were from Molecular Probes (Eugene, OR). $^3$H-AA was from Perkin Elmer (Boston, MA). Protein concentration was measured using the BioRad DC Protein assay (Hercules, CA). Calpeptin was from Biomol (Plymouth Meeting, PA). Most other chemicals used were from Sigma Chemical Company (St Louis, MO). The iron:NTA complex (1:3 Fe/NTA) was prepared as previously described (32).

Culture and treatment of cells: Two human hepatoma HepG2 cell lines described in (29), were used as models in this study: E47 cells, which constitutively express human CYP2E1, and C34 cells, which are HepG2 cells transfected with the empty pCI vector. Both cell lines were grown in MEM containing 10% fetal bovine serum and 0.5 mg/mL of G418 supplemented with 100 units/mL of penicillin and 100 µg/mL of streptomycin, in a humidified atmosphere in 5% CO$_2$ at 37°C. Cells were subcultured at a 1:5 ratio once a week. For the experiments, cells were plated at a density of 50000 cells/mL and incubated for 12 h, in MEM supplemented with 5% fetal bovine serum and 100 units/mL of penicillin and 100 µg/mL of streptomycin (MEM$_{exps}$). After this period, the medium was replaced with MEM$_{exps}$ supplemented with arachidonic acid (from 0 to 5 µM). For assays of PLA2 activity in situ, the medium was additionally supplemented with 0.2 µCi/ml $^3$H-AA (around 1 nM $^3$H-AA). After 12 h of incubation at 37 °C, the medium was removed and the cells were washed once with PBS to remove unincorporated arachidonic acid. The cells were incubated for an additional 12 h period with MEM$_{exps}$. Then, the cells were washed and the medium was replaced according to the experimental protocol in use. That medium was supplemented with any additions (e.g. antioxidants, inhibitors) for 1h, prior to the addition of buffer or Fe-NTA (25 µM), which was considered as the initiation of the cellular toxicity phase (time= 0h). The cells were incubated for variable periods (up to 12h) before the biochemical analyses. This basic protocol, i.e., preloading with arachidonic acid, washing, adding the appropriate medium, and initiating the toxicity phase by addition of Fe-NTA, was used for all experiments.
**Measurement of PLA2 activity in situ:** Phospholipase A₂ activation was monitored in cultured cells using tritiated arachidonic acid as described in Briand et al. (1998) (35). 50000 cells were plated onto 24 well plates in MEM exps. Cells were first incubated with 2 µM AA and labeled with 0.2 µCi/ml ³H-AA, in MEM exps for 12h. Cells were washed with PBS, and incubated with MEM exps for an additional 12h-period. After this, the cells were washed 4 times with PLA2 assay buffer: PBS pH 7.2 supplemented with 5.5 mM glucose, 0.8 mM MgSO₄, 0.1% fatty acid free BSA and 0 or 1 mM CaCl₂. 500 µl of PLA2 assay buffer at 37 °C was added per well. Cells were put in a shallow water bath at 37 °C for 15 min, to allow equilibration of the cells. Then, where indicated, inhibitors were added, and the cells incubated for 1h.

After this time, buffer or Fe-NTA (25 µM) was added, and ³H-AA release was evaluated at several time points (0.5 to 3h). To evaluate ³H-AA release, the medium containing the released ³H-AA was removed, placed in eppendorf tubes and centrifuged for 2 min at 5000 rpm. The supernatant was transferred to scintillation vials and the pellet containing detached cells was kept. The cells in each well were resuspended by adding 500 µl of 0.1% Triton X-100. The content of each well was transferred to the eppendorf tubes containing the corresponding cellular pellet, and vortexed. Finally, the suspension was transferred to scintillation vials. 4 ml of scintillation fluid was added to each vial, and after vigorously shaking, the radioactive content was determined by scintillation counting using a β-counter. The data were expressed as percentage of cellular ³H-AA released: 100x[³H-AA released/(³H-AA incorporated+³H-AA released)]. 24±4% of the initial ³H-AA was taken up by the cells. 86±4% of the radioactivity taken up by the cells was esterified in phospholipids, and free AA represented only 0.4% of the total radioactivity incorporated in the cells. ³H-AA was found to account for 79±6% of the total radioactivity recovered in the medium. Cell extraction was performed by the method of Folch et al. (1957) (36). The extract was washed two times with 0.5 ml of a rinse solution containing chloroform:methanol:water (3:48:47, by volume) (37). The lipid phase (concentrated under N₂ and redissolved in 15 µl chloroform) was applied to a Silicagel 60 TLC plate and developed in petroleum ether/ethylic ether/methanol/acetic acid (80:20:2.5:1,
by volume) to resolve radiolabelled AA and other lipids. The lipid zones were located with I₂ vapor; they were then scraped into scintillation vials, and the radioactivity was measured after the addition of scintillation fluid. Identification of the lipids was based on a comparison of their TLC mobilities with those of authentic unlabelled standards (specifically R_f=0 for phospholipids, and R_f=0.43 for AA).

**Identification of the location of arachidonic acid in phospholipids:** This control was performed following the protocol of Kim and Southard (1999) (37). Lipids from cells labeled with ³H-AA were extracted, and the dried extract redissolved in 1 ml of 0.1 mM Tris-HCl buffer containing 15 mM CaCl₂ (pH 8.5). After 15 seconds sonication on ice, half of the sample was treated with 100 IU snake venom PLA₂ and incubated at 37 °C for 1 h. The other half was also incubated for 1 h but in the absence of the enzyme. The reaction was terminated with 5 ml chloroform:methanol (2:1, by volume), the lipids extracted and chromatographed on silicagel 60 TLC plates with petroleum ether:ethyl ether:methanol:acetic acid (80:20:2.5:1, by volume) to separate phospholipids (R_f=0) from AA (R_f=0.43). After exposure to iodine vapor, the spots were identified and scraped into vials containing 4 ml scintillation fluid. In samples treated with PLA₂, 99% of the radioactivity was located in the AA spot. This suggests that most of the arachidonic acid incorporated into phospholipids occupies the sn-2 position.

**Cytotoxicity measurements:** 5 x 10⁴ cells were plated onto 24 well plates and after the corresponding treatment, the medium was removed, and cell viability was evaluated by the MTT test (38) as previously described (39). Another index of cytotoxicity used was the leakage of lactate dehydrogenase (40). LDH activity was assessed in a medium containing 50 mM KPi, pH 7.4, 170 µM NADH, and 562 µM pyruvate. Cytotoxicity was expressed as % LDH release: (100 x (LDHout/LDH out+LDH in)).

**Measurement of intracellular calcium:** The intracellular calcium levels were determined with the fluorescent calcium indicator fluo3-AM by flow cytometry. 5x 10⁵ cells were plated in 10 mm-Petri dishes and at the end of the various treatments the medium was replaced with 3 mL of MEM exps without fetal bovine serum plus 2.5 µM fluo3-AM and 0.02% pluronic acid (stock solution x1000 in DMSO). Cells were incubated for 30 min at 37°C. After loading, the cells were washed in PBS (to remove any dye non-
specifically associated with the cell surface), trypsinized, and resuspended in 1 mL of MEM exps without fetal bovine serum plus 5 µg of propidium iodide. Propidium iodide was used to assay for the viable cell population as these cells exclude this dye, whereas non-viable cells take up this dye. The measurement of 

$[\text{Ca}^{2+}]_i$ was performed by flow cytometry analysis of 5000 cells using Cell Quest software. Intracellular calcium level was evaluated as Fluo3 fluorescence intensity in propidium iodide negative (i.e. viable) cells (41). 10 µM ionomycin was applied to one sample before each experiment to check for correct loading of the cells and thus served as a positive control. The inhibitors tested did not interfere with the quantification of the fluorescence (488/525 nm excitation/emission) of a standard fluo3-Ca$^{2+}$ solution. Quinacrine itself presented high fluorescence at 488/525 excitation/emission, and therefore was not used in fluorescence measurements at these wavelengths.

**Lipid peroxidation assay:** The production of thiobarbituric acid-reactive substances (TBARS) was assayed as previously described (38). The protein concentration of the cell suspension was determined using a protein assay kit based on the Lowry assay (BioRad DC kit). The concentration of MDA was calculated from a standard curve prepared using malonaldehyde bisdimethylacetal (42).

**Antioxidant activity of inhibitors:** The possible antioxidant activity of phospholipase A2 inhibitors was evaluated following the protocol described in Rodriguez et al. (2001) (43). Microsomal membranes from control rat liver (250 µg protein/0.10 mL) were mixed with 0.5 µL of each drug (stock solutions x 200), and preincubated for 10 min at 37 ºC in a reaction buffer consisting of 10 mM potassium phosphate, pH 7.2. Lipid peroxidation reactions were initiated by the addition of 0.05 mM FeCl$_3$ chelated by 1 mM ADP and 0.4 mM NADPH. At various times of incubation, the levels of TBARS were determined as described above. The compounds tested did not interfere with the quantification of MDA in the standard curve at the concentrations employed.

**CYP2E1 activity:** CYP2E1 activity was measured in liver microsomes from acetone-treated rats (1% acetone v/v in the drinking H$_2$O for 7 to 10 days), by the spectrophotometric analysis of p-nitrophenol hydroxylation as described in Dai et al. (1993) (44). The tested compounds did not interfere with the
quantification of 4-nitrocatechol.

**Measurement of calpain activity in situ:** The activity of calpain in intact cells was assessed with the use of the cell permeable fluorogenic substrate SUCC-LLVY-AMC (45) as previously described (34). The tested compounds at the concentrations used did not interfere with the quantification of AMC fluorescence.

**Flow cytometry analysis of the mitochondrial membrane potential:** The mitochondrial transmembrane potential was analyzed from the accumulation of rhodamine 123, a membrane-permeable cationic fluorescent dye. Cells were plated onto 6 well plates, and at the end of the treatment the medium was replaced with fetal bovine serum-free MEMEXP containing 5 µg/ml rhodamine 123, and incubated at 37 °C for 1 h. The cells were then harvested by trypsinization, washed with PBS, and resuspended in 1 mL of fetal bovine serum-free MEMEXP. The intensity of fluorescence from rhodamine 123 was determined using a BD FACS Calibur Flow Cytometer (San Jose, CA) as previously described (38).

**Statistics:** Data are expressed as mean ± standard error of the mean from 1 to 5 independent experiments run in duplicate. One-way analysis of variance (ANOVA) with subsequent post hoc comparisons by Scheffe was performed. A p < 0.05 was considered as statistically significant.
RESULTS

CYP2E1-dependent toxicity and protection by PLA2 inhibitors.

As previously described (38), addition of Fe+AA to CYP2E1-expressing HepG2 cells caused a rapid loss of cell viability (Fig 1A-E, 0 concentration of inhibitor). This mode of cell death was shown to be necrosis based upon assays of trypan blue uptake, release of lactic dehydrogenase and propidium iodide staining, and no DNA ladder formation (38). The effect of phospholipase A2 inhibitors on this toxicity of Fe+AA was tested. Aristolochic acid and quinacrine (two general non-specific PLA2 inhibitors) partially prevented the toxicity of Fe+AA in these cells, with optimal concentrations (maximal inhibition with lowest toxicity by itself) of 200 µM and 100 µM, respectively) (Fig 1A and 1B respectively). PTK, a specific inhibitor of cPLA2 and iPLA2, also showed significant protection against the toxicity, with 20 µM optimal concentration (Fig 1C). BPB (selective inhibitor of sPLA2) and BEL (selective inhibitor of iPLA2) did not show significant protection against the toxicity of Fe+AA (Fig 1D and E, respectively). Control HepG2 cells not expressing CYP2E1 did not show significant toxicity after exposure to Fe+AA and/or aristolochic acid (Fig 1F), confirming the role of CYP2E1 in this Fe+AA toxicity model.

CYP2E1-dependent toxicity and activation of PLA2.

As several PLA2 inhibitors partially protected the CYP2E1-expressing HepG2 cells from the toxicity of Fe+AA, we evaluated the activation of PLA2 in this system, by measuring in situ the release of $^3$H-AA as described in Materials and Methods. Cells were first loaded with 2 µM AA + 0.2 µCi/ml $^3$H-AA for 12h and after several washing steps, the percentage of release of $^3$H-AA was measured at different time points after exposure to Fe-NTA. E47 cells treated with Fe+AA showed a time-dependent increased release of $^3$H-AA (denoting increased PLA2 activity) with respect to cells which were treated only with AA (Fig 2A).

The non CYP2E1-expressing cells also showed an increased release of $^3$H-AA especially after 3h of exposure to Fe-NTA, but the release was lower than that measured in the cells expressing CYP2E1 (Fig 2A). These results suggest an increased activation of PLA2 by AA+Fe in the CYP2E1-expressing cells. As a non-CYP2E1 dependent control, C34 and E47 cells were preloaded with 2 µM AA + 0.2 µCi/ml $^3$H-AA...
and then exposed to a calcium ionophore, A23187 (a potent PLA2 activator), for up to 3h. Both cell lines showed comparable release of $^3$H-AA by the calcium ionophore at all time points evaluated (Fig 2B). A23187-activated release of $^3$H-AA was partially blocked (41%) by 200 µM aristolochic acid (not shown).

To evaluate simultaneously the kinetics of activation of PLA2 and the toxicity by Fe+AA, the release of $^3$H-AA and of LDH was measured in the same experimental sample, as described in Materials and Methods. $^3$H-AA release and LDH release proceeded simultaneously, both reactions starting after 1.25h of exposure of AA-loaded CYP2E1-expressing cells to Fe-NTA (Fig 3). Interestingly, a 5-fold increase in AA release (2% to 10% at 3h) was associated with a corresponding 5-fold increase in LDH release (5% to 25% at 3h).

If the tested PLA2 inhibitors prevented toxicity by effectively inhibiting PLA2 activity, then they should also block the AA+Fe-activated release of $^3$H-AA. Table 1 shows that aristolochic acid, quinacrine and PTK added at their optimal protective concentrations (Fig 1) significantly inhibited the release of $^3$H-AA triggered by Fe-NTA in AA-loaded cells expressing CYP2E1. BPB and BEL, inhibitors which did not block the toxicity of Fe+AA, also did not block the release of $^3$H-AA. α-Tocopherol, which was shown to completely block the toxicity of AA+Fe (38), completely blocked the Fe+AA-activated release of $^3$H-AA. For the inhibitors tested, controls for comparison were carried out using the solvent in which the inhibitor was dissolved (ethanol for PTK and α-tocopherol, DMSO for BPB and BEL, and water for quinacrine and aristolochic acid) (Table 1).

Activation of PLA2 by Fe-NTA was further validated by measuring the activity of cPLA2 in cell lysates, using arachidonoyl thio-PC as substrate, according to the manufacturer’s instructions (Cayman Chemical Company kit 765021, Ann Arbor, MI). In non-treated CYP2E1-expressing cells, cPLA2 activity was 0.31±0.05 nmol/min/mg protein while in AA+Fe-treated cells, cPLA2 activity was 0.58±0.06 nmol/min/mg protein (around 2-fold increase) (data not shown).

Experiments were carried out to check for the possible inhibitory effect of quinacrine, aristolochic acid and PTK on CYP2E1 activity and lipid peroxidation. CYP2E1 activity was measured in microsomes obtained...
from acetone-treated rats. The tested PLA2 inhibitors did not block CYP2E1 activity at the optimal concentration used, while a known CYP2E1 inhibitor, 50 µM 4-methyl pyrazole blocked CYP2E1 activity by 70% (data not shown). Since α-tocopherol blocked the AA+Fe toxicity and activation of PLA2, it was important to evaluate whether aristolochic acid, quinacrine or PTK had an antioxidant action similar to that of α-tocopherol. NADPH + (Fe)ADP-dependent lipid peroxidation, assessed as TBARS generation in microsomes obtained from control rats, was not blocked by the inhibitors tested, although 50 µM trolox significantly blocked (96%) TBARS generation (data not shown). The effect of the inhibitors on the TBARS content of homogenates from cells expressing CYP2E1 and exposed to Fe+AA was also tested to evaluate whether they exerted an antioxidant action in intact cells. As shown in Table 2, AA+Fe increased the production of TBARS 5 fold, an increase totally prevented by α-tocopherol. The PLA2 inhibitors did not have any effect on the content of TBARS of the Fe+AA-treated cells.

**Activation of PLA2 and calcium.**

Experiments were performed in order to test the Ca²⁺-dependence of the activation of PLA2 detected in the Fe+AA-treated E47 cells, since BEL, an inhibitor of the calcium-independent PLA2, did not protect against the AA+Fe toxicity or the ³H-AA release. Cells were preloaded with 2 µM AA + 0.2 µCi/ml ³H-AA, and after several washing steps the medium was replaced with PLA2 assay buffer containing 1 mM or 0 mM CaCl₂. Buffer or 25 µM Fe-NTA was added, and the release of ³H-AA was measured at different time points. As shown in Fig 4A, there was no statistical difference in ³H-AA release between Ca²⁺ containing or Ca²⁺-deficient medium in Fe+AA-treated cells, although after subtracting out the release of ³H-AA in the AA alone controls, a 42% decrease was found for the minus calcium medium. Nevertheless, substantial AA release occurs in the Ca²⁺-deficient medium. In order to minimize adventitious calcium contamination in the buffer, PBS was treated with 5% (w/v) Chelex-100 for 1h (46). The Chelex treatment did not significantly modify the release of ³H-AA triggered by Fe-NTA in 0 mM Ca²⁺ medium, suggesting that the effect was not due to contaminating extracellular calcium (data not
shown). The release of $^{3}$H-AA in Fe+AA treated cells in 0 mM Ca$^{2+}$ medium was efficiently blocked by aristolochic acid and α-tocopherol (Fig 4B), results similar to that observed in 1 mM Ca$^{2+}$ (Table 1).

Although the activation of PLA2 in the Fe+AA-treated cells may not entirely depend on extracellular calcium, it is possible that intracellular calcium stores may be involved in the activation. To evaluate for this possibility, cells were depleted of stored intracellular calcium before the addition of Fe-NTA. This was performed by first washing the AA-loaded cells with SMEM, then incubating in SMEM containing 10 μM A23187 and 500 μM EGTA for 30 min at 37 °C (47). The cells were finally washed with SMEM before the addition of Fe-NTA in 0 mM Ca$^{2+}$ assay buffer. Fig 4C shows that the Ca$^{2+}$ depletion protocol significantly inhibited $^{3}$H-AA release after exposure of AA-loaded cells to Fe-NTA in 0 mM Ca$^{2+}$ medium. With respect to effect on toxicity, Table 3 confirms the previous observation (34) that AA+Fe toxicity was much greater (51% loss of viability) in Ca$^{2+}$-containing medium (MEM) than in Ca$^{2+}$ deficient medium (SMEM, 25% loss of viability). However, 3h incubation of AA-loaded cells in 0 mM Ca$^{2+}$ medium, with depleted intracellular Ca$^{2+}$ stores, proved to be not toxic at all with respect to cells incubated in 0 mM and especially 1 mM Ca$^{2+}$ medium (Table 3). Removal of extracellular Ca$^{2+}$, and removal of extracellular calcium +intracellular calcium stores strongly reduced the toxicity of Fe+AA in the CYP2E1 expressing cells (Table 3).

An increase in intracellular calcium can be detected after exposure of these cells to Fe+AA in MEM but not in SMEM. This increase occurs prior to the developing toxicity and is inhibited by α-tocopherol, but not by calpeptin (34). Fig 5 shows that the PLA2 inhibitors aristolochic acid and PTK significantly inhibited the increase in $[Ca^{2+}]_{i}$ observed after incubating AA-loaded cells with Fe-NTA in MEM for 3h. This suggests that activation of PLA2 is required prior to the influx of extracellular Ca$^{2+}$ into the CYP2E1 expressing HepG2 cells.

**Activation of PLA2 and mitochondrial damage.**

Mitochondrial damage is a very early effect leading to necrosis in several experimental models (48). In the
CYP2E1-expressing HepG2 cells treated with Fe+AA, mitochondrial damage manifested as a decrease in mitochondrial membrane potential was a very early event that preceded the permeabilization of the plasma membrane (49). The effect of PLA2 inhibitors on this AA+Fe-induced mitochondrial damage was assessed. As shown in Fig 6, Fe+AA increased the number of cells in the M1 low membrane potential zone (low rhodamine 123 fluorescence). The PLA2 inhibitors aristolochic acid and PTK significantly prevented the decrease of mitochondrial membrane potential observed in the Fe+AA-treated E47 cells.

A control experiment was performed in order to test for a possible direct effect of PLA2 inhibitors on the mitochondrial permeability transition. Mitochondria were isolated, energized and loaded with Ca²⁺. After exposing mitochondria to 10 μM AA, the permeability transition-dependent swelling was measured on the basis of the absorbance changes at 540 nm, following the protocol described in (50). As was shown by Scorrano et al., (2001) (50), AA-dependent swelling was inhibited (60-80%) by 2 μM cyclosporin A, a specific inhibitor of the permeability transition (Fig 7). Stock solutions of cyclosporin A and AA were prepared in ethanol; control experiments showed that the vehicle alone did not induce any mitochondrial swelling and that the vehicle alone did not prevent the AA-induced swelling. As discussed in (51), inhibition and persistence of inhibition by cyclosporin A was dependent on the inducer of the mitochondrial permeability transition, ranging from complete to approximately 50%. AA-dependent swelling was not significantly inhibited by 20 μM PTK, 100 μM quinacrine, or 100 μM aristolochic acid, suggesting that PLA2 inhibitors do not directly affect the permeability transition (Fig 7). A PLA2 inhibitor, trifluoperazine, was reported to inhibit the mitochondrial permeability transition, however, this inhibition was related to a change of surface charge of the mitochondrial membrane and not inhibition of PLA2 (51). Thus, the ability of these inhibitors to prevent the decrease of the mitochondrial membrane potential by Fe+AA in the CYP2E1-expressing HepG2 cells is a reflection of their ability to inhibit PLA2 activity.

**CYP2E1-dependent activation of calpain and calcium.**

It has been previously shown that calpain is activated in the Fe+AA-treated cells expressing CYP2E1, and that toxicity is partially inhibited by 2 calpain inhibitors, calpeptin and E64d (34). Experiments were performed in order to evaluate if Fe+AA-activated calpain activity requires extracellular calcium, or if
intracellular calcium stores can play a role in the activation analogous to what was observed for PLA2 activation. Fe+AA-activated calpain activity assessed in buffer containing 1 mM CaCl₂ was significantly blocked by calpeptin and completely prevented by α-tocopherol (Table 4). When the experiment was performed in buffer containing 0 mM CaCl₂, Fe+AA again activated calpain activity; the net increase by Fe-NTA was 65% that in Ca²⁺-containing medium. This activation was also significantly inhibited by calpeptin and α-tocopherol (Table 4). These results suggest that the activation of calpain in the Fe+AA-treated cells does not require extracellular calcium, although it is still possible that calcium from intracellular stores may play a role in the activation. Thus, AA-loaded cells were depleted from calcium in intracellular stores with A23187+EGTA as described above, and Fe-NTA-activated calpain activity was assessed in these cells. Fe-NTA did not activate calpain activity in the Ca²⁺-depleted cells when incubated in Ca²⁺-deficient medium.

**PLA2 activation and toxicity of Fe+AA in Ca²⁺-deficient medium.**

PLA2 was activated by Fe+AA in the CYP2E1-expressing HepG2 cells in Ca²⁺-deficient medium (Fig 3). Toxicity by Fe+AA in Ca²⁺-deficient medium is much less than in Ca²⁺-containing medium (Table 3) especially at shorter incubation times e.g. <6h after addition of Fe-NTA, but significant toxicity is observed after 12h of exposure (34). This toxicity in SMEM at 12h incubation was not prevented by calpeptin (49). Is the activation of PLA2 in Ca²⁺-deficient medium related to the delayed toxicity of Fe+AA in these conditions? To test this, inhibitors of PLA2 were preincubated for 1h in AA-loaded cells, in SMEM, followed by the addition of Fe-NTA for 12h. As shown in Fig 8, Fe+AA caused an approximate 50% loss of cell viability in control incubations. The PLA2 inhibitors significantly blocked this toxicity.
DISCUSSION

Previous work (38,52,53) has shown that the toxicity of Fe+AA in CYP2E1-overexpressing HepG2 cells is principally mediated by oxidative stress. Toxicity is dependent on CYP2E1 expression, as HepG2 cells that do not express P450 activity show low levels of cytotoxicity by Fe+AA (38,53, and Fig 1). CYP2E1-mediated generation of ROS in an intracellular environment containing an active metal catalyst (Fe) and an oxidizable substrate (AA) is a key component of oxidative stress and toxicity in this system. Previous studies indicated that there was an early toxicity phase which required extracellular Ca$^{2+}$ and a later toxicity phase which occurred in Ca$^{2+}$-deficient medium (34). One Ca$^{2+}$-activated hydrolase, calpain, played a role in the early but not later toxicity phase (49). The current study was carried out to assess the role of another Ca$^{2+}$-activated hydrolase, PLA2, in the toxicity.

The CYP2E1-expressing HepG2 cells treated with Fe+AA showed increased PLA2 activity, measured as release of pre-labeled $^{3}$H-AA. Release of prelabeled $^{3}$H-AA is an appropriate measure of PLA2 activity because: i) prelabeled $^{3}$H-AA was mainly (86%) incorporated into phospholipids, in the sn-2 position predominantly, thus presenting an adequate substrate for PLA2 catalysis; ii) released radioactivity was shown to be mainly $^{3}$H-AA (79%); iii) release of $^{3}$H-AA was inhibited by the PLA2 inhibitors quinacrine, aristolochic acid, and PTK. The non CYP2E1-expressing HepG2 (C34) cells exposed to Fe+AA showed lower activation of PLA2, indicating that the higher activity of PLA2 in E47 cells is a CYP2E1-dependent effect. A possible lower content of PLA2 capable of being activated by Ca$^{2+}$ is not a cause for the low activation of PLA2 in C34 cells, as a calcium ionophore activated PLA2 to the same extent in both HepG2 cell lines.

Increased AA release in AA+Fe-treated cells expressing CYP2E1 is likely a reflection of increased PLA2 activity, and not simply an inhibition of AA reacylation due to ATP depletion because: i) cPLA2 activity in cell lysates of Fe+AA-treated cells, measured using a synthetic substrate in a reaction system not dependent on ATP, significantly increased, and ii) CYP2E1-expressing HepG2 cells exposed to AA+Fe in a medium lacking extracellular Ca$^{2+}$, showed increased AA release (Fig 4), although ATP levels did not
PLA2 activity and cytotoxicity appear to be causally related because inhibiting PLA2 activity with quinacrine, aristolochic acid and PTK significantly blocks toxicity, and PLA2 activation and LDH release are simultaneous early events. Interestingly, in contrast to calpain, PLA2 activation is an early event that is linked to cytotoxicity in cells exposed to Fe-NTA in a buffer lacking Ca\(^{2+}\), since significant release of \(^3\text{H}-\text{AA}\) occurred in Ca\(^{2+}\)-deficient medium, and the above PLA2 inhibitors also blocked the AA+Fe-induced toxicity. Thus, PLA2 plays a role in the AA+Fe early toxicity which occurs in Ca\(^{2+}\)-containing medium, and in the later toxicity which occurs in Ca\(^{2+}\)-deficient medium. Low activation of PLA2 in C34 cells treated with Fe+AA in buffer containing 1 mM Ca\(^{2+}\) was associated with lower toxicity. To our knowledge, this is the first report of a critical role for PLA2 in a CYP2E1-dependent model of toxicity. The specific isoform involved is probably cPLA2, since selective inhibitors of sPLA2 (BPB) and iPLA2 (BEL) were ineffective in blocking toxicity and PLA2 activity, while a selective inhibitor of i plus cPLA2 (PTK) was effective. Further studies to specifically validate this will be necessary. Toxicity and PLA2 activation were completely inhibited in Ca\(^{2+}\)-depleted cells, pointing to a requirement of Ca\(^{2+}\) for PLA2 activation. Many reports in the literature indicate that cPLA2 regulates the initiation of AA metabolism in response to stimuli that mobilize intracellular Ca\(^{2+}\) (54).

Although Ca\(^{2+}\) is necessary for PLA2 activation and toxicity, the results obtained suggest that the release of Ca\(^{2+}\) from intracellular stores is a probable major initial source of this Ca\(^{2+}\). The intracellular stores from which Ca\(^{2+}\) may be released consist of three major components: a Ca\(^{2+}\), Mg\(^{2+}\)-ATPase that sequesters calcium; Ca\(^{2+}\)-binding proteins that store Ca\(^{2+}\) (calsequestrin and calreticulin); and the specific IP\(_3\) and ryanodine receptors that release Ca\(^{2+}\) back to the cytosol (55-57). It is well documented that various oxidants including iron can inhibit the Ca\(^{2+}\)-Mg\(^{2+}\) ATPase; in contrast, oxidative modifications of the IP\(_3\) and ryanodine receptors leads to activation of both channels (58-60). Oxidative modifications
caused by Fe+AA in E47 cells may modify the activity of these enzymes and cause release of Ca\(^{2+}\) from intracellular stores, and subsequent activation of PLA2 in the absence of extracellular calcium. Further studies are planned to evaluate these possibilities after isolating microsomes from the E47 cells, analogous to experiments showing release of accumulated microsomal Ca\(^{2+}\) by Fe or by acetaminophen (61,62).

PLA2 activity in Fe+AA-treated E47 cells incubated in a medium with or without Ca\(^{2+}\) was blocked by \(\alpha\)-tocopherol (Table 1, Fig 4), suggesting an oxidant-dependent process was involved in the PLA2 activation. Control experiments indicated that the PLA2 inhibitors did not show an antioxidant activity under the condition utilized, and the PLA2 inhibitors did not block the generation of TBARS triggered by AA+Fe-NTA (Table 2).

A previous study showed that inhibition of calpain by calpeptin did not block the AA+Fe-induced influx of Ca\(^{2+}\) from the extracellular medium (49). However, the PLA2 inhibitors did block this increase in intracellular calcium (Fig 5). PLA2-mediated increase of intracellular calcium may represent a critical step in the mechanism of the early toxicity phase. A recent report shows that the Ca\(^{2+}\) influx in PC12 cells exposed to polychlorinated biphenyls was mediated through PLA2 activation (63). The mechanism for this PLA2-mediated increase in intracellular calcium may involve a direct effect on the plasma membrane by depletion of phospholipids or by free fatty acids and lysophospholipids that can increase membrane permeability to Ca\(^{2+}\) (64), or an indirect effect mediated by store operated channel activation caused by P450 metabolites of free AA. Such activation of Ca\(^{2+}\) channels by coupled cPLA2 plus P450 activity has been proposed as a possible mechanism for capacitative calcium entry (65). The fact that SKF 96365 (a store operated channel inhibitor) did not inhibit toxicity of Fe+AA in E47 cells (34) may indicate that direct effects on the plasma membrane are more important.

While PLA2 (Fig 4) and lipid peroxidation (34) are activated to similar or slightly different extents in AA-loaded cells exposed to Fe-NTA in 0 or 1 mM Ca\(^{2+}\) medium, toxicity was much lower in the buffer lacking Ca\(^{2+}\), and was delayed to longer incubation times e.g. 12h instead of 3h. Increased PLA2 activity
and lipid peroxidation in E47 cells treated with Fe-NTA in Ca\textsuperscript{2+}-deficient medium is not sufficient to produce rapid toxicity probably because of the lack of Ca\textsuperscript{2+} incorporation from the extracellular medium. The results obtained suggest that lipid peroxidation of cellular membranes is not sufficient per se for rapid toxicity, but that Ca\textsuperscript{2+} incorporation from the extracellular medium is, and peroxidation-induced PLA2 activity (but not lipid peroxidation alone) is a key mediator of this increase.

Toxicity of AA + Fe in SMEM develops at longer time periods (i.e. 12h). Toxicity is still blocked by α-tocopherol, but it is not associated with measurable increase in intracellular Ca\textsuperscript{2+} (49). PLA2 activity (Fig 4) and the later toxicity (Fig 8) in AA-loaded E47 cells exposed to Fe-NTA in a medium without Ca\textsuperscript{2+} were blocked by PLA2 inhibitors. This suggests that apart from increasing influx of Ca\textsuperscript{2+}, PLA2 activity is acting on other cellular targets that result in toxicity without requiring influx of extracellular Ca\textsuperscript{2+}. Possible targets are lysosomes and mitochondria. Destabilization of the lysosomal membrane and leakage of lysosomal contents were associated with activation of PLA2 in H\textsubscript{2}O\textsubscript{2}-treated J774 cells (66).

Phospholipids are important constituents of the mitochondrial membrane and are essential for activity of certain mitochondrial enzymes (64). Ischemia-reperfusion induced a PLA2-dependent mitochondrial damage in renal mitochondria (67). Structural alteration of the inner mitochondrial membrane was associated with activation of PLA2 (68). A decrease in the mitochondrial membrane potential by AA+Fe was shown to occur in both Ca\textsuperscript{2+}-containing and Ca\textsuperscript{2+}-deficient medium, and the decline preceded the early and late toxicity phases induced by AA+Fe (49).

Calpain activation by Fe+AA in E47 cells is Ca\textsuperscript{2+}-dependent because it was completely blocked in Ca\textsuperscript{2+}-depleted cells incubated in 0 mM Ca\textsuperscript{2+} (Table 4). AA-loaded cells exposed to Fe-NTA in 1 or 0 mM CaCl\textsubscript{2} exhibited increases of calpain activity (Table 4), suggesting that stored Ca\textsuperscript{2+} contributes to the calpain activation in this system. α-Tocopherol blocked calpain activation by Fe-NTA in the absence and presence of external calcium, linking calpain activation with oxidative stress in both media. Calpain
contributes to the early toxicity by AA+Fe in Ca$^{2+}$-containing medium because calpeptin and E64d partially blocked the toxicity and the decline in mitochondrial membrane potential (49). However, calpain, unlike PLA2, did not appear to play a role in the late toxicity phase in Ca$^{2+}$-deficient medium since calpeptin had no effect on the toxicity or the decline in membrane potential (49).

Nanji et al. (1993) (16) have shown that in rats treated with corn oil plus ethanol for 1 month (intragastric infusion model), a decrease in microsomal AA correlated with CYP2E1 induction, lipid peroxidation and increased phospholipase A$_2$ activity, with respect to controls fed glucose instead of ethanol. This in vivo correlation between loss of AA-CYP2E1 induction-lipid peroxidation-and PLA2 activity was reproduced in this work using an in vitro cellular model of CYP2E1 overexpression. This model suggests the scheme shown in Fig 9 as a possible mechanism for the AA release in CYP2E1-overexpressing cells, and the subsequent toxicity, with intracellular calcium playing a central role. The combination of CYP2E1 plus a polyunsaturated fatty acid such as AA plus Fe increases lipid peroxidation in the phospholipid environment; this is followed by activation of calpain and PLA2 probably by increased release of Ca$^{2+}$ from intracellular stores and structural alterations in the membranes. PLA2 activation contributes to an increase in the influx of Ca$^{2+}$ from the extracellular space. Increased Ca$^{2+}$, lipid peroxidation, calpain activity, and PLA2 activity are events that are suggested to converge on the mitochondria, inducing a profound bioenergetic failure and a rapid necrotic cell death. The scheme presented includes previous observations where CYP2E1+Fe+AA activated calpain through lipid peroxidation events, and activated calpain was involved in mitochondrial damage and cell death (34,49). The combination of increased lipid peroxidation and PLA2 activity, but not calpain, induced a delayed bioenergetic failure in the absence of increased Ca$^{2+}$ which results in cell death after more prolonged incubation. Given the importance of CYP2E1 in activating hepatotoxins such as acetaminophen, CCl$_4$, benzene, nitrosamines, and in producing ROS, and contributing to alcohol-induced liver injury, these studies suggest that the control of intracellular calcium release and inhibition of PLA2 activity may represent strategies to block early events of CYP2E1-dependent cytotoxicity.
ACKNOWLEDGEMENTS
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REFERENCES


FOOTNOTES

1 Abbreviations: PLA2, phospholipase A2; Fe-NTA, iron-nitrilotriacetate 1:3 complex; MTT, 3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; LDH, lactate dehydrogenase; TBARS, thiobarbituric acid reactive substances; AA, arachidonic acid; MDA, malondialdehyde; SUCC-LLVY-AMC, N-succinyl-leu-leu-val-tyr 7-amido-4-methylcoumarin; E47 cells, HepG2 cell line derived after transfection with pCI-neo vector containing the human CYP2E1 cDNA; C34 cells, HepG2 cell line derived after transfection with pCI-neo vector; MEM\textsubscript{exp}, MEM medium supplemented with 5% fetal bovine serum and 100 units/mL of penicillin and 100 µg/mL of streptomycin; SMEM, MEM medium lacking CaCl\textsubscript{2}; CYP2E1, cytochrome P450 2E1; PBS, phosphate buffered saline; AMC, 7-amido-4-methylcoumarin; BPB, 4-bromophenacyl bromide; BEL, bromoenol lactone; PTK, palmityl trifluoromethyl ketone; A.U., arbitrary units
Figure legends

Figure 1. Effect of PLA2 inhibitors on the toxicity of Fe+AA in E47 cells. E47 cells were preloaded without AA, or with 2 µM AA for 12h in MEM exps followed by washing with PBS and an additional 12h-period of incubation with MEM exps. After this, cells were preincubated for 1h with different concentrations of the following inhibitors: aristolochic acid (A, dissolved in H2O), quinacrine (B, dissolved in H2O), PTK (C, dissolved in ethanol), BPB (D, dissolved in ethanol), BEL (E, dissolved in DMSO). For controls, wells contained 1µl/ml of the solvent used to dissolve the inhibitor employed. A non-CYP2E1 control was run with C34 cells (F), using aristolochic acid as PLA2 inhibitor. Following this preincubation, Fe-NTA was added to cells preloaded with AA, followed by a 3h-incubation. After this, viability was assessed measuring MTT reduction. Empty circles: 0 µM AA + 0 µM Fe-NTA; filled circles: 2 µM AA + 25 µM Fe-NTA.
* Significantly different (p<0.05, ANOVA) with respect to cells incubated with AA+Fe in the absence of inhibitors.

Figure 2. Time course of 3H-AA release. HepG2 cells expressing (E47) or not expressing (C34) CYP2E1 were preloaded with 2 µM AA + 0.2 µCi/ml 3H-AA for 12 h in MEM exps. After this, cells were washed with PBS followed by an additional 12h-period of incubation in MEM exps. Cells were then washed 4 times and incubated for 15 min in assay buffer containing 1 mM CaCl2. (A) Buffer or 25 µM Fe-NTA was added to each well, and 3H-AA release was monitored as a function of time (0.5-3h). (B) 5 µMA23187 was added to each well, and 3H-AA release was monitored as a function of time (0.5-3h), in E47 or C34 cells.
* Significantly different (p<0.05, ANOVA) with respect to the same cell type incubated with AA for the same time period.

Figure 3. Time course of 3H-AA and LDH release activated by Fe+AA. E47 cells were preloaded with 2
µM AA + 0.2 µCi/ml ³H-AA for 12 h in MEM exps. After this, cells were washed with PBS followed by an additional 12h-period of incubation in MEM exps. Cells were then washed 4 times and incubated for 15 min in assay buffer containing 1 mM CaCl₂. Buffer or 25 µM Fe-NTA was added to each well, and ³H-AA and LDH release was monitored simultaneously as a function of time (1-3h).

**Figure 4. Ca²⁺-dependence of Fe+AA-activated ³H-AA release.** E47 cells were preloaded with 2 µM AA + 0.2 µCi/ml ³H-AA for 12 h in MEM exps. After this, cells were washed with PBS followed by an additional 12h-period of incubation in MEM exps. (A) Cells were then washed 4 times and incubated for 15 min in assay buffer containing 1 mM CaCl₂ (+Ca), or 0 mM CaCl₂ (-Ca). Buffer or 25 µM Fe-NTA was added to each well, and ³H-AA release was monitored as a function of time (0.5-3h). (B) Cells were washed 4 times and incubated for 15 min in incubation medium containing 1 mM CaCl₂ (+Ca), or 0 mM CaCl₂ (-Ca). Cells were preincubated for 1 h with 100 µM α-tocopherol, 200 µM aristolochic acid, or without any addition. Following this, Fe-NTA 25 µM was added to each well, and ³H-AA release was monitored as a function of time (0.5-3h). (C) AA-loaded cells were subjected to Ca²⁺ depletion (Ca depleted) by treatment with A23187+EGTA as described in Experimental Procedures. Non Ca²⁺-depleted cells were not treated with A23187+EGTA. Cells were then washed 4 times and incubated for 15 min in assay buffer containing 1 mM CaCl₂ (+Ca), or 0 mM CaCl₂ (Ca²⁺ depleted). Buffer or 25 µM Fe-NTA was added to each well, and ³H-AA release was monitored as a function of time (0.5-3h).

**Figure 5. Effect of PLA2 inhibitors on intracellular calcium.** E47 cells were preloaded with 2 µM AA for 12 h in MEM exps. After this, cells were washed with PBS followed by an additional 12h-period of incubation in MEM exps. Aristolochic acid, PTK, and a solvent control (ethanol) were preincubated for 1h, followed by the addition of buffer or 25 µM Fe-NTA. After 2h of incubation, intracellular calcium was
monitored by flow cytometry using Fluo3-AM + propidium iodide. Representative dot plots. FL1 stands for fluo3 fluorescence, and FL2 stands for propidium iodide fluorescence. Numerical values represent mean fluorescence (A.U.) in the FL1-channel, of the cells located in the lower right quadrant (i.e. propidium iodide negative cells).

**Figure 6. Effect of PLA2 inhibitors on mitochondrial damage by Fe+AA.** E47 cells were preloaded with 2 µM AA for 12 h in MEM exps. After this, cells were washed with PBS followed by an additional 12h-period of incubation in MEM exps. Aristolochic acid and PTK were preincubated for 1h, followed by the addition of buffer or 25 µM Fe-NTA. After 3h of incubation, mitochondrial membrane potential was monitored by flow cytometry using rhodamine 123. Representative histograms. FL1 stands for rhodamine 123 fluorescence. Numerical values represent percentage of cells in the M1 fraction (low rhodamine 123 fluorescence).

**Figure 7. Effect of PLA2 inhibitors on the permeability transition induced by arachidonic acid in isolated mitochondria.** The experimental protocol was essentially the one described by Scorrano et al., (2001) (50). The incubation medium contained 150 mM KCl, 10 mM Tris-MOPS, 5 mM glutamate Tris, 2.5 mM malate Tris, 1 mM Pi Tris, and 10 µM EGTA-Tris. Final volume was 2 ml, pH 7.4, 25 °C. The experiments were started by the addition of 1 mg of mitochondrial protein (isolated from rat liver by standard centrifugation techniques) (1 min incubation), followed by 20 µM Ca²⁺ (1 min incubation) and 0.5 mM EGTA-Tris (0.5 min incubation) (not shown). These additions did not significantly change the absorbance at 540 nm of the mitochondrial suspension (not shown). At time=0 sec, 10 µM AA was added, and the change in absorbance at 540 nm was evaluated for 300 sec. The vertical axes represent the absorbance at 540 nm, with 0.1 absorbance units per division of the scale; the horizontal axes represent time (0-300 sec), with 50 sec per division of the scale. Inhibitors were added before mitochondria: (A) upper curve, 2 µM cyclosporin A; lower curve, no inhibitor; (B) 20 µM PTK; (C) 100 µM quinacrine; (D) 100 µM aristolochic acid.

**Figure 8. Effect of PLA2 inhibitors on the toxicity of Fe+AA in Ca²⁺-deficient medium.** E47 cells were preloaded with 5 µM AA for 12 h in MEM exps. After this, cells were washed with PBS followed by an
additional 12h-period of incubation in MEM_{exp}. The medium was then replaced with SMEM_{exp}, and the cells were preincubated for 1h with several concentrations of PLA2 inhibitors (A, aristolochic acid; B, PTK; C, quinacrine). Then buffer or 25 µM Fe-NTA was added, the cells incubated for an additional 12h-period, and cell viability was assessed as MTT reduction activity. Empty circles: 0 µM AA + 0 µM Fe-NTA; filled circles: 5 µM AA + 25 µM Fe-NTA.

* Significantly different (p<0.05, ANOVA) with respect to cells incubated with AA+Fe in the absence of inhibitors.

**Figure 9.** Proposed mechanism for the peroxidation-dependent release of AA and cell death in CYP2E1-overexpressing cells. Please see text for details.
Table 1. Effect of inhibitors on Fe+AA-activated $^3$H-AA release. E47 cells were preloaded with 2 μM AA+0.2 μCi/ml $^3$H-AA for 12h in MEM$_{\text{exps}}$. Cells were washed with PBS and incubated for an additional 12h-period in MEM$_{\text{exps}}$. After this, cells were washed 4 times and incubated for 15 min in assay buffer containing 1 mM CaCl$_2$. Cells were preincubated for 1 h with several inhibitors: 200 μM aristolochic acid, 100 μM quinacrine, 20 μM PTK, 5 μM BPB, 10 μM BEL and 100 μM α-tocopherol. Control experiments were performed using the solvents in which the inhibitors were dissolved (water for aristolochic acid and quinacrine, ethanol for PTK and α-tocopherol, and DMSO for BPB and BEL) (1 μl/ml). Afterwards, buffer or 25 μM Fe-NTA was added to each well, and $^3$H-AA release was evaluated after 3h as described in Experimental Procedures.

<table>
<thead>
<tr>
<th>Addition</th>
<th>$^3$H-AA release (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>-Fe-NTA</td>
</tr>
<tr>
<td>AA</td>
<td>6.7 ± 0.7</td>
</tr>
<tr>
<td>AA + Ethanol</td>
<td>7.4 ± 0.4</td>
</tr>
<tr>
<td>AA + DMSO</td>
<td>5.4 ± 0.4</td>
</tr>
<tr>
<td>AA + Aristolochic Acid</td>
<td>3.2 ± 0.6</td>
</tr>
<tr>
<td>AA + Quinacrine</td>
<td>7.7 ± 0.4</td>
</tr>
<tr>
<td>AA + PTK</td>
<td>8.0 ± 1.6</td>
</tr>
<tr>
<td>AA + BPB</td>
<td>5.9 ± 0.5</td>
</tr>
<tr>
<td>AA + BEL</td>
<td>7.7 ± 0.4</td>
</tr>
<tr>
<td>AA + α-tocopherol</td>
<td>4.6 ± 0.9</td>
</tr>
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</table>

* significantly different (p<0.05, ANOVA) with respect to the release of AA in the presence of Fe-NTA in the appropriate solvent.
Table 2. Effect of inhibitors on the content of TBARS. E47 cells were preloaded with 2 µM AA for 12h in MEMexp. Cells were washed with PBS and incubated for an additional 12h-period in MEMexp. Cells were then preincubated for 1 h with several inhibitors: 200 µM aristolochic acid, 100 µM quinacrine, 20 µM PTK, and 100 µM α-tocopherol. Control experiments were performed using the solvents in which the inhibitors were dissolved (water for aristolochic acid and quinacrine, and ethanol (1 µl/ml) for PTK and α-tocopherol. Afterwards, buffer or 25 µM Fe-NTA was added to each well, the cells incubated for 3h, and the content of TBARS evaluated as described in Experimental Procedures.

<table>
<thead>
<tr>
<th>Addition</th>
<th>TBARS (nmol MDA/mg prot)</th>
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<tr>
<td></td>
<td>- Fe-NTA</td>
</tr>
<tr>
<td>AA</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>AA + Ethanol</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>AA + α-tocopherol</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>AA + Quinacrine</td>
<td>0.27 ± 0.03</td>
</tr>
<tr>
<td>AA + Aristolochic Acid</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>AA + PTK</td>
<td>0.17 ± 0.03</td>
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</table>

* significantly different (p<0.05, ANOVA) with respect to the content of TBARS in the presence of Fe-NTA in the appropriate solvent.
Table 3. Effect of Ca^{2+} manipulation on the toxicity of Fe+AA. E47 cells were preloaded with 2 µM AA for 12h in MEM_{exps}. Cells were washed with PBS and incubated for an additional 12h-period in MEM_{exps}. After this, cells were washed and the medium replaced with fetal bovine serum-free MEM_{exps} or SMEM_{exps}. To obtain Ca^{2+}-depleted cells, cells in SMEM_{exps} were supplemented with 5 µM A23187+500 µM EGTA, and the cells incubated for 30 min at 37°C. After this, cells were washed and the medium replaced with fetal bovine serum-free MEM_{exps} or SMEM_{exps}. Buffer or 25 µM Fe-NTA was added to each well, the cells incubated for 3h, and viability evaluated as MTT reduction activity.

<table>
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<th>Condition</th>
<th>Viability (%)</th>
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<tr>
<td>AA, MEM</td>
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<tr>
<td>AA + Fe, MEM</td>
<td>49 ± 10</td>
</tr>
<tr>
<td>AA, SMEM</td>
<td>93 ± 3</td>
</tr>
<tr>
<td>AA + Fe, SMEM</td>
<td>75 ± 10*</td>
</tr>
<tr>
<td>AA, SMEM, Ca^{2+}-depleted cells</td>
<td>88 ± 9*</td>
</tr>
<tr>
<td>AA + Fe, SMEM, Ca^{2+}-depleted cells</td>
<td>102 ± 7*</td>
</tr>
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</table>

* significantly different (p<0.05, ANOVA) with respect to the viability in the AA+Fe, MEM condition.
Table 4. Effect of calpeptin and calcium manipulation on the Fe+AA-mediated activation of calpain. E47 cells were preloaded with 2 µM AA for 12h in MEMexp. Cells were later washed with PBS and incubated for an additional 12h-period in MEMexp. To obtain Ca<sup>2+</sup>-depleted cells, cells in SMEMexp were supplemented with 5 µM A23187+500 µM EGTA, and the cells incubated for 30 min at 37°C. After this, cells were washed 4 times and incubated for 15 min in assay buffer containing 0 or 1 mM CaCl<sub>2</sub>. A group of cells were then preincubated for 1h with 50 µM calpeptin or 100 µM α-tocopherol. Afterwards, 25 µM SUCC-LLVY-AMC was added to each well, followed by a 5 min equilibration period. Fe-NTA (0 or 25 µM) was added to each well, the cells incubated for 3h, and AMC accumulation evaluated as described in Materials and Methods.

<table>
<thead>
<tr>
<th>Addition</th>
<th>[Ca&lt;sup&gt;2+&lt;/sup&gt;] (mM)</th>
<th>AMC fluorescence (A.U.)</th>
<th>Increase by Fe-NTA (A.U.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-Fe-NTA</td>
<td>+Fe-NTA 25 µM</td>
</tr>
<tr>
<td>AA</td>
<td>1</td>
<td>95±10</td>
<td>290±32</td>
</tr>
<tr>
<td>AA+Calpeptin</td>
<td>1</td>
<td>89±8</td>
<td>168±25*</td>
</tr>
<tr>
<td>AA+α-tocopherol</td>
<td>1</td>
<td>99±13</td>
<td>89±28*</td>
</tr>
<tr>
<td>AA</td>
<td>0</td>
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<tr>
<td>AA+α-tocopherol</td>
<td>0</td>
<td>126±16</td>
<td>146±31*</td>
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<tr>
<td>AA, Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>0</td>
<td>89±27</td>
<td>62±12*</td>
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</table>

* significantly different (p<0.05, ANOVA) with respect to the AMC fluorescence level in the presence of AA + Fe-NTA in 1 or 0 mM Ca<sup>2+</sup>-medium.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 6
Figure 7
Figure 8
CYP2E1+PUFA+Fe \\
\downarrow \\
Lipid peroxidation \\
\downarrow \\
Increased release of Ca^{2+} from intracellular stores \\
\hspace{1cm} i) Activation of PLA2 \\
\hspace{1cm} ii) Activation of calpain \\
\hspace{1cm} \rightarrow \text{Increased influx of Ca}^{2+} \text{ from extracellular space} \\
\hspace{1cm} \rightarrow \text{AA release} \\
\rightarrow \text{Mitochondrial damage} \\
\rightarrow \text{Necrosis} \\

Figure 9
Role of phospholipase A2 activation and calcium in CYP2E1-dependent toxicity in HepG2 cells
Andres A. Caro and Arthur I. Cederbaum

J. Biol. Chem. published online June 17, 2003

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