Role of p38 MAPK in CYP2E1-dependent Arachidonic Acid Toxicity*

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Polysaturated fatty acids such as arachidonic acid (AA) play an important role in alcohol-induced liver injury. AA promotes toxicity in rat hepatocytes with high levels of cytochrome P4502E1 (CYP2E1) and in HepG2 E47 cells, which express CYP2E1. The possible role of mitogen-activated protein kinase (MAPK) members in this process was evaluated. SB203580, a p38 MAPK inhibitor, and PD98059, an ERK inhibitor, but not wortmannin a phosphatidylinositol 3-kinase (PI3K) inhibitor, prevented AA toxicity in pyrazole hepatocytes and E47 cells. SB203580 prevented the enhancement of AA toxicity by salicylate. SB203580 neither lowered the levels of CYP2E1 nor affected CYP2E1-dependent oxidative stress. The decrease in mitochondrial membrane potential produced by AA was prevented by SB203580. Treating CYP2E1-induced cells with AA activated p38 MAPK but not ERK or AKT. This activation was blocked by antioxidants. AA increased the translocation of NF-κB to the nucleus. Salicylate blocked this translocation, which may contribute to the enhancement of AA toxicity by salicylate. SB203580 restored AA-induced NF-κB translocation, which may contribute to protection against toxicity. In conclusion, AA toxicity was related to lipid peroxidation and oxidative stress, and to the activation of p38 MAPK, as a consequence of CYP2E1-dependent production of reactive oxygen species. Activation of p38 MAPK by AA coupled to AA-induced oxidative stress may synergize to cause cell toxicity by affecting mitochondrial membrane potential and by modulation of NF-κB activation.

Polysaturated fatty acids (PUFA) such as arachidonic acid (AA) or its metabolites play an important role in a variety of biological processes, such as signal transduction, chemotaxis, and cell proliferation and differentiation (1–3). PUFA also play an important role in alcoholic liver injury (4–6). In the intragastric infusion model of ethanol feeding, liver injury occurs when the rats consume diets containing polysaturated fatty acid but not saturated fatty acid (7, 8). This model is associated with induction of high levels of CYP2E1 and greatly increased lipid peroxidation, which appear to contribute to the liver injury. AA induced toxicity in HepG2 E47 cells, a cell line that expresses CYP2E1 but not control HepG2 cells, which do not express CYP2E1 (9). AA also induced toxicity in pyrazole-induced rat hepatocytes with high levels of CYP2E1 but not saline control hepatocytes (10). This AA toxicity was prevented by inhibitors of CYP2E1 and by antioxidants (9, 10).

AA can activate mitogen-activated protein kinase (MAPK), a ubiquitous group of serine/threonine kinases, which play a crucial role in transmitting transmembrane signals required for cell growth, differentiation, and apoptosis (11–13). Members of the kinase family, originally found to be activated by mitogens, have now been found to be activated by a wide variety of mitogenic and non-mitogenic agents via a cascade of kinase/effector molecules which, in mammalian cells, includes protein kinase C, p21, Raf-1, and MEK (MAPK/extracellular signal-regulated protein kinase, ERK) (14–16). AA or its metabolites can activate MAPK members such as ERKs and JNKs/SAPKs, suggesting an important role for AA and its metabolites in mitogenic signaling events (17, 18).

We recently reported that salicylate can potentiate the toxicity of AA in CYP2E1-indurated hepatocytes (19). Salicylate was found to increase CYP2E1 levels, preventing its degradation, and this may be one mechanism by which salicylate increases AA-induced toxicity. Sodium salicylate and acetylsalicylic acid are non-steroidal anti-inflammatory agents that prevent activation of nuclear factor κB by inhibition of phosphorylation and subsequent degradation of IκB, or by direct inhibition of IκB kinase (20–23). Salicylate also interferes with MAPK and other kinase-dependent signaling pathways (24–26) and can affect mitochondrial function (27, 28). Damage to mitochondria plays an important role in AA plus CYP2E1-dependent toxicity (29). Whether MAPKs are involved in the AA-induced toxicity, and if so, how MAPKs induce or regulate this toxicity have not been reported, for cytochrome P450-dependent processes in general, and specifically for CYP2E1. In the present study, we characterized the possible role of the MAPKs, p38 and ERK, and P38 on AA or AA plus salicylate-induced toxicity in pyrazole-induced rat hepatocytes, human hepatocyte cultures, and HepG2 E47 cells. Effects of specific kinase inhibitors on cellular toxicity, CYP2E1 levels, lipid peroxidation, mitochondrial membrane potential, and NF-κB activation and the role of MAPKs in the salicylate enhancement of AA-induced toxicity were evaluated. Results show that AA or AA plus salicylate activated p38 MAPK but not ERK or P38, and that p38 MAPK plays a role in AA-induced cytotoxicity in CYP2E1-expressing cells.

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1 The abbreviations used are: PUFA, polysaturated fatty acid; AA, arachidonic acid; sal, salicylate; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; SAPK, stress-activated protein kinase; Rh123, rhodamine 123; P38, phosphatidylinositol 3-kinase; MMP, mitochondrial membrane potential; Trolox, (±)-hydroxy-2,5,7,8-teramethylchroman-2-carboxylic acid; PF, pyrazole; JNK, c-Jun NH2-terminal kinase; ANOVA, analysis of variance; PBS, phosphate-buffered saline; TBARS, thiobarbituric acid-reactive products; TBA, thiobarbituric; CYP2E1, cytochrome P4502E1; ROS, reactive oxygen species.
Role of p38 MAPK in CYP2E1 Toxicity

MATERIALS AND METHODS

Hepatocyte Isolation and Cell Culture—Rats received humane care, and experiments were carried out according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals and Institutional Animal Care and Use Committee approval. Male Sprague-Dawley rats, 150–170 g body weight were injected intraperitoneally with pyrazole, 200 mg/kg body weight, once a day for 2 days to induce CYP2E1. After overnight fasting, rat hepatocytes were isolated by a two-step collagenase perfusion method (30). Induction of CYP2E1 was validated by Western blot analysis and catalytic activity with p-nitrophenol. Cell viability was generally about 90%. Hepatocytes were seeded onto 100-mm culture dishes, which were coated with the basement membrane Matrigel (BD Biosciences) and cultured in serum-free HepatoZYME-SFM medium (Invitrogen) containing 1% penicillin and streptomycin. One to two hours after seeding, the medium was changed. Unattached cells were gently washed out, and the cell culture experiments were initiated. Human hepatocyte cultures plated on rat tail collagen-coated flasks (T-25) in serum-supplemented media were obtained from the Liver Tissue Procurement and Distribution System (University of Minnesota, Minneapolis, MN).

E47 cells were HepG2 cells that were transfected with a human CYP2E1 cDNA in the sense orientation and constitutively express CYP2E1. C34 cells are HepG2 cells that were transfected with the pCI vector only; these cells do not express CYP2E1 (9). The HepG2 cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum plus 100 units/ml penicillin plus 100 μg/ml streptomycin at 37°C.

Toxicity Determination—Hepatocyte or HepG2 cell cultures were treated with arachidonic acid (AA, 60 μM) with or without salicylate (sal, 5 mM) in the presence or absence of 10 μM SB203580 (a specific p38 MAPK inhibitor) or 10 μM PD98059 (a specific ERK inhibitor) (Calbiochem Inc.), or 0.1–10 μM wortmannin (a PI3K inhibitor, Sigma Chemical Co.) for 24 h. Inhibitors were dissolved in Me2SO, and controls were incubated with Me2SO (0.6% v/v final concentration). As a non-CYP2E1-HepG2 E47 cells, plus salicylate-induced toxicity in HepG2 E47 cells, rats were treated with AA or AA plus sal in the presence or absence of 10 μM SB203580, 10 μM PD98059, or 10 μM wortmannin for 10, 20, or 30 min. The cells were harvested and sonicated as described above. The cellular extract (15 μg of protein) was subjected to SDS-PAGE using an 8% gel. The blotted membranes were incubated with either polyclonal p38 or ERK MAPK antibodies or AKT polyclonal antibody to detect the total content of these kinases or incubated with the appropriate phosphorylated monoclonal antibodies, respectively (Santa Cruz), to determine the content of the activated, phosphorylated kinase. The blots were then incubated with either anti-rabbit IgG or anti-mouse IgG conjugated with horseradish peroxidase and fluorescence developed, and results were analyzed as described above.

Immunohistochemical Localization of the Translocation of NF-κB—Freshly isolated rat hepatocytes were grown on a glass slide placed in culture dishes. The cells were treated as described above. At the end of treatment, the cells on the glass slide were fixed with 4% paraformaldehyde, washed with PBS, and incubated with NF-κB antibody (Santa Cruz Biotechnology) for 2 h. After rinsing several times with PBS, the slides were incubated with anti-IgG antibody conjugated with fluorescein isothiocyanate. The slides were rinsed several times with PBS and mounted onto a microscopy glass slide with mounting medium for fluorescence (Vector Laboratories, Inc., Burlingame, CA). The localization of NF-κB in the nucleus or the cytosol was observed under a fluorescence microscope.

Electrophoretic Mobility Shift Assay—To determine the effect of AA or AA plus sal on NF-κB DNA binding activity, cells were treated with AA or AA plus sal in the presence or absence of 10 μM SB203580, 10 μM PD98059, or 10 μM wortmannin for 30 min or for 24 h. Cells were harvested and lysed with a lysis solution containing 0.32 M sucrose, 2 mM CaCl2, 2 mM MgCl2, 0.1 mM EDTA, 0.5% Triton X-100, 1 mM dithiothreitol, 2.5 μg/ml antipain, 2.5 μg/ml pepstatin, and 2.5 μg/ml aprotinin for 15 min. After centrifugation at 2500 rpm for 10 min, the pellet was collected and resuspended in 1 ml of lysis solution and centrifuged at 6000 rpm for 10 min. The pellet was resuspended in a buffer containing 0.42 mM NaCl, 20 mM Hepes, pH 7.5, 25% glycerol, 2 mM MgCl2, 0.2 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride for 1 min. After centrifugation at 2500 rpm for 10 min, the supernatant containing nuclear extract was collected, and 10 μg of nuclear extract was used to carry out the electrophoretic mobility shift assay with a kit (Promega) according to the protocol offered by the company. The NF-κB oligonucleotide in the kit was labeled with [32P]ATP, and bands on the gel were visualized by exposure to the Kodak film and then developed.

Mitochondrial Membrane Potential Analysis—Mitochondrial membrane potential (MMP) was analyzed from the accumulation of rhodamine 123. Hepatocytes were seeded onto six-well dishes for 1–2 h, and unattached hepatocytes were gently removed and replaced with fresh medium. The cells were treated with AA, or AA plus sal with or without 10 μM SB203580, 10 μM PD98059, or 10 μM wortmannin for 20 h. One hour before ending the treatment, rhodamine 123 (5 μg/ml) was added to the medium. Cells were harvested by trypsinization and resuspended...
in 0.5–0.8 ml of PBS. The intensity of the fluorescence from rhodamine 123 was determined with a FluorScan flow cytometer as previously described (29).

Statistics—One-way ANOVA (ANOVA with subsequent post hoc comparisons by Sheffé) was performed (Version 10.0, SPSS, Chicago, IL). p values of less than 0.05 were considered statistically significant; values reflect means ± S.E., and the number of experiments are given in the figure legends.

RESULTS

SB203580 Prevents AA or AA Plus Salicylate-induced Toxicity in E47 Cells—HepG2 E47 cells were treated with 60 μM AA or 60 μM AA plus 5 mM sal for 12, 24, or 36 h in the presence or absence of 10 μM SB203580, an inhibitor of p38 MAPK, and cell viability (trypan blue exclusion) was determined. As shown in Fig. 1, AA or AA plus sal induced significant toxicity in E47 cells in a time-dependent manner. Salicylate, which was not toxic by itself, increased the toxicity by AA as described previously (19). SB203580 by itself was not toxic to the E47 cells, however, SB203580 lowered the AA or AA plus sal-induced toxicity e.g. from 60 or 80% at 24 h in the absence of SB203580 to toxicity values of 20 or 30% (p < 0.05) in the presence of SB203580. Similar protection by SB203580 was observed in AA concentration dependence experiments, e.g. in the absence of SB203580, toxicity by 15, 30, or 60 μM AA at 24 h was 24 ± 3, 34 ± 7, and 60 ± 6%, respectively, whereas the toxicity in the presence of 10 μM SB203580 was lowered to values of 18 ± 2, 20 ± 3, and 22 ± 7% (p < 0.05) at 15, 30, or 60 μM AA, respectively (data not shown). C34 cells, which do not express CYP2E1, only exhibited a small response to AA or AA plus sal (Table 1) as compared with the E47 cells, which was not altered by SB203580. The effect of SB203580 on another model of toxicity, independent of CYP2E1, was evaluated. In contrast to results with AA or AA plus salicylate in E47 cells, the toxicity by tumor necrosis factor α plus cycloheximide was similar in E47 and C34 cells, and this toxicity was not altered by SB203580 (Table 1). Thus, there appears to be some specificity in the protective actions of SB203580.

| Treatment          | E47 cells Cytotoxicity % | C34 cells Cytotoxicity % |
|--------------------|--------------------------|--------------------------|
| None               | 4.8 ± 1.7                | 3.8 ± 1.0                |
| Salicylate         | 6.5 ± 1.0                | 5.3 ± 1.5                |
| SB203580           | 6.0 ± 1.8                | 6.5 ± 1.0                |
| AA                 | 31.5 ± 8.7               | 5.3 ± 1.0                |
| AA + Sal           | 57.0 ± 7.5               | 13.0 ± 2.9               |
| AA + SB203580      | 10.5 ± 2.3               | 9.5 ± 1.3                |
| AA + Sal + SB203580| 28.0 ± 7.5               | 12.0 ± 3.2               |
| TNF                | 5.0 ± 3.4                | 4.5 ± 1.7                |
| CHX                | 5.5 ± 1.7                | 6.0 ± 0.8                |
| TNF + CHX          | 37.3 ± 6.9               | 39.5 ± 3.9               |
| TNF + CHX + Sal    | 42.8 ± 6.9               | 47.3 ± 7.5               |
| TNF + CHX + Sal + SB203580 | 45.8 ± 7.9 | 44.0 ± 15.1 |

* Nova statistic analysis, p < 0.05 compared with control group.

** Nova statistic analysis, p < 0.05 compared with AA treatment group.

Fig. 2. SB203580 and PD98059 but not wortmannin decrease AA- or AA plus salicylate-induced toxicity in human hepatocyte cultures. Human hepatocyte cultures were treated with 60 μM AA or AA plus 5 mM sal in the presence or absence of 10 μM SB203580, 10 μM PD98059, or 10 μM wortmannin for 24 h. At the end of treatment, trypan blue exclusion was carried out to determine the percent toxicity. *, significantly different (p < 0.05) compared with control group; **, significantly different (p < 0.05) compared with the AA-treated group; ***, significantly different (p < 0.05) compared with the AA plus sal-treated group. Results are from four experiments.

Fig. 3. SB203580 does not lower the level of CYP2E1 in rat hepatocyte cultures. Pyrazole-induced rat hepatocytes were incubated with or without 5 mM sal or 10 μM SB203580 or 0.6% Me2SO (solvent control for SB203580) for 1 or 2 days. At the end of treatment the cells were harvested, microsomes were prepared, and levels of CYP2E1 were determined by Western blot analysis. The immunoblots were carried out by using anti-CYP2E1 antibody. A typical Western blot from three independent experiments is shown. The bands were scanned and the arbitrary unit values from the experiment are shown above the blots.
SB203580 and PD98059 Lower AA or AA Plus Salicylate-Induced Toxicity in Pyrazole Rat Hepatocytes and Human Hepatocytes—To extend the results with the HepG2 cells to primary liver cells, rats were treated with pyrazole (PY) to increase hepatic CYP2E1 levels. Hepatocytes from PY-induced rats were treated with AA or AA plus salicylate (sal), and cell viability was assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction. Cell viability was decreased to 44 ± 7/1006 or 31 ± 1/1006% after 24 h of culture with 60 ± 9262 M AA or 60 ± 9262 M AA plus 5 mM sal, respectively (data not shown). Adding SB203580, a p38 MAPK inhibitor, or PD98059, an ERK MAPK inhibitor, lowered this toxicity. In the presence of SB203580, cell viability increased to 84 ± 3/1006 (AA) and 75 ± 6/1006% (AA plus sal) (p < 0.05), whereas in the presence of PD98059, cell viability increased to 63 ± 6 (AA) and 78 ± 7% (AA plus sal) (p < 0.05), respectively. Wortmannin, a PI3K inhibitor, used at concentrations ranging from 0.1 to 10 ± 9262 M, failed to prevent AA- or AA plus sal-induced toxicity. SB203580, PD98059, or wortmannin had no effect on cell viability; neither did the Me2SO (0.6%) solvent control.

Human hepatocyte cultures were treated with AA or AA plus salicylate in the presence or absence of SB203580, PD98059, or wortmannin for 24 h. AA (60 μM) or AA plus 5 mM sal caused a 50 ± 2/1006 or 84 ± 1% loss of cell viability, respectively (Fig. 2). SB203580 lowered toxicity to 23 ± 7 or 50 ± 2% (p < 0.05), whereas PD98059 lowered toxicity to 36 ± 4 or 63 ± 10% (p < 0.05), respectively. Wortmannin failed to prevent AA- or AA

Fig. 4. MAPK or PI3K inhibitors do not affect AA- or AA plus salicylate-induced lipid peroxidation. A, pyrazole-induced rat hepatocytes were treated with 60 μM AA or AA plus 5 mM sal in the presence or absence of 10 μM SB203580, 10 μM PD98059, or 10 μM wortmannin (or 0.6% Me2SO) for 24 h, respectively. Cells were harvested, and a cellular lysate was prepared by sonication. Cell extract containing 0.2 mg of protein was incubated with a trichloroacetic acid-TBA-HCl solution to carry out the lipid peroxidation analysis. The production of TBARS (malondialdehyde) was measured at 535 nm. *, significantly different (p < 0.05) compared with control. There was no significant effect of SB203580, PD98059, or wortmannin on the AA- or AA plus sal-induced increase in lipid peroxidation. Results are from four experiments. B, rat liver microsomes (0.2 mg) were incubated with Me2SO (0.6%), SB203580 (10 μM), in Me2SO (0.6%) or Trolox (100 μM) for the indicated time periods.

The production of TBARS (malondialdehyde) was measured at 535 nm. *, significantly different (p < 0.05) compared with control. There was no significant effect of SB203580, PD98059, or wortmannin on the AA- or AA plus sal-induced increase in lipid peroxidation. Results are from four experiments.
plus sal-induced toxicity in human hepatocyte cultures (p > 0.05, Fig. 2). Generally, with both PY rat hepatocytes and the human hepatocytes, SB203580 was more effective than PD98059 in preventing the CYP2E1 plus AA toxicity.

Effect of SB203580 on CYP2E1 Protein Levels—Changes in the level of CYP2E1 could be one mechanism by which SB203580 protects against AA toxicity in the hepatocytes or E47 cells. Pyrazole-induced rat hepatocytes were incubated with 10 μM SB203580 for 1 or 2 days, and levels of CYP2E1 were determined by Western blot analysis. The immunoblots showed the expected decrease in CYP2E1 levels when hepatocytes are placed in culture (30) (Fig. 3, lanes 1, 2, and 6). SB203580 did not lower CYP2E1 protein levels as compared with control incubations (Fig. 3, lanes 4 and 8); actually, CYP2E1 levels were increased, but this increase was due to the Me2SO solvent used to solubilize the SB203580 (Fig. 3, lanes 5 and 9). Me2SO is a CYP2E1 ligand that protects against CYP2E1 degradation. This suggests that SB203580 prevention of AA- or AA plus sal-induced toxicity in hepatocytes is not mediated via lowering the levels of CYP2E1. As previously shown (19), salicylate helped to partially maintain CYP2E1 levels at 24 h in the tissue culture (Fig. 3, compare lanes 3 and 7 with lanes 2 and 6), and this maintenance was not altered by SB203580 (not shown).

Effect of SB203580 on Lipid Peroxidation—Treating the cells with AA increased lipid peroxidation in hepatocytes about 2-fold, and this was further elevated in the presence of salicylate (Fig. 4A). Salicylate alone, as previously shown (19), had no effect on lipid peroxidation. Antioxidants such as Trolox, which prevent the AA-induced lipid peroxidation, were previously shown to protect against the AA toxicity (29); hence, if SB203580 had any antioxidant action, its ability to prevent lipid peroxidation could explain its protective effects against AA-induced toxicity. However, SB203580, PD98059, or wortmannin (or Me2SO) did not reduce lipid peroxidation induced by AA or AA plus sal (Fig. 4A). To further exclude a possible antioxidant effect of SB203580, we incubated rat liver microsomes with Me2SO, Me2SO plus SB203580, or, as a positive control, the antioxidant Trolox (100 μM) and determined lipid peroxidation (Fig. 4B). SB203580 did not affect lipid peroxidation of rat microsomes compared with its Me2SO solvent control. In contrast, Trolox completely inhibited the lipid peroxidation (Fig. 4B). These results suggest that the prevention by SB203580 of AA- or AA plus sal-induced toxicity is not mediated by an antioxidant action that lowers lipid peroxidation.

Effect of Kinase Inhibitors on AA- or AA Plus Salicylate-induced Reduction of the MMP—Mitochondria appear to be a critical target for damage by AA as well as a major organelle for the production of reactive oxygen species. AA was previously

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Fig. 5. SB203580 prevents AA- or AA plus salicylate-induced reduction of MMP. Pyrazole-induced rat hepatocyte cultures were treated with 60 μM AA or AA plus 5 mM sal in the presence or absence of 10 μM SB203580, 10 μM PD98059, or 10 μM wortmannin for 20 h, respectively. One hour before ending of treatment, Rh123 (5 μg/ml) was added. Cells were then harvested, and the MMP was analyzed by flow cytometry. M₁ and M₂ refer to two cell populations with low or high membrane potential. The percentage of cells in the M₁ zone is indicated in the cytogram.
shown to decrease MMP, a decrease that was intensified by salicylate; moreover, cyclosporin A, an inhibitor of the mitochondrial permeability transition protected against AA or AA plus salicylate toxicity (29). Therefore, it was important to study whether the inhibition of p38 MAPK by SB203580 could protect against the AA or AA plus sal reduction of the MMP. Cells were treated with AA or AA plus sal in the presence or absence of kinase inhibitors for 24 h, and the cells were then treated with Rh123 for 1 h. Flow cytometry was carried out to determine the extent of Rh123 fluorescence, an index of the MMP. The percentage of cells with low Rh123 fluorescence (M1 population; reflective of low MMP) was increased from 12 to 31% by AA or to 49% by AA plus sal (Fig. 5). However, in the presence of SB203580, the decline of MMP was prevented as the cells with low MMP decreased to 10% (AA) or 16% (AA plus sal) \((p < 0.05, \text{Fig. 5})\). PD98059 showed only a modest preventive effect (21% or 33% M1 cells), whereas wortmannin had no protective effect against the decline in MMP produced by AA or AA plus salicylate (Fig. 5).

Activation of p38 MAPK Phosphorylation by AA or AA Plus Salicylate—The prevention of AA toxicity by SB203580 suggests a role for p38 MAPK in the overall toxicity mechanism of AA. One method to assess activation of p38 MAPK is by determining its extent of phosphorylation. Treatment of rat hepatocytes with AA, sal, or AA plus sal significantly increased the level of phosphorylated p38 MAPK. After 20-min treatment with AA, sal, or AA plus sal, the extent of p38 MAPK phosphorylation increased 2.6-, 2.9-, or 5.2-fold, respectively (Fig. 6A), compared with lane 1, respectively, whereas the extent of phosphorylation increased 3.1-, 3.7-, or 3.9-fold, respectively, after 30-min incubation (Fig. 6A, lanes 4, 2, and 5 compared with lane 1, respectively). AA or AA plus sal did not activate p38 MAPK phosphorylation activity after 30-min treatment in saline hepatocytes with lower levels of CYP2E1 (data not shown). AA, sal, or AA plus sal also induced p38 MAPK phosphorylation in human hepatocyte cultures (Fig. 6B, top panels, lanes 4, 2, and 5 compared with lane 1, respectively). The ratio of phosphorylated p38 MAPK to total p38 MAPK was increased to values of 2.7, 2.1, and 4.2 after 30-min treatment with salicylate, AA, or AA plus salicylate, respectively, over the control values of 1.0. In contrast to p38 MAPK, AA or AA plus salicylate did not promote the phosphorylation of either ERK or PI3K in PY hepatocytes (data not shown) or human hepatocytes (Fig. 6B, bottom panels, ERK ratios of phosphorylated ERK to total ERK between 0.9 to 1.2). SB203580 lowered the level of phosphorylated p38 MAPK induced by AA or AA plus salicylate in the pyrazole hepatocytes (lanes 6 and 7 compared with lanes 4 and 5 in Fig. 6A) and the human hepatocytes (lanes 6 and 8 compared with lanes 4 and 5, Fig. 6B, top panels), validating the ability of this compound to inhibit p38 MAPK activity (ratios of phosphorylated p38 MAPK to total MAPK of 0.5 to 0.7 in the presence of AA or AA plus salicylate plus SB203580).

Antioxidants Prevent AA or AA Plus Salicylate Activation of p38 MAPK—In E47 cells or pyrazole hepatocytes with high levels
of CYP2E1, AA produces radical stress and lipid peroxidation (10, 19, 29). Oxidative stress is known to modulate signal transduction pathways (33). To test whether oxidative stress produced by AA treatment is related to the activation of p38 MAPK, pyrazole-induced rat hepatocytes were preincubated with the antioxidants Trolox or catalase, and then the cells were treated with AA or AA plus salicylate for 30 or 60 min. The p38 MAPK phosphorylation level was determined by Western blot using the phosphorylated p38 MAPK antibody. AA increased p38 phosphorylation at 30 min but not after 60 min; salicylate potentiated the AA-induced increase in p38 MAPK at 30 or 60 min (Fig. 7, lanes 4 and 7 compared with lane 1). Both antioxidants blocked AA (lanes 5 and 6)—or AA plus salicylate (lanes 8 and 9)—induced activation of p38 MAPK to the control levels (Fig. 7). SB203580, as a positive control, also blocked the activation of p38 MAPK (lanes 10–12). These results suggest that AA-induced oxidative stress and not AA per se played a role in the activation of p38 MAPK in hepatocytes with high levels of CYP2E1.

**Effect of AA, Salicylate, or SB203580 on the Translocation of NF-κB**—The activation of NF-κB may inhibit apoptosis by increasing NF-κB-dependent gene expression to produce products that prevent cellular toxicity. Inhibition of NF-κB activation enhances toxicity in various models, including hepatocytes (33, 34). In hepatocytes, p38 MAPK can inhibit the activation of NF-κB, and it was therefore considered that such effects might contribute to the role of p38 MAPK in AA and AA plus salicylate toxicity and in the protection afforded by SB203580. The possibility that AA could, at least initially (prior to the onset of significant toxicity), activate NF-κB by promoting its translocation into the nucleus was therefore evaluated. Pyrazole-induced rat hepatocytes were treated with AA, sal, or SB203580 for 24 h, and the translocation of NF-κB into the nucleus was
detected by immunohistochemistry. In control, non-treated cells, little or no NF-κB was detected in the nucleus in the absence or presence of salicylate (Fig. 8, A and B). After 24-h treatment, AA induced translocation of NF-κB into the nucleus (Fig. 8C). Salicylate inhibited this translocation of NF-κB (Fig. 8D), which may contribute to the salicylate enhancement of AA toxicity. In the presence of salicylate plus SB203580, the translocation of NF-κB was restored (Fig. 8E), which may play a role in the protective effects of SB203580. SB203580 alone has no significant effect on the translocation of NF-κB (Fig. 8F). An electrophoretic mobility shift assay was carried out to evaluate the effect of AA, sal, or SB203580 on NF-κB DNA binding activity. Control NF-κB binding activity in hepatocytes in the absence of additions was low and assigned a value of 100 arbitrary densitometric units (Fig. 9, lane 5). Treatment with 60 μM AA for 24 h increased NF-κB binding with DNA to 360 ± 20 units (Fig. 9, lane 8). Salicylate inhibited this increased binding produced by AA to levels of 100 ± 15 units (lanes 10 and 11). SB203580 partially restored the NF-κB binding level in the presence of AA plus salicylate to 200 ± 20 units (lanes 15 and 16). AA did not increase the NF-κB binding level after a 30-min treatment in contrast to the increase found after the 24-h treatment (data not shown).

DISCUSSION

Mitogen-activated protein kinases (MAPKs) are regulated by distinct signal transduction pathways that control many aspects of mammalian cellular physiology, including cell proliferation, differentiation, inflammation, and apoptosis (35, 36). The activation of MAPK also plays a role in cell toxicity effects (37–39). We are not aware of any studies on the possible role of MAPKs in cytochrome P450-dependent toxicities, especially with respect to CYP2E1-dependent actions.

Arachidonic acid has been shown to induce the phosphorylation of MAPKs (40–42), but whether such activation can be related to AA toxicity and the mechanism through which MAPK plays a role in AA toxicity have not been addressed. Exogenous polysaturated fatty acids (PUFAs) such as AA exert a wide range of effects on cells of diverse origin, such as regulation of gap junctions, permeability between adherent cells, neutrophil secretion and migration, NADPH oxidase activities, expression of cell-surface receptors, gene transcription, cytotoxic T cell function, and modulation of the activities of components of intracellular signaling, PUFAs such as AA also play an important role in chronic alcohol-induced liver injury, where correlations between injury, lipid peroxidation, and CYP2E1 have been reported (4–7). Our previous work has demonstrated that AA can induce apoptosis and necrosis in pyrazole-induced rat hepatocytes with high levels of CYP2E1 to a much greater extent than in saline control rat hepatocytes, or in E47 cells as compared with non-CYP2E1-expressing HepG2 cells (19, 29). Salicylate was recently found to have a synergistic effect in enhancement of AA toxicity and AA-induced lipid peroxidation (19).

In the current study, AA or AA plus salicylate was shown to induce the phosphorylation of p38 MAPK 2.0- to 5.0-fold as compared with non-treated controls in pyrazole-induced rat hepatocyte or human hepatocyte cultures. SB203580, a specific p38 MAPK inhibitor, lowered the AA or AA plus salicylate-induced toxicity. These results suggest that p38 MAPK plays a role in AA- and AA plus salicylate-induced toxicity. Although PD98059, an ERK inhibitor, also lowered AA- or AA plus salicylate-induced toxicity, generally, this compound was not as effective as SB203580, and ERK phosphorylation activity was not induced by AA or AA plus salicylate. Thus, activation of ERK does not appear to play an important role in AA toxicity in hepatocyte cultures, although why PD98059 is somewhat protective remains unclear. AA or AA plus salicylate did not increase AKT phosphorylation, and the PI3K inhibitor wortmannin over a wide concentration range did not block AA- or AA plus salicylate-induced toxicity, indicating that PI3K does not play a role in this toxicity process. p38 MAPK inhibitors such as SB203580 or SB202190 are pyridinyl imidazole compounds that inhibit p38 MAPK function by binding to the ATP-substrate binding pocket. In the present study, SB203580 alone had no effect on CYP2E1 levels nor did it act as an antioxidant. Importantly, SB203580 did not affect AA- or AA plus salicylate-induced lipid peroxidation suggesting that the inhibitor was functioning downstream from the generation of lipid peroxidation, which results from the interaction of CYP2E1 with AA.

It was reported that AA alone, or its epoxycagen or ω-hydroxylase products, can activate the MAPK superfamily members C-Jun NH2-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) in rabbit proximal tubule cells (43, 44). In the present study, AA or AA plus salicylate activated p38 MAPK in pyrazole-induced rat hepatocytes, which contain a 3- to 4-fold higher CYP2E1 level than normal rat hepatocytes; AA or AA plus salicylate did not activate p38 MAPK in saline control rat hepatocytes, which contain very low levels of CYP2E1. Catalase and Trolox block the activation of p38 MAPK by AA in the pyrazole-induced rat hepatocytes. These results suggest that the activation of p38 MAPK by AA or AA plus salicylate is CYP2E1-dependent, and ROS and lipid peroxidation metabolites, but not AA itself, promote the phosphorylation of p38 MAPK. Further studies to evaluate how CYP2E1-dependent ROS production activates p38 MAPK, e.g., the role of upstream regulators such as Ras, are in progress. Interestingly, the antioxidants Trolox and catalase did not prevent the activation of p38 MAPK by salicylate, indicating that ROS and lipid peroxidation did not appear to play a role in the salicylate activation. This is consistent with the lack of effect of salicylate on lipid peroxidation. The mechanism by which salicylate activates p38 MAPK is unknown (21, 24).

How does activation of p38 MAPK contribute to the CYP2E1 plus AA toxicity? Activation of p38 MAPK has been shown to cause apoptosis and cell toxicity (37–39). This may be mediated by downstream mediators such as e-Jun NH2-terminal kinase; preliminary studies have shown that a JNK II inhibitor could partially protect against the AA or AA plus salicylate toxicity in pyrazole hepatocytes, and further studies to identify p38 MAPK downstream mediators are in progress. Clearly, activation of p38 MAPK alone is not sufficient to account for the AA-induced toxicity, because salicylate, which activated p38 MAPK to an equal or even greater extent than did AA, was not toxic under conditions in which AA was toxic. AA-induced lipid peroxidation and ROS generation are critical for the developing toxicity, and other cell targets besides p38 MAPK must play a role in the AA-induced toxicity, although activation of this kinase potentiates or is permissive for the developing toxicity. We have recently shown that damage to mitochondria plays an important role in the AA- or AA plus salicylate-induced toxicity in pyrazole hepatocytes, because there was an early reduction of mitochondrial membrane potential prior to toxicity, and cyclosporin A, an inhibitor of the mitochondrial permeability transition, partially prevented the toxicity (29). The decrease in mitochondrial membrane potential produced by AA (but not salicylate) was confirmed in the current study (Fig. 5). Importantly, SB203580 prevented the AA- or AA plus salicylate-induced lowering of the mitochondrial membrane potential. We hypothesize that mitochondrial membrane potential is sensi-
tive to the combined actions of activated p38 MAPK coupled to ROS and lipid peroxidation-induced oxidative stress. The AA and the salicylate potentiation of AA toxicity were shown to be a mixed mode of cell death, because both necrosis and apoptosis could be observed (19, 29). Damage to the mitochondria and a decrease in membrane potential would result in a decline in ATP levels, which would promote a necrotic mode of cell death. AA induced a membrane permeability transition, which could result in release of apoptotic-inducing factors such as cytochrome c and activation of caspase-3. These events have been shown to occur in E47 cells and in pyrazole hepatocytes treated with AA or AA plus salicylate (19, 29). Hence, the prevention by SB203580 of the decline in mitochondrial membrane potential and, ultimately, cellular toxicity induced by AA suggests that a critical target of activated p38 MAPK are the mitochondria.

A second target of activated p38 MAPK may be the NF-\(\kappa\)B system. p38 MAPK is one of the more important regulators in the cellular response to inflammation. p38 MAPK can inhibit the degradation of I\(\kappa\)B kinase (45, 46) and, thereby, inhibit the translocation of NF-\(\kappa\)B into the nucleus. NF-\(\kappa\)B is a key component of innate immunity (47), promoting the expression of a set of genes involved in host defense (48, 49). NF-\(\kappa\)B is normally constitutively present in the cytosol, because the interaction with inhibitory I\(\kappa\)B masks the nuclear localization domain of the complex (50). NF-\(\kappa\)B-dependent gene transcription requires the phosphorylation of I\(\kappa\)B by IKK2, which then releases this inhibitory component from the dimer of Rel proteins, followed by degradation of the phospho-I\(\kappa\)B by the proteasome (51, 52). Inhibiting the degradation of I\(\kappa\)B will prevent NF-\(\kappa\)B translocation into the nucleus, and this generally increases susceptibility of the hepatocyte to cytotoxicity by various agents, including pro-oxidants. Sodium salicylate and acetyl salicylic acid (aspirin) inhibit the activation of NF-\(\kappa\)B by preventing the phosphorylation of I\(\kappa\)B and its degradation by the ubiquitin-proteasome pathway (20, 53, 54). It has been shown that sodium salicylate prevents the phosphorylation of I\(\kappa\)B through activation of the p38 MAPK pathway (21). AA did not activate NF-\(\kappa\)B when incubated with pyrazole hepatocytes for short time periods, e.g. 15 min. This time point is associated with maximum activation of p38 MAPK (Fig. 6), which may prevent any potential activation of NF-\(\kappa\)B by AA. At longer time periods, e.g. 24 h, AA did activate NF-\(\kappa\)B translocation into the nucleus and increased DNA binding activity (Figs. 8 and 9). This may reflect an attempt to protect the hepatocyte against the ensuing oxidant stress and toxicity. Salicylate, which itself induced the activation of p38 MAPK, blocked this AA-induced NF-\(\kappa\)B translocation and decreased the NF-\(\kappa\)B DNA binding activity (Figs. 8 and 9). This may contribute to the enhancement of AA toxicity by salicylate. SB203580 counters these actions of salicylate and restores NF-\(\kappa\)B translocation and DNA binding activity by inhibiting p38 MAPK activity, and this results in partial protection against toxicity. These results suggest that the activation of p38 MAPK may eventually modulate the activation of NF-\(\kappa\)B and affect NF-\(\kappa\)B-dependent gene expression, which subsequently weakens response of the cell to stress and oxidative toxicity induced by AA.

In summary, the CYP2E1-dependent toxicity of AA in HepG2 cells expressing CYP2E1 and in pyrazole hepatocytes is mediated in part, via activation of p38 MAPK. A model to accommodate current and previous results is shown in Fig. 10. This activation is due to CYP2E1 plus AA production of ROS and lipid peroxidation metabolites, because antioxidants block the activation of p38 MAPK by AA, and the ensuing toxicity. Enhancement of AA toxicity by salicylate, previously shown to be due in part to salicylate increasing the half-life of CYP2E1 by decreasing its turnover, is likely also due to salicylate activation of p38 MAPK. Activated p38 MAPK alone is not likely to promote significant cellular toxicity, because salicylate alone was not toxic. However, the combination of activated p38 MAPK and oxidative stress synergizes to promote cellular toxicity, perhaps by effects on the mitochondrial membrane potential, and may prevent activation of NF-\(\kappa\)B and production of cellular protective agents. Although further studies are necessary to identify upstream and downstream mediators involved in the activation of p38 MAPK by AA, and in the toxic actions associated with activated p38 MAPK, this is the first report on the role of stress-associated MAPK in CYP2E1-dependent toxicity. In view of the critical role of polyunsaturated fatty acids in alcohol-induced liver injury, the possible therapeutic effectiveness of MAPK inhibitors such as SB203580 against CYP2E1-
dependent toxicity and alcohol-dependent toxicity may be worthwhile to consider.

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