Role of Phosphatidylinositol 3-Kinase/AKT as a Survival Pathway against CYP2E1-Dependent Toxicity

Andres A. Caro and Arthur I. Cederbaum

Department of Pharmacology and Biological Chemistry, Mount Sinai School of Medicine, New York, New York

Received February 13, 2006; accepted April 18, 2006

ABSTRACT

The objective of this work was to evaluate the possible role of PI3-kinase/AKT as a survival pathway against CYP2E1-dependent toxicity. E47 cells (HepG2 cells transfected with human CYP2E1 cDNA) exposed to 25 μM iron-nitrilotriacetate + 5 μM arachidonic acid (AA) developed higher toxicity than C34 cells (HepG2 cells transfected with empty plasmid). Toxicity was associated with increased oxidative stress and activation of calcium-dependent hydrolases calpain and phospholipase A2. Treatment of E47, but not C34, cells, with arachidonic acid and iron (AA+Fe) led to a decrease in the phosphorylation state of AKT. 2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride (LY294002), a specific inhibitor of PI3-kinase, produced a further decrease of phosphorylated AKT in AA+Fe-treated E47 cells. LY294002 and down-regulation of endogenous AKT with small interference RNAs increased the toxicity of AA+Fe in E47 cells. Toxicity of AA+Fe in rat hepatocytes was also increased by LY294002. LY294002 did not affect phospholipase A2 or calpain activation, CYP2E1 activity, or lipid peroxidation elicited by AA+Fe. α-Tocopherol prevented both AA+Fe and AA+Fe+LY294002-induced toxicity and decrease of phosphorylated AKT. LY294002 potentiated AA+Fe-induced loss of mitochondrial membrane potential and ATP, whereas overexpression of constitutively active AKT partially prevented mitochondrial impairment and toxicity. Mitochondrial permeability transition inhibitors prevented both AA+Fe and AA+Fe+LY294002-induced toxicity and decrease of mitochondrial membrane potential. These results suggest that: i) AA+Fe+CYP2E1-induced oxidative stress decreases AKT activation; ii) AKT inactivation induces mitochondrial impairment associated with opening of the permeability transition pore but is not dependent on the activation state of bad, glycogen synthase kinase-3β, mammalian target of rapamycin, or bcl-xL; and iii) PI3-kinase/AKT may serve as a survival pathway against CYP2E1-dependent toxicity.

Recent data have implicated phosphatidylinositol 3-kinase (PI3-kinase) and their phospholipid products in promoting cell survival downstream of extracellular stimuli (Datta et al., 1999). In brief, PI3-kinase converts the plasma membrane lipid phosphatidylinositol 4,5-bisphosphate to phosphoinositide-dependent kinase 1 directly bind to phosphatidylinositol 3,4,5-trisphosphate through their pleckstrin-homology domain. This association at the membrane brings these proteins into proximity and facilitates phosphorylation of AKT. 2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride (LY294002), a specific inhibitor of PI3-kinase, produced a further decrease of phosphorylated AKT in AA+Fe-treated E47 cells. LY294002 and down-regulation of endogenous AKT with small interference RNAs increased the toxicity of AA+Fe in E47 cells. Toxicity of AA+Fe in rat hepatocytes was also increased by LY294002. LY294002 did not affect phospholipase A2 or calpain activation, CYP2E1 activity, or lipid peroxidation elicited by AA+Fe. α-Tocopherol prevented both AA+Fe and AA+Fe+LY294002-induced toxicity and decrease of phosphorylated AKT. LY294002 potentiated AA+Fe-induced loss of mitochondrial membrane potential and ATP, whereas overexpression of constitutively active AKT partially prevented mitochondrial impairment and toxicity. Mitochondrial permeability transition inhibitors prevented both AA+Fe and AA+Fe+LY294002-induced toxicity and decrease of mitochondrial membrane potential. These results suggest that: i) AA+Fe+CYP2E1-induced oxidative stress decreases AKT activation; ii) AKT inactivation induces mitochondrial impairment associated with opening of the permeability transition pore but is not dependent on the activation state of bad, glycogen synthase kinase-3β, mammalian target of rapamycin, or bcl-xL; and iii) PI3-kinase/AKT may serve as a survival pathway against CYP2E1-dependent toxicity.

ABBREVIATIONS: PI3-kinase, phosphatidylinositol 3-kinase; FFK506, tacrolimus; ROS, reactive oxygen species; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; siRNA, small interference RNA; TBARS, thiobarbituric acid reactive substances; DCFH-DA, 2′-(4-piperazinyl)-8-phenyl-1(4H)-benzopyran-4-one dihydrochloride; Fe-NTA, iron-nitrilotriacetate 1:3 complex; E47 cells, HepG2 cell line derived after transfection with human CYP2E1 cDNA; C34 cells, HepG2 cell line derived after transfection with empty plasmid. Toxicity was associated with increased oxidative stress and activation of calcium-dependent hydrolases calpain and phospholipase A2. Treatment of E47, but not C34, cells, with arachidonic acid and iron (AA+Fe) led to a decrease in the phosphorylation state of AKT. 2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride (LY294002), a specific inhibitor of PI3-kinase, produced a further decrease of phosphorylated AKT in AA+Fe-treated E47 cells. LY294002 and down-regulation of endogenous AKT with small interference RNAs increased the toxicity of AA+Fe in E47 cells. Toxicity of AA+Fe in rat hepatocytes was also increased by LY294002. LY294002 did not affect phospholipase A2 or calpain activation, CYP2E1 activity, or lipid peroxidation elicited by AA+Fe. α-Tocopherol prevented both AA+Fe and AA+Fe+LY294002-induced toxicity and decrease of phosphorylated AKT. LY294002 potentiated AA+Fe-induced loss of mitochondrial membrane potential and ATP, whereas overexpression of constitutively active AKT partially prevented mitochondrial impairment and toxicity. Mitochondrial permeability transition inhibitors prevented both AA+Fe and AA+Fe+LY294002-induced toxicity and decrease of mitochondrial membrane potential. These results suggest that: i) AA+Fe+CYP2E1-induced oxidative stress decreases AKT activation; ii) AKT inactivation induces mitochondrial impairment associated with opening of the permeability transition pore but is not dependent on the activation state of bad, glycogen synthase kinase-3β, mammalian target of rapamycin, or bcl-xL; and iii) PI3-kinase/AKT may serve as a survival pathway against CYP2E1-dependent toxicity.
mediated by choline deficiency through PI3K/AKT activation (Albright et al., 2005), and the same occurs for insulin (Bilodeau et al., 2004), hepatocyte growth factor (Schulze-Bergkamen et al., 2004), lysophosphatidic acid (Sautin et al., 2001), and interleukin-6 (Kuo et al., 2001), which inhibit apoptosis mediated by transforming growth factor-β, CD95, tumor necrosis factor-α/galactosamine, and retinoic acid, respectively, through PI3K/AKT activation. Furthermore, other agents promote liver cell apoptosis through suppression of PI3-kinase/AKT: sphingosine (Chang et al., 2001), celecoxib (Leng et al., 2003), and FTY720 (Lee et al., 2004). Several targets of the PI3-kinase AKT pathway may underlie the ability of this regulatory cascade to promote survival, including bad, caspase 9, transcription factors of the Forkhead family, and IκB kinase, which regulates nuclear factor-xB, and GSK-3β (Datta et al., 1999). There is more limited experimental evidence for the role of PI3-kinase/AKT in the protection against liver necrosis. The expression of MyrAKT reduces hepatocyte necrosis in the rat liver central vein following ischemia/reperfusion (Harada et al., 2004). Ischemic preconditioning decreases the number of necrotic and apoptotic liver cells after ischemia/reperfusion (Harada et al., 2004). Ischemic preconditioning decreases the number of necrotic and apoptotic liver cells after ischemia/reperfusion through activation of AKT (Izuishi et al., 2003). Loss of all isoforms of PI3-kinase p85α results in perinatal lethality in knockout mice, characterized by extensive hepatic necrosis (Frueman et al., 2000).

In liver cells, oxidative stress-promoting conditions, such as sulfur amino acid deprivation (Kang et al., 2001b) tert-butythiol hydroquinone (Kang et al., 2001a) and ischemia reperfusion (Harada et al., 2004), activate PI3-kinase and AKT. Following oxidative stress conditions in liver cells, activation of PI3-kinase/AKT was responsible for the induction of glutathione S-transferase A2 mediated by the antioxidant response element (Kang et al., 2001a), for the induction of microsomal epoxide hydrolase (Kang et al., 2001b), and for increased phosphorylation of bad and decreased release of cytochrome c (Harada et al., 2004), events associated with protection against injury. Microsomes represent a potential source of reactive oxygen species (ROS) via cytochrome P450 (Zargar et al., 2004). In comparison with other isoforms of cytochrome P450, CYP2E1 exhibits increased NADPH oxidation and capacity to induce ROS and lipid peroxidation. CYP2E1 overexpression in HepG2 cells and primary rat liver cells is associated with increased cytotoxicity of arachidonic acid (AA), glutathione (GSH) depletion, iron-nitritolactate (Fe-NTA), and ethanol. Increased production of ROS is a main cause of the increased cytotoxicity, which was associated with increased lipid peroxidation and early intracellular 2′,7′-dichlorofluorescein diacetate (DCFH-DA) oxidation, and was inhibited by antioxidants. Toxicity in these models show morphological and biochemical features of necrosis and apoptosis, depending on the nature of the insult (Caro and Cederbaum, 2004).

Considering the role of the PI3-kinase/AKT pathway in liver cell survival, particularly following oxidative stress, and the cellular toxicity caused by overexpression of CYP2E1 in liver cells, the objectives of this work were: i) to evaluate possible changes in AKT levels and activation after exposure of HepG2 cells overexpressing CYP2E1 to AA+Fe-NTA, an oxidative stress- and CYP2E1-dependent toxicity model that is mainly necrotic in nature; ii) to evaluate a possible role of PI3-kinase/AKT in the protection of liver cells against CYP2E1-dependent necrosis; and iii) to study possible mechanisms involved in the effects of PI3-kinase/AKT.

**Materials and Methods**

**Chemicals.** Phosphate-buffered saline (PBS) was from Roche (Newark, NJ). G418 was from Invitrogen (Carlsbad, CA). Fluoro-AM and pluronic acid were from Molecular Probes (Eugene, OR). [3H]AA was from PerkinElmer (Boston, MA). Protein concentration was measured using the Bio-Rad DC protein assay (Hercules, CA). Most of the other chemicals used were from Sigma Chemical Co. (St Louis, MO). Antibodies for immunoblot were from Santa Cruz Biotechnology (Santa Cruz, CA), with the exception of antiseroplin, which was from Chemicon (Temecula, CA). siRNAs against AKTs 1, 2, and 3 were purchased from New England Biolabs (Beverly, MA), and siRNA transfection reagent DharmaFeet 1 was from Dharmacon (Lafayette, CO).

**Culture and Treatment of Cells.** Two human hepatoma HepG2 cell lines described in Chen and Cederbaum (1998) were used as models in this study: 1) E47 cells, which constitutively express human CYP2E1, and 2) C34 cells, which are HepG2 cells transfected with the empty pcI vector. Both cell lines were grown in minimal essential medium (MEM) containing 10% fetal bovine serum and 0.5 mg/ml G418 supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere in 5% CO2 at 37°C. Cells were subcultured at a 1:5 ratio once a week. For the experiments, cells were plated at a density of 50,000 cells/ml and incubated for 12 h in MEM supplemented with 5% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (MEMexp). After this period, the medium was replaced with MEMexp supplemented with AA (from 0 to 5 μM). 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 MEMexp. The medium then was supplemented with various additions (e.g., antioxidants, inhibitors) for 1 h prior to the addition of buffer or Fe-NTA (25 μM), which was considered as the initiation of the cellular toxicity phase (t = 0 h). The cells were incubated for variable periods (up to 3 h) before the biochemical analyses. This basic protocol, i.e., preloading with arachidonic acid, washing, adding inhibitors, and initiating the toxicity phase by the addition of Fe-NTA, was used for all experiments (Caro and Cederbaum, 2001).

Selected experiments were carried out using primary hepatocytes from male Sprague-Dawley rats purchased from In Vitro Technologies (Baltimore, MD). In this case, rat hepatocytes were plated on MEMexp at a density of 30,000 cells/ml, and after 6 h, AA was added (0–25 μM) for 12 h. After this, the cells were washed once with PBS, and MEMexp was added followed by a 1-h preincubation with inhibitors. This was followed by the addition of Fe-NTA for 6 h, after which biochemical analyses were performed on the cells.

**Measurement of PLA2 Activity in Situ.** Phospholipase A2 (PLA2) activation was monitored in cultured cells using tritiated arachidonic acid as described previously (Caro and Cederbaum, 2003). Cells (50,000) were plated onto 24-well plates in MEMexp. Cells were first incubated with 2 μM AA and labeled with 0.2 μCi/ml [3H]AA in MEMexp for 12 h. Cells were washed with PBS and incubated with MEMexp for an additional 12-h period. After this, the cells were washed four times with PLA2 assay buffer: PBS, pH 7.2 supplemented with 5.5 mM glucose, 0.8 mM MgSO4, 0.1% fatty acid free bovine serum albumin, and 1 mM CaCl2. This PLA2 assay buffer (500 μl) 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. Where indicated, inhibitors then were added, and the cells incubated for 1 h. After this time, buffer or Fe-NTA (25 μM) was added, and [3H]AA release was evaluated after 2 h. 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 contain-
ing 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. Scintillation fluid (4 ml) was added to each vial, and after vigorous shaking, the radioactive content was determined by scintillation counting using a β-counter. The data were expressed as percentage of cellular [3H]AA released: 100 × ([3H]AA released/ ([3H]AA incorporated + [3H]AA released)]. Validation of this method and evidence that the released radioactivity was indeed [3H]AA were previously studied (Caro and Cederbaum, 2003).

**Cytotoxicity Measurements.** Cells (5 × 10⁴) were plated onto 24-well plates, and after the corresponding treatment, the medium was removed, and cell viability was evaluated by the MTT test (Caro and Cederbaum, 2003).

**Measurement of Intracellular Calcium.** The intracellular calcium levels were determined with the fluorescent calcium indicator fluo3-AM by flow cytometry. Cells (5 × 10⁴) were plated in 10-mm Petri dishes, and at the end of the various treatments, the medium was replaced with 3 ml of MEMexpw without fetal bovine serum plus 2.5 μM fluo3-AM and 0.02% pluronic acid (stock solution × 1000 in DMEM). Cells were incubated for 30 min at 37°C. After loading, the cells were washed in PBS (to remove any dye nonspecifically associated with the cell surface), trypsinized, and resuspended in 1 ml of MEMexpw without fetal bovine serum plus 5 μg of propidium iodide (PI). PI was used to assay for the viable cell population because these cells exclude this dye, whereas nonviable cells take up this dye. The measurement of [Ca²⁺]i, was performed by flow-cytometry analysis of 5000 cells using CellQuest software (BD Biosciences, San Jose, CA). Intracellular calcium level was evaluated as Fluor3 fluorescence intensity in PI-negative (i.e., viable) cells (Caro and Cederbaum, 2003). Ionomycin (10 μM) 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-Ca²⁺ solution.

**Lipid Peroxidation Assay.** The production of thiobarbituric acid-reactive substances (TBARS) was assayed as described previously (Caro and Cederbaum, 2001). The protein concentration of the cell suspension was determined using a protein assay kit based on the Lowry assay (Bio-Rad DC protein assay kit). Protein samples (30 μg) were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and incubated with primary and secondary antibodies. Antibodies were used at the following dilutions: anti-phospho-AKT (Thr308) (1:1000), anti-AKT (1:1000), anti-phospho-bad (Ser136) (1:200), anti-bad (1:100), anti-β-catenin (1:250), anti-β-actin (1:10,000), antipectrin (1:1000), anti-phospho-GSK-3β (Ser9) (1:1000), anti-GSK-3β (1:1000), anti-AMPK (1:1000), anti-AMPK-mammalian target of rapamycin (mTOR) (Ser2448) (1:1000), and anti-mTOR (1:1000).

**Adenovirus Infection.** Adenovirus vectors expressing constitutively active forms of AKT tagged with the hemagglutinin epitope (ADV-MyrAKT) or β-galactosidase (ADV-LacZ) under the control of the cytomegalovirus promoter were obtained from Vector Biolabs (Philadelphia, PA). The constitutively active AKT has the c-Src myristoylation sequence fused in-frame to the N terminus of the wildtype AKT coding sequence that targets the fusion protein to the membrane. Cells destined to be infected were first loaded with AA following the standard protocol (12-h incubation with 5 μM AA or PBS in the case of controls, wash with PBS, and 12-h incubation with MEMexpw). For infection, recombinant adenovirus was diluted in MEM containing 2% fetal bovine serum and added to the cells at 37°C for 1 h. After the addition of an equal volume of MEM with 10% fetal bovine serum, cells were incubated for an additional 5-h period. After this, the medium was replaced with MEMexpw, and the cells incubated for 18 h followed by the addition of Fe-NTA (or buffer in the case of controls). Recombinant adenoviruses were used at a multiplicity of infection of 10.

**Statistics.** Data are expressed as mean ± S.E.M. from three to four independent experiments run in duplicate. One-way analysis of variance with subsequent post hoc comparisons by Scheffe was performed. A p < 0.05 was considered as statistically significant.

**Results**

**Effect of AA+Fe and Inhibition of the PI3-Kinase/AKT Pathway, on AKT Levels and Cellular Toxicity in CYP2E1-Expressing Liver Cells.** We have previously re-
ported that E47 cells (HepG2 cells transfected with a plasmid encoding the human CYP2E1 cDNA) exposed to 5 μM arachidonic acid + 25 μM Fe-NTA (AA+Fe) showed significant cytotoxicity with respect to C34 cells (HepG2 cells transfected with the empty plasmid not expressing any cytochrome P450) (Caro and Cederbaum, 2001). The first objective of this work was to investigate whether cell death in this CYP2E1-dependent model of cytotoxicity was associated with changes in the levels or activation of AKT. Figure 1A (left) shows representative immunoblots for phosphorylated AKT and total AKT in lysates prepared from E47 cells loaded with arachidonic acid and exposed to iron for 0 to 3 h. A time-dependent decrease of phosphorylated AKT levels was observed, whereas total AKT levels remained relatively constant. These changes are reflected by a significant decrease in the relative ratio of phosphorylated AKT (pAKT) over total AKT with time (Fig. 1A, left, numbers in parentheses below the blot). After 3 h of incubation with AA+Fe, the pAKT/AKT ratio was decreased by 30%, compared with the 0-h vehicle control incubation, and almost 50%, compared with the 3-h vehicle control incubation (0.7 versus 1.3). Similar kinetic experiments were done after preincubation with 10 μM LY294002, a specific cell-permeable PI3-kinase inhibitor (Fig. 1B, left). There was also a decrease in phosphorylated AKT with time of incubation with Fe-NTA and nonsignificant changes in total AKT; however, the decrease in phosphorylated AKT was more pronounced than in the absence of LY294002. These changes are reflected by an early and pronounced decrease in the relative ratio of pAKT over total AKT with time (Fig. 1B, left, numbers in parentheses below the blot). This decrease in pAKT/AKT occurs at time points, e.g., 0.5, 1 h prior to cell death (discussed below; Fig. 2A). Several control experiments were performed. 1) In the presence of the vehicle alone (0.1% DMSO) (Fig. 1A, right two panels) or in the presence of only 10 μM LY294002 (Fig. 1B, right two panels) but without AA+Fe, no significant changes in phosphorylated or total AKT were observed after 3 h of incubation; 2) AA-loaded C34 cells exposed to Fe-NTA did not show significant changes in phosphorylated or total AKT after 3 h of incubation with Fe-NTA, either in the absence or presence of 10 μM LY294002 (Fig. 1C); and c) LY303511, an inactive analog of LY294002, did not produce significant changes in AKT levels or activation in AA+Fe-treated E47 cells as LY294002 did (data not shown).

E47 cells loaded with 5 μM arachidonic acid and exposed to 25 μM Fe-NTA showed a time-dependent toxicity that started after 1 h of exposure to Fe-NTA (Fig. 2A). E47 cells exposed similarly to AA+Fe, but preincubated with 10 μM LY294002, showed an increased loss of viability with respect to cells exposed to AA+Fe in the absence of the inhibitor. LY294002 (10 μM) was not toxic by itself in these conditions (Fig. 2A). E47 cells preincubated with 10 μM of the inactive analog LY303511 and exposed to AA+Fe did not show a significant change in cellular toxicity with respect to AA+Fe-treated cells (data not shown). AA+Fe added to C34 cells did not produce significant toxicity (Fig. 2B). C34 cells preincubated with 10 μM LY294002 and exposed to AA+Fe showed a small increase in cellular toxicity with respect to C34 cells exposed to AA+Fe in the absence of LY294002 (Fig. 2B). However, toxicity in C34 cells exposed to LY294002 and AA+Fe was significantly less than in E47 cells incubated in the same conditions. Other inhibitors of protein kinases, such as SB203580 (p38 MAPK inhibitor) and PD98059 (extracellular signal-regulated kinase inhibitor), did not increase AA+Fe toxicity in E47 cells (data not shown). Interestingly, SB203580 was previously shown (Wu and Cederbaum, 2003) to inhibit the slowly developing (24–48 h) toxicity of AA alone or buthionine sulfoximine alone in the E47 cells but not the rapid (1–3 h) toxicity found with AA plus Fe. It seems that different signaling pathways play a role in the toxicity mechanisms in the E47 cells, depending on the toxin and time course for development of toxicity. Morphologically, toxicity by AA+Fe in E47 cells was characterized by cell swelling and membrane blebs, vacuolization, and cytoplasmic degradation (Fig. 2C). In cells preincubated with 10 μM LY294002 and treated with AA+Fe, similar morphological changes were observed, although they were much more pronounced than in the absence of LY294002 (Fig. 2C). These experiments suggest that inhibition of the PI3-kinase/AKT pathway potentiates AA+Fe toxicity in the E47 cells.

In the following set of experiments, we tested the effect of other inhibitors of the PI3-kinase/AKT pathway, structurally or functionally unrelated to LY294002, on the toxicity of AA+Fe in E47 cells. AKT inhibitor IV is an inhibitor of AKT phosphorylation and activation that targets a kinase down-
stream of PI3-kinase. AKT inhibitor IV (at 0.5–2 µM) increased the toxicity of AA+Fe without showing significant toxicity by itself (Fig. 3A, top). AKT inhibitor IV also lowered the level of phosphorylation of AKT in the presence of AA+Fe without affecting total AKT levels, with respect to E47 cells treated with AA+Fe alone (Fig. 3A, bottom). Deguelin is an inhibitor of PI3-kinase and activated AKT, and in the same way, increased the toxicity of AA+Fe in the concentration range of 1 to 10 µM, without showing significant toxicity by itself (Fig. 3B, top). Deguelin also lowered the level of phosphorylation of AKT in the presence of AA+Fe without affecting total AKT levels, with respect to E47 cells treated with AA+Fe alone (Fig. 3B, bottom). Another approach was the use of siRNAs to lower pAKT as well as total AKT levels. E47 cells pre-exposed to a pool of siRNA against AKTs 1, 2, and 3 within 24 h developed higher AA+Fe toxicity than E47 cells pre-exposed to a control, nontargeting siRNA and treated with AA+Fe (Fig. 3C, top). E47 cells pre-exposed to AKT1/2/3 siRNA and treated with AA+Fe showed a significant decrease of total AKT levels and a significant decrease of phosphorylated AKT, with respect to cells pre-exposed to a control, nontargeting siRNA and treated with AA+Fe (Fig. 3C, bottom). Thus, the use of three different chemical inhibitors and siRNA shows that the PI3-kinase/AKT pathway is protective against AA+Fe toxicity in the E47 cells. On the contrary, wortmannin, also a cell-permeable inhibitor of PI3-kinase, did not show any effect on AA+Fe toxicity in the concentration range of 0.1 to 10 µM (Fig. 3D, top). However, wortmannin did produce a decrease in phosphorylated AKT levels in cells treated with AA+Fe with respect to cells treated with AA+Fe alone, suggesting that it is an inhibitor of PI3-kinase in this system (Fig. 3D, bottom). The specificity of wortmannin was evaluated to try to explain the lack of potentiation of AA+Fe toxicity as found with the other PI3-kinase/AKT inhibitors. There are data that wortmannin, at the same range of concentrations where it is an effective inhibitor of PI3-kinase, also effectively inhibits PLA2 (Cross et al., 1995). PLA2 plays a critical role in the toxicity by AA+Fe in E47 cells (Caro and Cederbaum, 2003). PLA2 activity in E47 cells in situ was determined by measuring the release of preloaded [3H]AA into the medium. AA+Fe produced an increase in the release of [3H]AA, which was inhibited by a general PLA2 inhibitor, 100 µM quinacrine (Fig. 4). Wortmannin (0.1–10 µM) inhibited both the basal release of [3H]AA and the increased release of [3H]AA after treatment with AA+Fe. This suggests that, in our system, wortmannin acts both as a PI3-kinase and a
PLA2 inhibitor. Whereas the former should potentiate toxicity, the latter blocks toxicity, hence explaining the failure of wortmannin to alter AA+Fe toxicity.

We investigated the possible role of phosphatases in the dephosphorylation of AKT after exposure of E47 cells to AA+Fe in the presence or absence of LY294002. Specific...
cell-permeable inhibitors of serine/threonine phosphatases were used, okadaic acid for PP1C and PP2A and FK506 for PP2B. These inhibitors at concentrations regularly used for cell studies and that did not show significant toxicity by themselves (0–100 nM for okadaic acid and 0–20 μM for FK506) did not affect the toxicity of AA+Fe in the absence or presence of LY294002 (data not shown). Thus, stimulation of phosphatases, such as PP1C, PP2A, and PP2B, by ROS produced from the treatment with AA+Fe does not seem to be responsible for the deactivation of AKT.

**Hepatocyte Studies.** Studies were performed to extend the observations made in CYP2E1-expressing HepG2 cells to nontransformed, intact, CYP2E1-expressing rat hepatocytes. Hepatocytes showed stable levels of CYP2E1 protein (assessed by Western blot) and detectable CYP2E1 activity, assessed through metabolism of 7-methoxy-4-trifluoromethylcoumarin in situ (data not shown). Hepatocytes were cultured in MEM supplemented as described under Materials and Methods. AA+Fe produced a synergistic effect in intact hepatocytes (viability 99 ± 6, 84 ± 4, and 54 ± 2% with AA 25 μM alone, Fe-NTA 100 μM alone, and the combination of AA+Fe, respectively). As in E47 cells, preincubation of intact hepatocytes with 50 μM LY294002 produced a decrease in cellular viability after exposure to 25 μM AA + 100 μM Fe-NTA (viability 71 ± 1, 92 ± 3, and 54 ± 2% with AA 25 μM + Fe-NTA 100 μM, 50 μM LY294002 alone, and AA+Fe + 50 μM LY294002, respectively). Intact hepatocytes preincubated with 50 μM LY294002 and further treated with AA+Fe for 4 h showed lower levels of pAKT (pAKT/AKT ratio = 0.7) than intact hepatocytes incubated with AA+Fe alone (pAKT/AKT ratio = 1.0; data not shown).

**Role of Oxidative Stress on Cellular Toxicity and AKT Deactivation.** Oxidative stress and lipid peroxidation are the primary cause of cell death in this model of CYP2E1-dependent toxicity (Caro and Cederbaum, 2001, 2002). The next set of experiments (Fig. 5) investigated whether the decrease in phosphorylated AKT after AA+Fe treatment of E47 cells depended on increased oxidative stress and lipid peroxidation. In Fig. 5A, a representative Western blot of phosphorylated AKT of lysates from E47 cells preincubated with α-tocopherol (αT), an effective liposoluble antioxidant, LY294002 or the combination of both and further exposed to Fe-NTA (0–3 h), is shown. As presented in Fig. 1A, the AA-loaded E47 cells treated with Fe-NTA for 3 h showed a small decrease in phosphorylated AKT with respect to zero-time controls; preincubation with LY294002 caused a further decrease in pAKT levels (Fig. 5A). The decrease in pAKT by AA+Fe in the absence and presence of LY294002 was completely prevented by preincubation with α-tocopherol (Fig. 5A). AA+Fe caused ~50% loss of viability in E47 cells, and this toxicity was further increased to an 80% loss of viability by preincubation with LY294002 (Fig. 5B). α-Tocopherol completely prevented both AA+Fe and AA+Fe+LY294002-induced toxicity (Fig. 5B).

**Effect of LY294002 on Upstream Mediators of AA/Fe Toxicity in E47 Cells.** We previously suggested that CYP2E1-dependent toxicity induced by AA+Fe is mediated through an increase in lipid peroxidation that triggers an increase in intracellular calcium and activation of Ca2+-dependent hydrolases, such as calpain and PLA2 (Caro and Cederbaum, 2002, 2003). Considering that LY294002 increased AA+Fe toxicity, the possibility that LY294002 may affect upstream mediators of toxicity was investigated (Table 1). TBARS content, an index of lipid peroxidation, increased ~4-fold after AA+Fe treatment of E47 cells; the same increase in lipid peroxidation levels was observed in the presence of AA+Fe+LY294002 (Table 1). Intracellular calcium was measured by flow cytometry using fluo3 and gating for the PI-negative, i.e., viable E47 cells. AA+Fe caused a 2-fold increase in intracellular calcium; the same increase was detected in the presence of AA+Fe+LY294002. Release of preloaded [3H]AA (a measurement of in situ PLA2 activity) increased from 9.3 to 14.6% after treatment with AA+Fe, whereas in the presence of LY294002, a similar increase was detected. Calpain activation was evaluated by analyzing spectrin degradation products. α-Spectrin is cleaved by calpain to two fragments of 150 and 145 kDa and by caspase 3 to two fragments of 150 and 120 kDa. AA+Fe treatment in

---

**Fig. 5.** Effect of α-tocopherol on cytotoxicity and AKT activation in AA+Fe-treated E47 cells. A, E47 cells were preloaded with 5 μM AA and preincubated with either 50 μM α-tocopherol (αT) or 10 μM LY294002 (LY) or the combination of 50 μM α-tocopherol + 10 μM LY294002 (αT+LY) for 1 h. Cells were immediately collected for Western blot analysis of phospho-AKT (0 h results) or incubated with 25 μM Fe-NTA for 3 h before collection of protein samples for the Western blot. The ratio of pAKT/total AKT was calculated (number in parenthesis below the blots, standard error <10%). †, significantly different (p < 0.05) with respect to cells incubated in the same conditions at 0 h. ‡, significantly different (p < 0.05) with respect to cells incubated in the same conditions without α-tocopherol. B, E47 cells were preloaded with 5 μM AA and either exposed to 25 μM Fe-NTA for 3 h (Fe+AA) or left untreated. Preincubation was performed with 50 μM α-tocopherol (αT) or 10 μM LY294002 (LY) or the combination of 50 μM α-tocopherol + 10 μM LY294002 (LY+αT). Viability was assessed by the MTT reduction assay. *, significantly different (p < 0.05) with respect to cells incubated in the same conditions, without AA+Fe. ††, significantly different (p < 0.05) with respect to cells incubated in the same conditions, without α-tocopherol.
TABLE 1
Effect of AA + Fe and inhibition of PI3 kinase on upstream mediators of toxicity in E47 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TBARS</th>
<th>[Ca^{2+}]</th>
<th>PLA2</th>
<th>Calpain</th>
<th>CYP2E1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (2 h)</td>
<td>0.08 ± 0.04</td>
<td>1.0</td>
<td>9.3 ± 1.0</td>
<td></td>
<td>22 ± 5</td>
</tr>
<tr>
<td>AA+Fe+vehicle (2 h)</td>
<td>0.33 ± 0.05</td>
<td>2.1 ± 0.1</td>
<td>14.6 ± 1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LY294002 (2 h)</td>
<td>0.08 ± 0.04</td>
<td>0.9 ± 0.1</td>
<td>8.9 ± 0.3</td>
<td>18 ± 3</td>
<td></td>
</tr>
<tr>
<td>AA+Fe+LY294002 (2 h)</td>
<td>0.33 ± 0.05</td>
<td>1.7 ± 0.2</td>
<td>13.9 ± 0.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

E47 cells produced an increased cleavage of α-spectrin to the 145-kDa calpain-specific fragment (top arrow), and the same pattern and intensity of cleavage were observed in the presence of LY294002. Practically no cleavage of α-spectrin to the caspase 3-specific 120-kDa fragment (bottom arrow) was observed in the absence or presence of LY294002, suggesting that cell death in this model is primarily due to necrosis, not apoptosis (also reflected by cell swelling, formation of plasma membrane blebs, vacuolization of cytoplasm, early depletion of ATP, lack of DNA ladder formation, and absence of caspase activation) (Caro and Cederbaum, 2001). To further show that the AA+Fe toxicity in the presence of LY294002 was mainly necrosis, an annexin V/PI flow-cytometry experiment was carried out. At early time points (2–3 h), AA+Fe did not cause annexin V staining in the absence or presence of LY294002, whereas PI staining was elevated (data not shown). CYP2E1 activity in situ assessed through the oxidation of a specific fluorogenic substrate did not change by incubation with LY294002 (Table 1). These results suggest that the increased toxicity promoted by LY294002 is downstream of the CYP2E1-dependent oxidative stress and activation of calcium-dependent hydrolases.

Role of Mitochondrial Damage in AA+Fe-Induced Cytotoxicity. We suggested that in AA+Fe-treated E47 cells, lipid peroxidation, intracellular calcium, calpain, and PLA2 activity are events that converge on mitochondria, inducing a profound bioenergetic failure and a rapid necrotic cell death (Caro and Cederbaum, 2002, 2003). Considering the key role of mitochondrial damage in cytotoxicity, the effect of LY294002 on mitochondrial function in the presence of AA+Fe was studied. Mitochondrial damage is initially manifested by a decrease in mitochondrial membrane potential followed by ATP depletion (Orrenius et al., 1996). Mitochondrial membrane potential was assessed by measuring potential-driven mitochondrial accumulation of rhodamine 123 by flow cytometry. Kinetic studies were done measuring the percentage of the M1 population (cells showing low rhodamine fluorescence, directly related to mitochondrial membrane potential) with time. E47 cells preincubated with LY294002 and treated with AA+Fe showed a higher percentage of cells in the M1 fraction than cells treated only with AA+Fe at all of the time points evaluated (Fig. 6A). Similar experiments were done with MitoTracker Orange (Invitrogen) instead of rhodamine123/PI. In this case, E47 cells preincubated with LY294002 and treated with AA+Fe showed a higher percentage of cells in the M1 fraction (low MitoTracker Orange fluorescence, 27 ± 1%) than in cells treated only with AA+Fe (15 ± 1%) after 3 h of exposure to Fe-NTA (data not shown). Thus, using two different probes, LY294002 was shown to enhance the AA+Fe-mediated decline in mitochondrial membrane potential. ATP content followed a similar trend: E47 cells exposed to AA+Fe showed an early decrease in ATP concentration that precedes the toxicity, and the decrease in ATP levels caused by AA+Fe was higher in the presence of LY294002 (Fig. 6B).

The oxidative stress status of the cells was assessed through the intracellular oxidation of DCFH by ROS and the levels of glutathione, the principal water-soluble cellular antioxidant. DCFH oxidation was quantified in PI-negative (i.e., viable) cells, thus assuring that any change observed occurs before the onset of cell death. In AA+Fe-treated cells, a very early increase in DCFH fluorescence and decrease in total glutathione levels was observed. Cells treated with LY294002 and AA+Fe did not show major differences in DCFH oxidation and GSH levels with respect to cells treated with AA+Fe alone at all of the time points evaluated (Fig. 6C and D). Thus, LY294002 is not modulating the AA+Fe+CYP2E1-generated oxidant stress.

Two major processes are probable candidates as mechanisms for a loss of mitochondrial membrane potential: 1) nonspecific damage to the inner mitochondrial membrane or 2) the mitochondrial permeability transition (MPT), a more specific process, due to the opening of the MPT pore (Susin et al., 1998). Inhibitors of the MPT, such as cyclopholin D in-
hibitor cyclosporin A, adenine nucleotide translocator inhibitor bongkrekic acid, and fructose/trifluoperazine, a combination proven to provide a fairly specific inhibition of the MPT in iron-treated hepatocytes (Rauen et al., 2004), partially inhibited AA+Fe-induced toxicity in E47 cells (Fig. 7A, bars 1–8). The decreased toxicity caused by treatment with AA+Fe in the presence of LY294002 was also blocked by bongkrekic acid, and fructose/trifluoperazine, a combination known to inhibit cyclosporin A, adenine nucleotide translocator inhibitor and phosphatidylinositol 3-kinase (PI3-kinase) inhibitors (

**Fig. 6.** Effect of AA+Fe and PI3-kinase inhibition on mitochondrial function in CYP2E1-expressing HepG2 cells. A, E47 cells were either exposed to vehicle alone (white circles), 5 μM AA + 25 μM Fe-NTA + vehicle (black circles), 10 μM LY294002 alone (white squares), or 5 μM AA + 25 μM Fe-NTA + 10 μM LY294002 (black squares). After a toxicity phase of 1 to 3 h, mitochondrial membrane potential was monitored by flow cytometry using rhodamine 123. The percentage of the cell population in the M1 fraction (low-rhodamine 123 fluorescence) is represented versus time. #, significantly different (p < 0.05) with respect to cells incubated in the same conditions, without AA+Fe. *, significantly different (p < 0.05) with respect to cells incubated in the same conditions, without LY294002. B, ATP content was monitored at several times during the toxicity phase (0–3 h) as described in A. *, significantly different (p < 0.05) with respect to cells incubated in the same conditions, without AA+Fe. #, significantly different (p < 0.05) with respect to cells incubated in the same conditions, without LY294002. C and D, E47 cells were exposed to 5 μM AA + 25 μM Fe-NTA (AA+Fe) or 5 μM AA + 25 μM Fe-NTA + 10 μM LY294002 (AA+Fe+LY). After a toxicity phase of 0 to 3 h, DCFH fluorescence in PI-negative cells (C) or glutathione content (D) was evaluated as described under Materials and Methods. *, significantly different (p < 0.05) with respect to cells incubated in the same conditions, at time = 0 h.

**Fig. 6.** Effect of AA+Fe and PI3-kinase inhibition on mitochondrial function in CYP2E1-expressing HepG2 cells. A, E47 cells were either exposed to vehicle alone (white circles), 5 μM AA + 25 μM Fe-NTA + vehicle (black circles), 10 μM LY294002 alone (white squares), or 5 μM AA + 25 μM Fe-NTA + 10 μM LY294002 (black squares). After a toxicity phase of 1 to 3 h, mitochondrial membrane potential was monitored by flow cytometry using rhodamine 123. The percentage of the cell population in the M1 fraction (low-rhodamine 123 fluorescence) is represented versus time. #, significantly different (p < 0.05) with respect to cells incubated in the same conditions, without AA+Fe. *, significantly different (p < 0.05) with respect to cells incubated in the same conditions, without LY294002. B, ATP content was monitored at several times during the toxicity phase (0–3 h) as described in A. *, significantly different (p < 0.05) with respect to cells incubated in the same conditions, without AA+Fe. #, significantly different (p < 0.05) with respect to cells incubated in the same conditions, without LY294002. C and D, E47 cells were exposed to 5 μM AA + 25 μM Fe-NTA (AA+Fe) or 5 μM AA + 25 μM Fe-NTA + 10 μM LY294002 (AA+Fe+LY). After a toxicity phase of 0 to 3 h, DCFH fluorescence in PI-negative cells (C) or glutathione content (D) was evaluated as described under Materials and Methods. *, significantly different (p < 0.05) with respect to cells incubated in the same conditions, at time = 0 h.

**Possible Downstream Mediators of AKT Action.** Possible targets of active AKT with relevance for mitochondrial protection are, among others, GSK-3β, bad, mTOR, and bcl-xL (Juhaszova et al., 2004; Hu and Sayeed, 2005). Immunoblots for GSK-3β and bad show that its phosphorylation levels (phosphorylated protein/total protein associated with cell protection and viability) were not significantly affected by AA+Fe (data not shown). Control experiments showed that LY294002 inhibited phosphorylation of AKT and GSK-3β by hepatocyte growth factor, thus validating that LY294002 was inhibiting AKT activity/function. bcl-xL is an antiapoptotic member of the bcl-2 family. The protein levels of bcl-xL did not show significant changes with time in cells incubated with AA+Fe in the absence or presence of LY294002 (data not shown). The mTOR is considered a critical player for cell signaling pathways from translational machinery, cell growth, and metabolism to survival/apoptosis in many cell types (Li et al., 2005). Phosphorylation of mTOR may be triggered by activation of its upstream molecules,
PI3-kinase and Akt. We evaluated the possible role of mTOR in the protection against toxicity in AA+Fe-treated E47 cells. Rapamycin (up to 100 μM) did not affect the toxicity of AA+Fe in the absence or presence of LY294002 (data not shown). Immunoblots for mTOR show that its phosphorylation levels were not significantly affected by AA+Fe in the absence or presence of LY294002 (data not shown).

Discussion

The following experimental evidence suggests that PI3-kinase/AKT is a survival pathway against CYP2E1 plus AA/Fe-dependent toxicity. 1) Treatment of E47 cells with AA+Fe, a CYP2E1-dependent model of cell death, is associated with an early decrease in AKT activation; AA+Fe produced an early deactivation of AKT in CYP2E1-expressing cells but not in control cells that do not express any cytochrome P450, an event that paralleled toxicity. 2) Toxicity by AA+Fe, evaluated as plasma membrane permeabilization to propidium iodide, was inhibited by expression of active AKT. 3) Toxicity of AA+Fe in CYP2E1-expressing HepG2 cells was enhanced by inhibition of PI3-kinase/AKT. Inhibition of PI3-kinase/AKT was performed with structurally unrelated chemical inhibitors or siRNAs directed against AKTs 1, 2, and 3 and was confirmed by AKT deactivation.

AKT deactivation required AA+Fe and CYP2E1 overexpression. Without AA+Fe or with AA+Fe in control, non-P450 expressing HepG2 cells, no deactivation of AKT was observed. This requirement is linked to oxidative stress and lipid peroxidation, as α-tocopherol blocked the AA+Fe-induced AKT deactivation in E47 cells. Possible mechanisms may include PI3-kinase deactivation, activation of serine/threonine phosphatases that can dephosphorylate AKT directly (Luo et al., 2003), or specific oxidation of AKT by ROS that may modify phosphorylation sites. Phosphatase inhibitors, such as okadaic acid (PP1C and PP2A inhibitor) or FK506 (PP2B inhibitor), did not affect the toxicity of AA+Fe in the absence or presence of LY294002, suggesting that okadaic acid/FK506-sensible phosphatases are not involved in the mechanisms of toxicity. The underlying mechanism for the decrease in AKT activation after AA+Fe depends on...
increased oxidative stress, but how oxidative stress is promoting deactivation of AKT requires additional investigation.

Several studies suggest that the MPT pore opening in mitochondria is a factor in the pathogenesis of necrotic cell death. According to this hypothesis, the opening of the MPT pore is associated with mitochondrial impairment and cell death in our model. Overexpression of active AKT decreased the loss of mitochondrial membrane potential associated with AA +Fe in E47 cells and partially prevented toxicity, without affecting CYP2E1 activity, ROS levels, or intracellular calcium with respect to AA +Fe-treated E47 cells infected with a control adenovirus. This suggests that the protection afforded by active AKT is downstream of the CYP2E1-dependent oxidative stress and the increase in intracellular calcium. Inhibition of PI3K/AKT with LY294002 in AA +Fe-treated E47 cells produced increased cytotoxicity and a more pronounced loss of ATP and mitochondrial membrane potential, effects that are blocked by fructose/trifluoperazine. LY294002 did not significantly affect the Fe +AA-induced activation of PLA2 and calpain, the increase in oxidative stress indices, such as lipid peroxidation, DCFH oxidation, and depletion of GSH content, or CYP2E1 activity. Thus, the increased toxicity promoted by LY294002 is downstream of the CYP2E1-dependent oxidative stress and activation of calcium-dependent hydrolases. We suggest that AKT is partially preventing cell toxicity by AA +Fe +CYP2E1 through inhibition of the MPT pore opening and the loss of mitochondrial membrane potential and of ATP, because LY294002 potentiated the decline in mitochondrial membrane potential and ATP produced by AA +Fe, whereas adenoviral expression of active AKT protected against this loss of mitochondrial membrane potential and of ATP. This is a critical mechanistic aspect, because although in several systems AKT can mediate rescue from cell death, AKT can exert its effects upstream (Kennedy et al., 1999) or downstream (Zhou et al., 2000) of mitochondria.

CYP2E1-dependent toxicity in our model does not seem to depend on the protein levels or activation of bad, GSK-3β, or bcl-xL, as we could not detect significant early changes in the levels or phosphorylation states of these proteins after AA +Fe treatment in the absence or presence of LY294002. Akt modulates energy homeostasis by maintaining the level of ATP in cells (Hahn-Windgassen et al., 2005). The effect of Akt on the generation of ATP occurs via an increase in glycolysis and oxidative phosphorylation, although the one or more exact mechanisms by which Akt affects these processes are not known (Hahn-Windgassen et al., 2005). Considering the effects of AKT activation and inhibition on ATP levels and mitochondrial membrane potential in our system, experiments evaluating the modulation of mitochondrial bioenergetics by AKT will be important to try to identify downstream mediators of AKT-dependent inhibition of mitochondrial damage in CYP2E1-expressing cells.

This work is the first report for a role of PI3-kinase/AKT in the protection against CYP2E1-dependent cell necrosis. Alcoholic liver disease is associated with CYP2E1 induction and an increase in the number of necrotic and apoptotic liver parenchymal cells. Several reports in the literature suggest a role for PI3-kinase/AKT in the protection of liver cells against ethanol-induced toxicity. Chronic ethanol exposure activated caspase 3 in hepatocytes, and this was associated with reduced levels of PI3-kinase, AKT, and increased levels of
probably mediated by opening of the mitochondrial permeability transition pore but does not seem to be mediated by changes in GSK-3β, bad, bcl-xl, or mTOR, and the exact target(s) regulated by AKT remains to be defined. LY294002 potentiates the toxicity of AA+Fe in E47 cells by causing a further deactivation of AKT. The deactivation of AKT by AA+Fe is downstream of CYP2E1 and CYP2E1-catalyzed lipid peroxidation and is independent of the increase in intracellular calcium and activation of PLA2 and calpain. α-Tocopherol, which prevents the lipid peroxidation, prevents all of the downstream events associated with the increased lipid peroxidation, such as the increase in Ca2+ and PLA2 (Caro and Cederbaum, 2003), the deactivation of AKT (Fig. 5A), and the decline in mitochondrial membrane potential and ATP, and hence, prevents cell death.

References


Address correspondence to: Andres A. Caro, Department of Pharmacology and Biological Chemistry, Box 1603, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029. E-mail: andres.caro@mssm.edu