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# Sodium Salicylate Increases CYP2E1 Levels and Enhances Arachidonic Acid Toxicity in HepG2 Cells and Cultured Rat Hepatocytes

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## ABSTRACT

Sodium salicylate and acetylsalicylic acid are drugs used as anti-inflammatory agents. Salicylate prevents nuclear factor-κB activation and can cause apoptosis. However, salicylate, a substrate of CYP2E1, is also an antioxidant and can scavenge reactive oxygen species. Experiments were carried out to evaluate whether salicylate can modulate CYP2E1-dependent toxicity. Addition of a polyunsaturated fatty acid such as arachidonic acid (AA) to HepG2 cells resulted in loss of cell viability, especially in cells expressing CYP2E1 (E47 cells). Toxicity was enhanced by the addition of 1 to 10 mM salicylate to the E47 cells but not to control HepG2 cells or HepG2 cells expressing CYP3A4. Salicylate alone was not toxic, and the enhanced toxicity by AA in the presence of salicylate was prevented by diallyl sulfide, a CYP2E1 inhibitor, and by the antioxidant (±)6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid. Salicylate potentiated AA-induced lipid peroxidation in the E47 cells, a reaction blocked by diallyl sulfide. CYP2E1 levels were elevated by salicylate at concentrations (<5 mM), which did not increase CYP2E1 mRNA levels. This increase was associated with a decrease of CYP2E1 turnover by salicylate in the presence of cycloheximide. Salicylate also potentiated AA toxicity in hepatocytes isolated from pyrazole treated rats with high levels of CYP2E1 and from saline controls. In view of the potential role of CYP2E1 in contributing to alcohol-induced oxidative stress and liver injury, the potentiation of CYP2E1-dependent toxicity and the elevation of CYP2E1 levels by salicylate may be of clinical significance and merit caution in the use of salicylate and salicylate precursors such as acetylsalicylic acid with certain other drugs.

The production of reactive oxygen species and generation of a state of oxidative stress seems to be one of the mechanisms by which ethanol produces cellular toxicity (Dianzani, 1985; Nordmann et al., 1992). Several pathways may play a role in contributing to ethanol-induced oxidative stress (Cederbaum, 1989). Induction of CYP2E1 by ethanol is one pathway, which continues to receive much interest as CYP2E1 has been shown to generate superoxide and $H_2O_2$ upon reduction by NADPH (Ekstrom and Ingelman-Sundberg, 1989; Rashba-Step et al., 1993). This reduction is not influenced by the presence of substrates (Guengerich and Johnson, 1997). In the intragastric infusion model of alcohol administration, close association between levels of CYP2E1 and liver damage has been observed in many studies (Castillo et al., 1992; Morimoto et al., 1994; Nanji et al., 1994), but not all (Kono et al., 1999).

One consistent feature in all studies with the intragastric infusion model is that liver injury occurs when the rats consumed a diet containing polyunsaturated fatty acid (PUFA) but not saturated fatty acid. In this model, large increases in lipid peroxidation were observed which were shown to correlate with CYP2E1 levels (Tsukamoto et al., 1990; French, 1992; Morimoto et al., 1994; Nanji et al., 1994). One general hypothesis to account for liver injury with this model was that elevated production of reactive radical species caused by the induction of CYP2E1 resulted in enhanced lipid peroxidation when the diet was supplemented with PUFA. In previous studies, we have shown that arachidonic acid (AA) as a representative PUFA caused toxicity to HepG2 cells expressing CYP2E1 at concentrations and times of incubation that were not toxic to the control C34 cells, which do not express CYP2E1 (Chen et al., 1997). Arachidonic acid also induced significant cytotoxicity in cultures of rat hepatocytes isolated from pyrazole-induced rats, with high levels of CYP2E1 compared with hepatocytes from saline control

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**ABBREVIATIONS:** PUFA, polyunsaturated fatty acid; AA, arachidonic acid; Trolox, (±)6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; DAS, diallyl sulfide; salicylate, sodium salicylate; PI, propidium iodide; CHX, cycloheximide; MEM, minimal essential medium; HPLC, high-performance liquid chromatography; E47 cells, HepG2 cells transfected with pCI-neo vector containing human CYP2E1 cDNA; C34 cells, HepG2 cells transfected with pCI-neo vector.



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rats with lower levels of CYP2E1 (Wu and Cederbaum, 2000). This cytotoxic effect was related to oxidant stress and was dependent upon CYP2E1 because the antioxidant Trolox and the CYP2E1 inhibitor, DAS, could effectively block the AA toxicity (Chen et al., 1997; Wu and Cederbaum, 2000).

Sodium salicylate and acetylsalicylic acid have been used as nonsteroidal anti-inflammatory agents for decades. Acetylsalicylic acid is rapidly deacetylated to salicylate by esterases, present in the gastrointestinal tract, the liver, and serum (Flower et al., 1985). One major mechanism to explain salicylate action as an anti-inflammatory agent is the prevention of activation of nuclear factor-κB by inhibition of phosphorylation and subsequent degradation of IκBα or direct inhibition of IκB kinase (Kopp and Ghosh, 1994; Schwenger et al., 1996; McDade et al., 1999; Alpert and Vilcek, 2000). Salicylate also interferes with mitogen-activated protein kinase and other kinase-dependent signaling pathways (Schwenger et al., 1996; 1997; Chen et al., 1999; Wong et al., 2000), can inhibit transcription of certain genes [e.g., *iNOS* (Farivar and Brecher, 1996)], and affects mitochondrial function and calcium homeostasis (Biban et al., 1995; Trost and Lemasters, 1997). Our interest in salicylate was sparked by recent reports that CYP2E1 (and CYP3A4) can hydroxylate salicylate in the 5-position to produce 2,5-dihydroxybenzoic acid (Dupont et al., 1999) and that acetylsalicylic acid can induce hepatic CYP2E1 when administered in vivo to rats (Damme et al., 1996). In view of the widespread use of salicylate and acetylsalicylic acid, it seemed of interest to evaluate whether salicylate could modulate CYP2E1-dependent toxicity. Salicylate, as an antioxidant, would be expected to protect against oxidative damage produced by CYP2E1; e.g., 2 mM salicylate was shown to protect against reperfusion injury of rat liver (Colantoni et al., 1998) and salicylate and its hydroxylated metabolites are iron chelators (Graziano et al., 1974). On the other hand, salicylate has been shown to induce apoptosis of cells, including rat hepatocytes (Van Antwirp et al., 1996; Trost and Lemasters, 1997; Bellosillo et al., 1998; Klampfer et al., 1999), and inhibition of nuclear factor-κB activation generally promotes cytotoxicity because of the prevention of activation of protective genes. The goal of the current study was to evaluate the effects of salicylate on AA toxicity to HepG2 cells expressing CYP2E1 or to hepatocytes from pyrazole-induced rats and compare these effects with those on control cells that did not express CYP2E1 or hepatocytes that express low CYP2E1.

## Materials and Methods

Sodium salicylate (salicylate), arachidonic acid (AA), Trypan Blue, propidium iodide (PI), diallyl disulfide (DAS), cycloheximide (CHX) and collagenase type II were from Sigma Chemical Co (St. Louis, MO). Trolox was from Aldrich. Fetal bovine serum, MEM, antibiotics, G418, hepatocyte culture medium and HepatoZYME-SFM were from Life Technologies, Inc (Gaithersburg, MD). Enhanced chemiluminescence Western blot detection reagent, cDNA labeling kit, [$^{32}$P]dCTP, and nylon membranes were from Amersham Pharmacia Biotech (Piscataway, NJ). Human CYP2E1 polyclonal antibody raised in rabbits was kindly provided by Dr. Jerry Lasker (Mount Sinai School of Medicine, New York, NY). A CYP2E1 cDNA probe was excised from plasmid P91023 (B)-2E1 (kindly provided by Dr. F. J. Gonzales, National Cancer Institute, Bethesda, MD); this probe contains the full-length human cytochrome P4502E1 cDNA.

**Cell Lines and Cell Cultures.** Three human HepG2 sublines, HepG2-E47 cells, HepG2-C34 cells, and HepG2–8A-13 cells were used as cell culture models in this study. HepG2 E47 cells, a human hepatoma cell line that constitutively expresses CYP2E1, was established by transfection with plasmid pCI-NEO containing CYP2E1 cDNA in the sense orientation (Chen and Cederbaum, 1998). HepG2 C34 cells were established by transfection with pCI-NEO; these cells do not express CYP2E1 (or CYP3A4). HepG2–8A-13 cells, which express human CYP3A4, were obtained from Dr. Dennis Feierman (Mount Sinai School of Medicine). All cell lines were grown in MEM

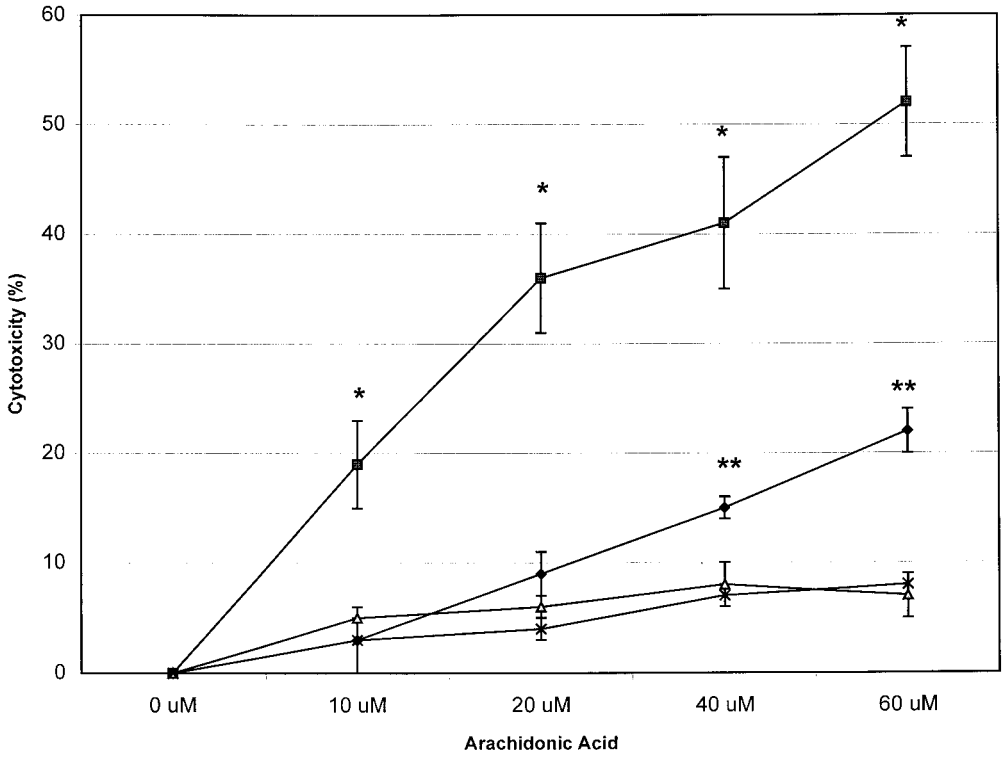


**Fig. 1.** Dose-response curve of AA toxicity in the absence and presence of salicylate. HepG2 E47 cells and control C34 cells were treated with 0, 10, 20, 40, and 60 μM AA in the presence or absence of 10 mM salicylate for 24 h. Cells were harvested by trypsinization and cytotoxicity was determined by a Trypan Blue exclusion method. *Significant difference ($P < 0.05$) compared with other groups. **Significant difference ($P < 0.05$) compared with C34 groups. $n = 4$. ◆, E47-AA; ■, E47-AA-Sal; △, C34-AA; ×, C34-AA-Sal.

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containing 10% fetal bovine serum and 0.4 mg/ml G418 supplemented with 100 U/ml of penicillin, 100 $\mu$g/ml of streptomycin and 0.01% fungizone antibiotics in a humidified atmosphere in 5% $CO_2$ at 37°C. Cells were subcultured at a 1:5 ratio once a week.

Male Sprague-Dawley rats, 150 to 170 g body weight, were intraperitoneally injected with pyrazole at a dose of 200 mg/kg of body weight once a day for 2 days to induce CYP2E1. Control rats were injected with saline only. Rat hepatocytes were isolated by a two-step collagenase perfusion method as described previously (Wu et al., 1990). Hepatocytes were cultured in HepatoZYME-SFM medium as described previously (Wu and Cederbaum, 2000).

For comparative purposes, the content of human CYP2E1 in the E47 cells was about 50 pmol/mg of microsomal protein, whereas the content of rat CYP2E1 after the pyrazole treatment was about 200 to 300 pmol/mg of microsomal protein. Rates of oxidation of *p*-nitrophenol were about 0.2 to 0.3 and 1.5 to 2 nmol/min/mg of protein for microsomes from E47 cells or from pyrazole-treated rats, respectively.

**Cytotoxicity Determination.** A Trypan Blue exclusion method was used to determine cytotoxicity. HepG2 cell lines were seeded onto six-well dishes at a density of $10 \times 10^4$ cells/well for 24 h. The medium was replaced with fresh medium containing a reduced serum concentration of 2.5%. Cells were treated with 0 to 60 $\mu$M AA for 24 h in the presence or absence of 0 to 10 mM salicylate. The AA was dissolved in fetal bovine serum and diluted with MEM. At the end of treatment, the medium was collected and the cells were treated with 0.25% trypsin for 5 min. The collected medium was centrifuged at 800*g* for 5 min to resuspend any detached cells. Cell pellets were collected and washed with PBS. One milliliter of 0.4% Trypan Blue was added to the cells and stained or unstained cells were counted to determine the percentage of necrotic cells in the total cell population.

**Apoptosis Determination.** Cells undergoing apoptosis after the various treatments were determined by a flow cytometry assay (Wu and Cederbaum, 1999). Cells were treated with AA in the absence or presence of salicylate. Some experiments included an antioxidant, Trolox (100 $\mu$M), or the CYP2E1 inhibitor DAS (100 $\mu$M). After 24 h of incubation, the cells were harvested and fixed with 70% ethanol, washed with PBS, and then incubated with 50 $\mu$g/ml RNase A for 2 h at room temperature. Cells were stained with PI (50 $\mu$g/ml) for 30 min followed by analysis of fluorescence (excitation, 488 nm; emission, 575 nm) using an EPICS profile II Analyzer flow cytometer. The cells undergoing apoptosis were calculated from the sub G hypodiploid area programmed by the analyzer.

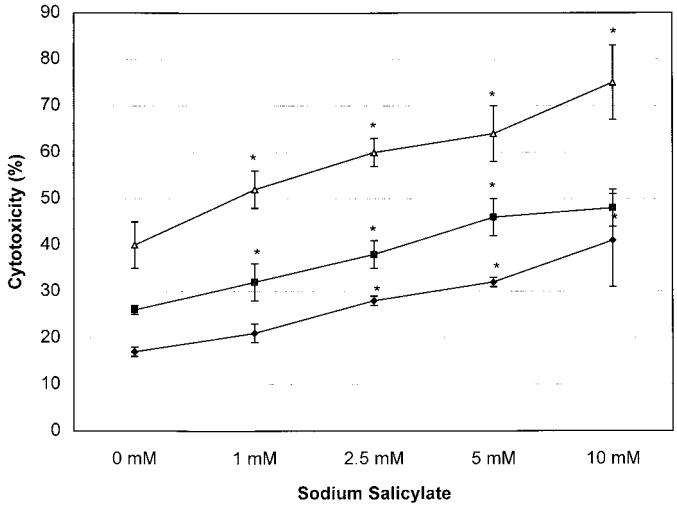


**Fig. 2.** Salicylate concentration curve for enhancement of AA cytotoxicity. HepG2 E47 cells were treated with 0, 1, 2.5, 5, and 10 mM salicylate in the presence of 10, 30, or 60 $\mu$M AA for 24 h. At the end of each treatment, cells were harvested by trypsinization and cytotoxicity was determined by a Trypan Blue exclusion method. *Significant difference ($P < 0.05$) compared with the preceding group. $n = 4$. ◆, AA 10$\mu$M; ■, AA 30 $\mu$M; △, AA 60$\mu$M.

**Immunoblot Analysis.** E47 cells were treated with salicylate (0 to 10 mM) for 24 h or with 5 or 10 mM salicylate for 12, 24, 36, or 48 h. At the end of treatment, cells were harvested and disrupted by sonication and microsomes were prepared by differential centrifugation. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out using an 8.0% gel and 20 $\mu$g of microsomal protein. Samples were blotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA) and incubated with human CYP2E1 polyclonal antibody, followed by incubation with goat anti-rabbit antibody conjugated with horseradish peroxidase. Fluorescence from the binding of the secondary antibody to the CYP2E1 antibody was developed and exposed using the enhanced chemiluminescence immunoblot-detecting reagent. The membranes were exposed to Kodak X-ray film for 5 to 30 s. Densitometry was determined with a computer software program.

**Northern Blot Determination.** HepG2-E47 cells were treated with 0 to 10 mM salicylate for 12, 24, 36, and 48 h. Total mRNA was isolated with the Trizol reagent (Life Technologies, Grand Island, NY). Twenty micrograms of total RNA was electrophoresed in a 1.2% agarose/formaldehyde gel. RNA was then transferred onto a Hybond-XL nylon membrane (Amersham Phamacia Biotech) and hybridized with a full-length human CYP2E1 cDNA probe labeled with [$^{32}$P]dCTP at 42°C overnight. The membranes were washed and exposed to X-OMAT AR Kodak diagnostic X-ray film at −70° overnight and the results were analyzed with a computer software program.

**Lipid Peroxidation Analysis.** E47 cells were treated with 30 or 60 $\mu$M AA in the absence or presence of 10 mM salicylate for 24 h. In some experiments, 100 $\mu$M Trolox or 100 $\mu$M DAS was also added. Cells were harvested and sonicated for 10 s in an ice bath with a Heat System-Ultrasonics. Inc. W-375 sonicator (50% duty cycle, output at 4) and the cellular lysate was collected. A 0.2-ml sample of cell extract containing 0.2 to 0.3 mg of protein was incubated with 0.4 ml of TCA-TBA-HCl solution (15% w/v trichloroacetic acid, 0.375% w/v thiobarbituric acid, 0.25N hydrochloric acid) in a boiling water bath for 15 min. After cooling in an ice bath, the samples were centrifuged at 1000*g* for 10 min. The formation of thiobarbituric acid-reactive components in the reaction was determined at 535 nm, using an extinction coefficient of $1.56 \times 10^5$/M/cm to calculate malondialdehyde equivalents.

**HPLC Measurements of Arachidonic Acid.** To determine the remaining concentration of AA in the incubation medium after the C34 or E47 cells were incubated with AA in the absence or presence of salicylate, HPLC analysis for AA was performed with a Water model 510 liquid chromatograph (Milford, MA). Separation was achieved with a C-18 reverse phase column (4.6 mm × 25 cm, 5 $\mu$m, 100 A; Microsorb; Rainin Instrument Company, Inc., Emeryville, CA). The mobile phase was saturated with helium and contained methanol with 20% (v/v) ethanol for analysis of arachidonic acid. The elution was carried out at a flow rate 1 ml per min. UV detection of arachidonic acid was conducted with a Shimadzu SPD-M10AVP photodiode detector at 205 nm. A 0.020-ml injection loop was used for all experiments. The retention time for AA under these HPLC conditions was 3.6 min.

**CYP2E1 Protein Degradation Analysis.** E47 cells were treated with salicylate at concentrations of 0, 1, 2.5, 5, or 10 mM for 12, 24, and 48 h in the presence of 100 $\mu$M CHX to inhibit synthesis of new CYP2E1 protein. Cells were harvested and sonicated. Immunoblots to detect the remaining CYP2E1 were carried out using 20 $\mu$g of total cell extract protein as described above.

**Statistics.** One–way analysis of variance (analysis of variance) with subsequent post hoc comparisons by Sheffé was performed (ver. 10.0; SPSS, Chicago, IL). *P* values < 0.05 were considered statistically significant; values reflect means ± S.E., and the number of experiments are given in the figure legends.

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## Results

**Salicylate Enhances AA-Induced Cytotoxicity in E47 Cells.** AA was used as a representative PUFA. AA was previously shown to cause toxicity to HepG2 cells expressing CYP2E1 and to pyrazole-induced rat hepatocytes (Chen et al., 1997; Wu and Cederbaum, 2000). A short reaction time of 24 h was used in the current study to allow evaluation of any potential change in AA toxicity. Indeed, salicylate was found to significantly enhance AA-induced cytotoxicity in E47 cells. Figure 1 shows that treatment of E47 cells with 10 to 60 $\mu$M AA for 24 h caused a concentration-dependent increase in cell toxicity. This toxicity was increased in the presence of 10 mM salicylate; for example, in the presence of 40 $\mu$M AA, 13% of E47 cells were stained with Trypan Blue, whereas in the presence of 40 $\mu$M AA plus 10 mM salicylate, 43% of E47 cells accumulated Trypan Blue (Fig. 1). AA induced less cytotoxicity to the C34 control subline, which does not express CYP2E1. Salicylate did not enhance cytotoxicity induced by AA in the C34 cells. Salicylate by itself was not cytotoxic to the HepG2 cells, at least at concentrations up to 10 mM.

A salicylate concentration curve for the enhancement of AA toxicity to the E47 cells is shown in Fig. 2. Treatment of E47 cells with 10, 30, or 60 $\mu$M AA plus 1, 2.5, 5, or 10 mM salicylate gradually increased cell toxicity; e.g., at 60 $\mu$M AA, the percentage cells accumulating Trypan Blue increased from a value of 40 $\pm$ 6% in the absence of salicylate to values of 52 $\pm$ 4, 60 $\pm$ 3, 64 $\pm$ 6, and 74 $\pm$ 7% in the presence of 1, 2.5, 5, and 10 mM salicylate, respectively.

**Trolox and DAS Protect against the Salicylate Enhancement of AA Toxicity.** Morphological observations under the light microscope confirmed the Trypan Blue results on salicylate enhancement of AA toxicity in the E47 cells. As shown in Fig. 3, E47 cells incubated with 30 $\mu$M AA for 12 h (Fig. 3B) or 24 h (data not shown) underwent morphological changes indicating some cell toxicity. The cell membrane lost its smooth appearance, cells rounded up and granules appeared in the cytoplasm. Treating the cells with 30 $\mu$M AA plus 10 mM salicylate for 12 h (D) significantly increased these morphological changes as many cells underwent necrosis. In the presence of 100 $\mu$M Trolox (a vitamin E

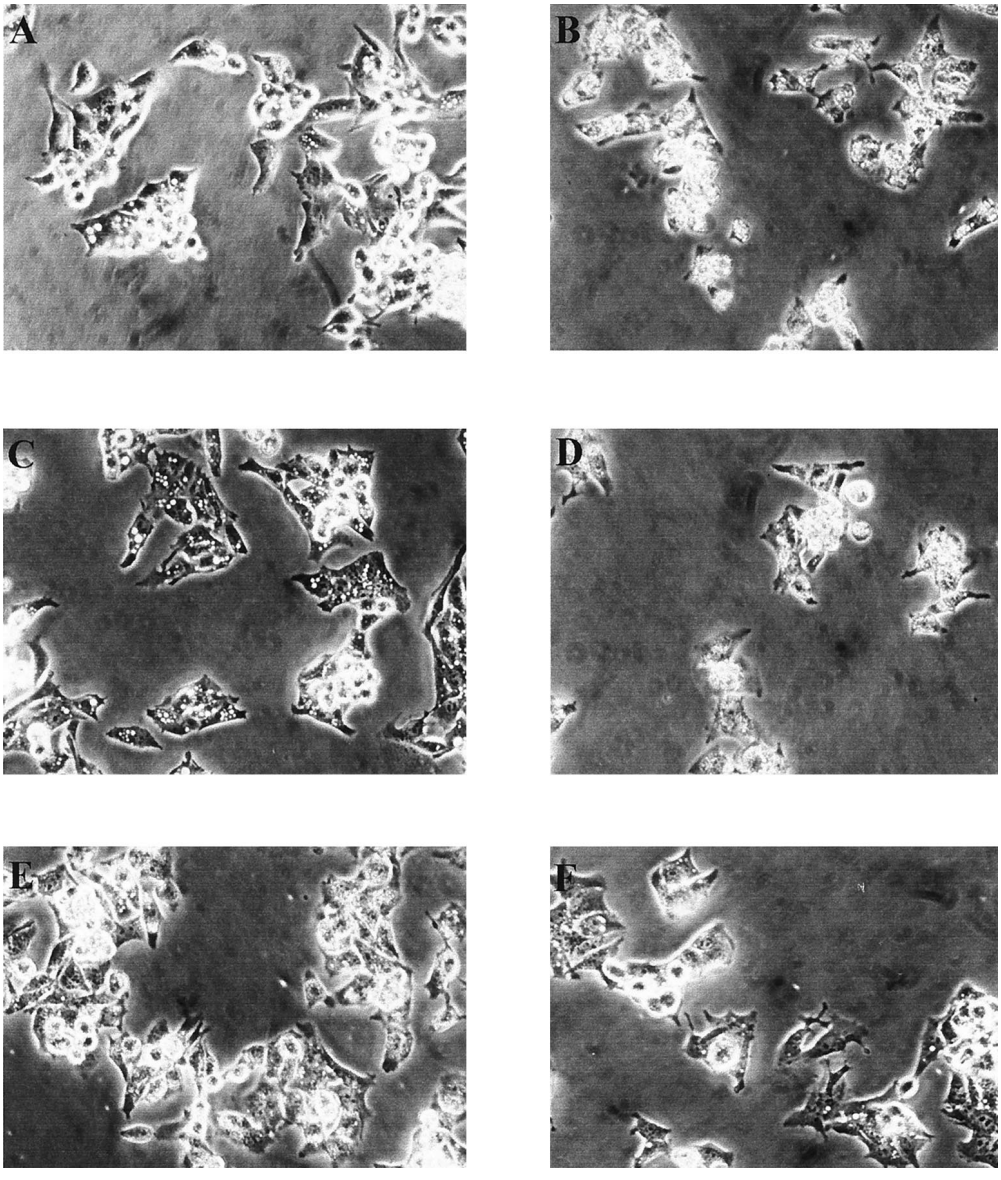


**Fig. 3.** Trolox or DAS prevents the salicylate enhanced cytotoxicity of AA in E47 cells. E47 cells were treated with 30 $\mu$M AA or AA plus 10 mM salicylate in the presence or absence of 100 $\mu$M Trolox or 100 $\mu$M DAS for 12 or 24 h, respectively. Cytotoxic morphologic changes were observed under the light microscope (10×20). A to F, cells were treated for 12 h in the presence of control (A); 30 $\mu$M AA (B); 10 mM salicylate (C); 30 $\mu$M AA plus 10 mM salicylate (D); 30 $\mu$M AA plus 10 mM salicylate plus 100 $\mu$M Trolox (E); and 30 $\mu$M AA plus 10 mM salicylate plus 100 $\mu$M DAS (F). Essentially similar results were observed for the 24-h treatments (data not shown).

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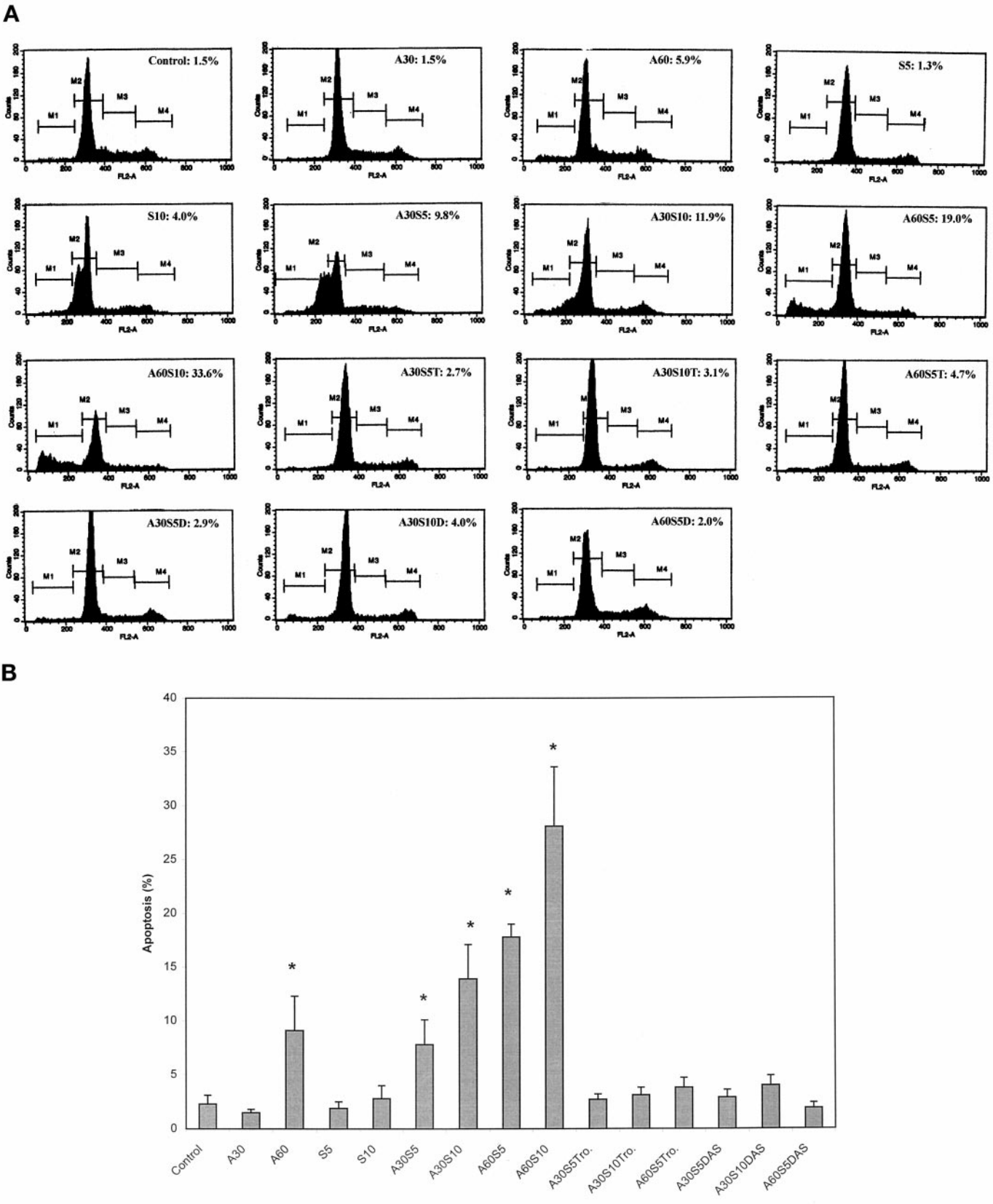


**Fig. 4.** Salicylate enhances AA-induced DNA fragmentation in E47 cells. E47 cells were treated with 30 or 60 $\mu$M AA (abbreviated A30 or A60), or AA plus 5 or 10 mM salicylate (abbreviated S5 or S10) in the presence or absence of 100 $\mu$M Trolox or 100 $\mu$M DAS for 24 h. Cells were harvested by trypsinization, fixed with 70% ethanol, and treated with 50 $\mu$g/ml RNase A for 2 h. Cells were then stained with 50 $\mu$g/ml PI for 30 min. DNA fragmentation was determined by flow cytometry. A, apoptotic cells were counted in the subG1 hypodiploid zones (M 1 in the figure). B, summary of results from four experiments. *Significant difference ($P < 0.05$) compared with control.

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analog) or 100 $\mu$M DAS (an inhibitor of CYP2E1) these morphological changes were prevented (Fig. 3, compare E and F with D).

**Salicylate Enhancement of AA-Induced Apoptosis.** Incubating E47 cells with 30 or 60 $\mu$M AA for 24 h resulted in only a low level of apoptosis (Fig. 4A for a typical experiment, Fig. 4B for summary of four experiments). AA-induced apoptosis was increased in the presence of either 5 or 10 mM salicylate, whereas salicylate by itself did not trigger apoptosis. Trolox and DAS prevented the apoptosis observed in the presence of AA plus salicylate. The prevention by DAS validates the role of CYP2E1 in the AA plus salicylate toxicity, whereas the protection by Trolox suggests that lipid peroxidation-related processes might be important in causing the cellular toxicity.

**Salicylate Potentiates AA-Induced Lipid Peroxidation.** To evaluate whether salicylate modifies AA induced lipid peroxidation, E47 cells were treated with AA or AA plus salicylate in the presence or absence of 100 $\mu$M Trolox or 100 $\mu$M DAS for 24 h. Lipid peroxidation was determined from the formation of malondialdehyde, which results from the break down of oxidized polyunsaturated fatty acids. Treatment of E47 cells with 60 $\mu$M AA for 24 h increased the formation of malondialdehyde from 1.8 $\pm$ 0.5 nmol/mg of cell protein to values of 3.2 $\pm$ 0.8 nmol/mg of cell protein (Fig. 5). Salicylate (10 mM) significantly increased the AA-induced lipid peroxidation to 6.7 $\pm$ 1.3 nmol/mg. Salicylate also significantly increased lipid peroxidation after incubation of E47 cells with 30 $\mu$M AA (Fig. 5). Trolox and DAS completely blocked the potentiation by salicylate of AA-induced lipid peroxidation in E47 cells. This prevention of lipid peroxidation by Trolox or DAS may account for the ability of these two agents to protect against the synergistic toxicity observed for the combined addition of AA plus salicylate to E47 cells.

We determined the concentration of AA remaining in the incubation medium after 24-h incubation of 60 $\mu$M AA in the absence or presence of 10 mM salicylate. Results were as follows: C34 cells, 25 $\mu$M AA remaining; C34 cells plus salicylate, 28 $\mu$M AA remaining; E47 cells, 19 $\mu$M AA remaining; E47 cells plus salicylate, 23 $\mu$M AA remaining. These results indicate that similar amounts of AA (35 to 40 $\mu$M) were taken up and incorporated in C34 and E47 cells, and that salicylate had no effect on this uptake by either cell line. The lipid peroxidation data (Fig. 5), however, suggests that salicylate increases the rate of metabolism of the incorporated AA by peroxidative reactions.

**Effect of Salicylate on AA Toxicity in HepG2 Cells Expressing CYP3A4.** HepG2 8A-13 is a transfected HepG2 subline that constitutively expresses CYP3A4. To study whether salicylate has a synergistic toxic effect with AA in a cell line expressing a different cytochrome P450 enzyme rather than CYP2E1, we treated HepG2 8A-13 cells with 60 $\mu$M AA in the presence or absence of 10 mM salicylate for 24 h. Salicylate did not potentiate toxicity of 60 $\mu$M AA in the CYP3A4-expressing HepG2 cells (percentage cytotoxicity: control, 7 $\pm$ 2; salicylate, 5 $\pm$ 1; AA, 15 $\pm$ 4; AA plus salicylate, 18 $\pm$ 2, $n$ = 4).

**Effect of Salicylate on CYP2E1 Protein Levels in E47 Cells.** To evaluate possible mechanisms by which salicylate acts to potentiate AA toxicity and why this may be more robust in cells expressing CYP2E1, the effect of salicylate on levels of CYP2E1 was determined. Addition of salicylate to E47 cells resulted in an increase in steady-state levels of CYP2E1 (Fig. 6A). CYP2E1 protein was increased by 1.5-, 2-, 2.5-, and 3.5-fold after 24 h of incubation with 1, 2.5, 5, and 10 mM salicylate, respectively. Salicylate also produced a time-dependent increase in CYP2E1 levels; 10 mM salicylate elevated CYP2E1 levels by 1.4-, 2.8-, 3.2-, and 6.1-fold after 12, 24, 36, or 48 h of incubation, respectively (Fig. 6, B and C). Increases in the level of CYP2E1 may play an important role in the potentiation of AA toxicity by salicylate.

To study why CYP2E1 levels were increased by salicylate,

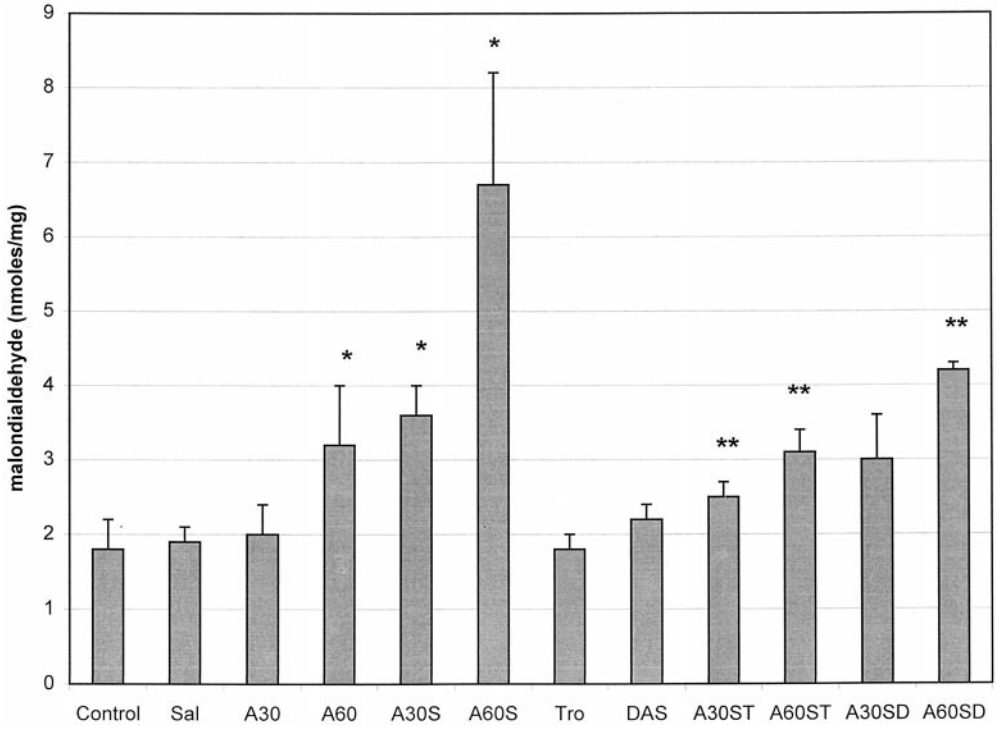


**Fig. 5.** Salicylate enhances AA induced lipid peroxidation in E47 cells. E47 cells were treated with 30 or 60 $\mu$M AA (A30 or A60) in the absence or presence of 10 mM salicylate (sal or S). In some experiments, 60 $\mu$M Trolox (Tro or T) or 100 $\mu$M DAS (D) were added. After 24 h, cells were collected and sonicated. Lipid peroxidation was determined using 0.15 to 0.25 mg of lysate protein as described under *Materials and Methods*. The production of malondialdehyde was determined at 535 nm. *Significant difference ($P < 0.05$) compared with control. **Significant difference ($P < 0.05$) compared with same arachidonic acid plus sodium salicylate dose groups. $n$ = 4.

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CYP2E1 mRNA levels were determined by Northern blot analysis. Salicylate at a concentration of 5 mM did not change CY2E1 mRNA levels over a 48-h incubation (Fig. 7). Salicylate (10 mM) produced an increase of ~2-fold in CYP2E1 mRNA levels at all time periods evaluated (Fig. 7); this concentration of salicylate, however, also increased the levels of the loading control, glyceraldehyde 3-phosphate dehydrogenase (data not shown). The ratio CYP2E1/GAPDH mRNA was not increased by 10 mM salicylate. The reasons for this effect by 10 mM salicylate are not known.

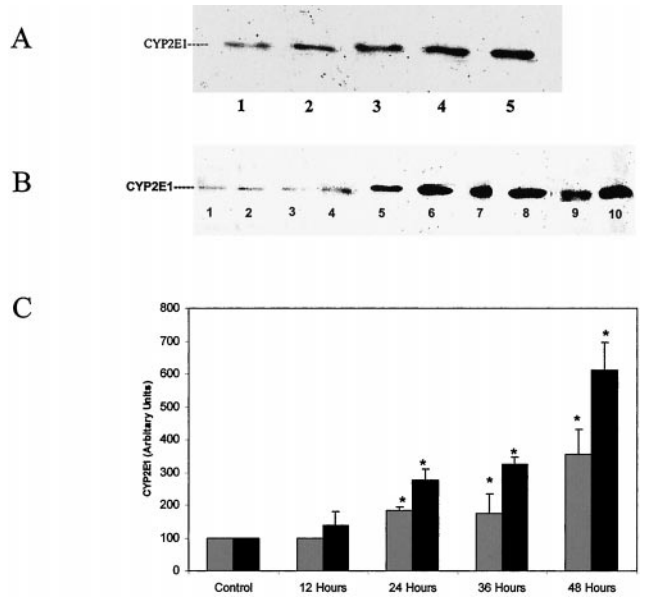


**Fig. 6.** Salicylate increases the content of CYP2E1. A, E47 cells were treated with 0, 1, 2.5, 5, and 10 mM salicylate for 24 h (Lanes 1, 2, 3, 4, and 5, respectively). B, time course-increase of CYP2E1 in E47 cells by salicylate. Cells were either not treated with salicylate (lanes 1 and 2) or treated with 5 or 10 mM salicylate for 12 (lanes 3 and 4), 24 (lanes 5 and 6), 36 (lanes 7 and 8), or 48 h (lanes 9 and 10). Cells were harvested and microsomes were prepared and the immunoblots were carried out using 20 $\mu$g of microsomal protein. C, the results were scanned with a computer scanner and levels of CYP2E1 were expressed in arbitrary units as shown in the bar graphs. Control refers to levels of CYP2E1 in the absence of salicylate. *Significant difference ($P < 0.05$) compared with control. $n = 4$. ▨, 5 mM; ■, 10 mM.

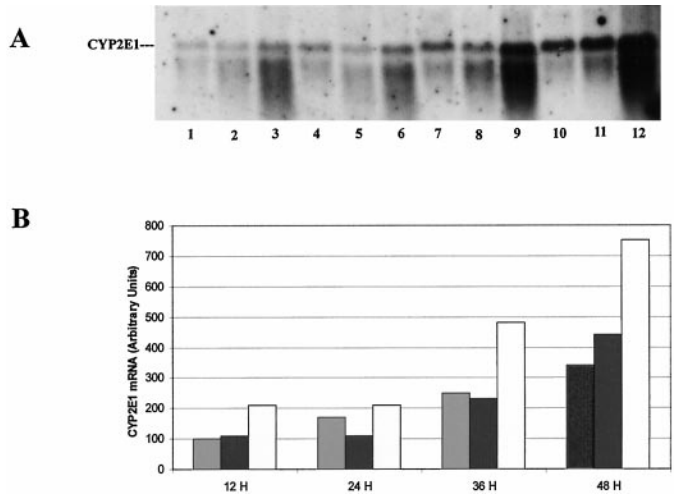


**Fig. 7.** Effect of salicylate on CYP2E1 mRNA in E47 cells. E47 cells were treated with 5 or 10 mM salicylate for 12, 24, 36, or 48 h. Total mRNA was isolated and Northern blot analysis was carried out with the use of a probe containing the full- length human CYP2E1 cDNA (top). The bands were scanned with computer software program and results were expressed as fold increase as shown in the bottom panel. Lanes 1, 4, 7, and 10 are 12, 24, 36, and 48 h in the absence of salicylate; lanes 2, 5, 8, and 11 are 12, 24, 36, and 48 h in the presence of 5 mM salicylate; lanes 3, 6, 9, and 12 are 12, 24, 36, and 48 h in the presence of 10 mM salicylate. Levels of GAPDH mRNA were not affected by 5 mM salicylate but were increased by 50 to 100% at 10 mM salicylate (data not shown). ▨, O mM; ■, 5 mM; □, 10 mM.

**Effect of Salicylate on CYP2E1 Degradation.** Ethanol and other CYP2E1 ligands such as acetone and 4-methylpyrazole increase levels of CYP2E1 largely by stabilizing the enzyme against degradation (Song et al., 1986; Koop and Tierney, 1990). Because 5 mM salicylate increased CYP2E1 levels (Fig. 6) without increasing CYP2E1 mRNA levels (Fig. 7), the possibility that salicylate could increase CYP2E1 by preventing or slowing its degradation was considered. E47 cells were treated with cycloheximide to inhibit new protein synthesis and the rate of decline in CYP2E1 protein was determined by immunoblot analysis. As shown in Fig. 8, CYP2E1 levels rapidly declined after the addition of cycloheximide with less than 20% remaining after 24 h of incubation. Salicylate produced a concentration-dependent decrease in the degradation of CYP2E1; e.g., more than 50% CYP2E1 was still present 24 h after the addition of cycloheximide in the presence of 5 or 10 mM salicylate (Fig. 8).

Because salicylate may be metabolized by CYP2E1 (Dupont et al., 1999), it would be of interest to assess what the effect of induction of CYP2E1 by salicylate would have on the toxicity of AA in the absence of the competitive substrate salicylate. However, if the major mechanism of the salicylate potentiation of AA toxicity was elevated CYP2E1 content as a consequence of salicylate stabilization against degradation, removal of salicylate would prevent this stabilization effect, cause CYP2E1 levels to fall, and result in AA toxicity comparable with no added salicylate values. An experiment was carried out to increase CYP2E1 by a 24-h incubation with 10 mM salicylate, followed by removing the salicylate and washing the cells, and then adding AA and studying cell viability after a 24-h incubation. Results are shown in Table 1. The addition of 30 or 60 $\mu$M AA to E47 cells that had been preincubated only with buffer resulted in losses of cell viability of 21 and 37%, respectively, after 24-h incubation. Addition of 30 or 60 $\mu$M AA to E47 cells previously incubated with salicylate, followed by removal of the salicylate, resulted in losses of cell viability of 25 and 42%, respectively, after 24-h incubation. Thus, the preincubation, followed by removal of salicylate did not potentiate AA toxicity. We have previously shown that the half-life of CYP2E1 in the HepG2 cells was about 4 to 6 h (Yang and Cederbaum, 1997). The failure of the salicylate preincubation to potentiate AA toxicity is probably caused by the rapid deinduction of CYP2E1 once the salicylate stabilizer is removed, coupled to the rapid turnover of CYP2E1 in the absence of a stabilizing ligand or substrate. As shown in Table 1, once the salicylate is added to either the buffer-preincubated cells or the salicylate-preincubated cells, AA toxicity is now potentiated. AA toxicity was considerably lower in the C34 cells than E47 cells preincubated with either buffer or salicylate, and addition of salicylate did not potentiate AA toxicity in the C34 cells (Table 1).

**Salicylate Increases AA Toxicity in Cultured Rat Hepatocytes.** To extend the results of salicylate increasing AA toxicity in HepG2 cells to intact liver cells, hepatocytes

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were isolated from rats treated with pyrazole, to increase CYP2E1 levels, and from saline control rats and placed into culture for 24 or 48 h. As described previously (Wu and Cederbaum, 2000), AA was more toxic to the hepatocytes from the pyrazole-treated rats than toward the control hepatocytes (Fig. 9), consistent with CYP2E1 playing a role in potentiating AA toxicity. Salicylate, which by itself had no effect on cell viability, increased AA toxicity by about 50% in both hepatocyte cultures and at both time points (Fig. 9). Similar results were found when examining cellular morphology. Salicylate, at a concentration of 10 mM, in the presence of 30 or 60 $\mu$M AA, caused almost complete loss of viability of hepatocytes from pyrazole-treated rats (Fig. 10, E and F) compared with incubations in the absence of any addition (Fig. 10A) or salicylate alone (Fig. 10B) or 30 or 60 $\mu$M AA alone (Fig. 10, C and D). The salicylate-plus-AA–treated hepatocytes lost their "hepatocyte" morphology and had swollen plasma membranes, without a distinct nucleus. Similar but less dramatic changes in morphology were observed in hepatocytes from saline-treated rats (data not shown).

## Discussion

The goal of the present study was to evaluate whether sodium salicylate, a widely used nonsteroidal anti-inflammatory agent, can alter arachidonic acid- induced cytotoxicity to cells expressing CYP2E1. Arachidonic acid, as a representative PUFA, was toxic to cells that express CYP2E1 but not to cells that did not express CYP2E1 (Chen et al., 1997). AA was also found to be toxic to rat hepatocytes isolated from pyrazole-treated rats but had very low toxicity to hepatocytes from saline-injected control rats (Wu and Cederbaum, 2000). These increases in cytotoxicity were associated with in-

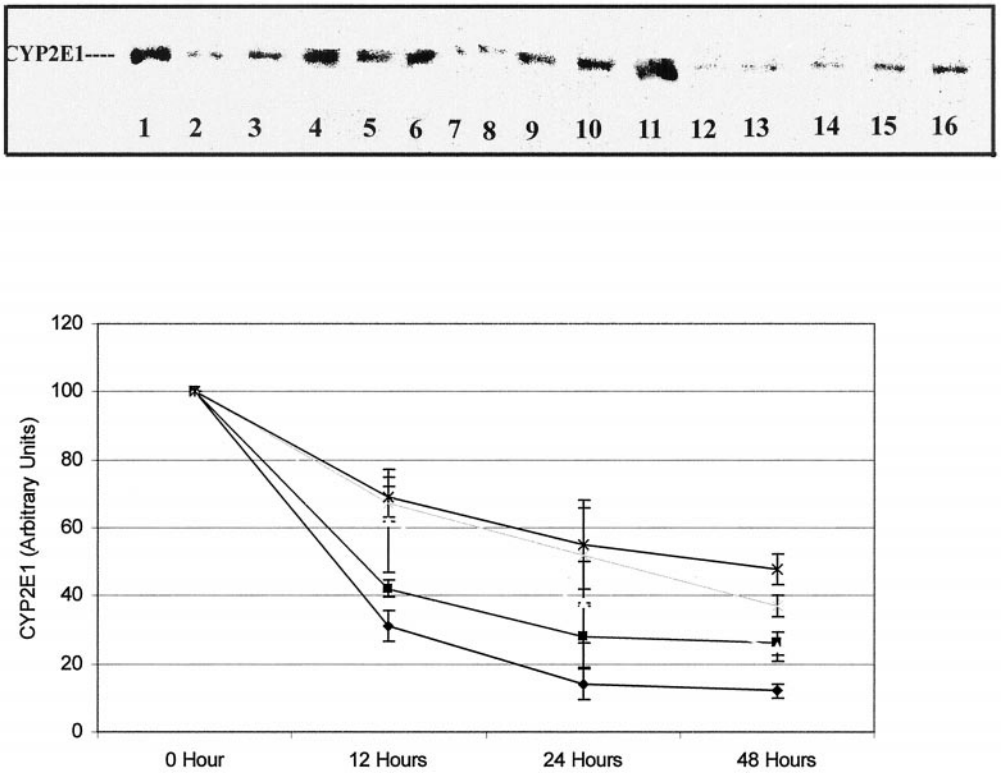


**Fig. 8.** Salicylate stabilizes CYP2E1 protein from degradation. E47 cells were treated with 0, 1, 2.5, 5, and 10 mM salicylate in the presence of 100 $\mu$M CHX for 12, 24, and 48 h. Cells were harvested and sonicated. Immunoblot analysis was carried out using 20 $\mu$g of lysate protein. The blots were scanned with computer software program and results expressed as arbitrary units as shown in the bottom panel. Lanes 2 to 6 are 12 h in the presence of 0, 1, 2.5, 5, and 10 mM salicylate, respectively; lanes 7 to 11 are 24 h in the presence of 0, 1, 2.5, 5, and 10 mM salicylate, respectively; lanes 12 to 16 are 48 h in the presence of 0, 1, 2.5, 5, and 10 mM salicylate, respectively. Lane 1 refers to cells before the addition of cycloheximide. ◆, 0 mM; ■, 1 mM; gray ✕, 2.5 mM; ✕, 5 mM; ✷ 10 nM.

TABLE 1

Effect of preincubation with salicylate on AA toxicity in HepG2 cells.

E47 cells (A, B) or C34 cells (C, D) were preincubated with buffer (A, C) or with 10 mM sodium salicylate (B, D) for 24 hours. The medium was removed, the cells washed, and the indicated additions were made to all cells, followed by viability assays (Trypan Blue uptake) after 24 h of incubation.

| Addition | Percent Toxicity | | | |
|---|---|---|---|---|
| | A | B | C | D |
| | *E47 (buffer)* | *E47 (Sal)* | *C34 (buffer)* | *C34 (Sal)* |
| None | 2.8 ± 2.1 | 5.8 ± 2.5 | 9.5 ± 3.0 | 9.0 ± 4.2 |
| Salicylate (5 mM) | 5.2 ± 3.0 | 5.5 ± 1.9 | 11.8 ± 1.5 | 6.3 ± 1.0 |
| Salicylate (10 mM) | 3.0 ± 0.0 | 4.3 ± 2.6 | 10.8 ± 2.9 | 6.5 ± 2.4 |
| AA (30 $\mu$M) | 21.3 ± 4.6* | 25.0 ± 8.4* | 12.0 ± 2.7 | 11.8 ± 1.0 |
| AA (60 $\mu$M) | 37.0 ± 5.1* | 42.0 ± 4.4* | 12.3 ± 2.9 | 16.5 ± 1.3 |
| AA 30 $\mu$M + Sal (5 mM) | 43.5 ± 9.0** | 53.3 ± 3.5** | 7.8 ± 3.0 | 11.8 ± 1.0 |
| AA 60 $\mu$M + Sal (5 mM) | 60.8 ± 2.2** | 65.5 ± 3.1** | 16.0 ± 6.2 | 8.1 ± 4.0 |
| AA 30 $\mu$M + Sal (10 mM) | 61.5 ± 7.4** | 68.3 ± 6.2** | 11.0 ± 4.5 | 11.0 ± 5.0 |
| AA 60 $\mu$M + Sal (10 mM) | 75.0 ± 10.0** | 82.5 ± 6.5** | 10.5 ± 5.3 | 13.3 ± 0.5 |

*$p < 0.05$ compared with no addition. $n = 4$.
**$p < 0.05$ compared with AA without addition of salicylate. $n = 4$.

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creases of lipid peroxidation and were found to be both necrotic and apoptotic in nature. These studies with cell culture models seemed to extend results with the continuous intragastric infusion model of ethanol feeding, where correlations between induction of CYP2E1, lipid peroxidation, and ethanol induced liver injury were observed (Castillo et al., 1992; Morimoto et al., 1994; Nanji et al., 1994).

Salicylate increased AA toxicity in the CYP2E1-expressing E47 cells with no significant effect in HepG2 cells, which do not express CYP2E1. To evaluate whether salicylate might enhance toxicity of AA in cell lines expressing other cytochrome P450 enzymes, a HepG2 cell line that expresses CYP3A4 was used. Salicylate did not potentiate toxicity of AA in the CYP3A4-expressing cells. Salicylate is a substrate for hydroxylation by CYP2E1 and by CYP3A4 (Dupont et al., 1999). Salicylate alone, at the concentrations used and under these reaction conditions, was not toxic to the control HepG2 cells or the cells expressing CYP2E1 or CYP3A4, although there are reports that salicylate promotes apoptosis of several cell lines (Van Antwirp et al., 1996; Trost and Lemasters, 1997; Bellosillo et al., 1998; Klampfer et al., 1999). The potentiation of AA toxicity by salicylate in the E47 cells was demonstrated by several approaches, including cell morphology, Trypan Blue uptake and DNA fragmentation analysis. The salicylate-potentiated toxicity of AA was blocked by DAS, thus validating that CYP2E1 was important in the overall mechanisms leading to toxicity in the E47 cells.

The strong protection against loss of cell viability by Trolox indicated that lipid peroxidation-dependent mechanisms played a key role in the enhanced toxicity, which may explain why salicylate potentiated AA toxicity. Actual assays of lipid peroxidation indicated that salicylate enhanced AA-induced lipid peroxidation in the E47 cells and that this elevation of lipid peroxidation was dependent on CYP2E1 because it was blocked by DAS. Although salicylate is a scavenger of the hydroxyl radical and an antioxidant, clearly the pro-oxidant actions of salicylate under these conditions were greater than any antioxidant effects, such that AA-induced lipid peroxidation was increased not decreased.

To try to understand why salicylate would promote a state of increased rather than decreased oxidative stress, we determined the effects of salicylate on CYP2E1 levels in the E47 cells. Salicylate, at concentrations as low as 1 mM, increased steady-state levels of CYP2E1 protein; CYP2E1 mRNA levels were not increased by concentrations of salicylate up to 5 mM. Damme et al. (1996) reported that in vivo treatment with salicylate increased CYP2E1 mRNA. In our HepG2 cell models, the cytomegalovirus promoter regulates control of CYP2E1 expression because only CYP2E1 cDNA is incorporated into the pCI plasmid. Future experiments with hepatocytes in culture will assess the effects of salicylate on CYP2E1 mRNA. Because salicylate is a substrate for CYP2E1 (Dupont et al., 1999), the increase in CYP2E1 protein but not CYP2E1 mRNA by $< 5$ mM salicylate is likely to reflect substrate or ligand stabilization of the enzyme against proteolysis, analogous to that which has been observed for "induction" of CYP2E1 by ethanol and many other low-molecular-weight ligands (Song et al., 1986; Koop and Tierney, 1990; McGehee et al., 1994; Roberts et al., 1994). We have previously shown that these agents can increase CYP2E1 levels in HepG2 E9 cells, which also express CYP2E1, by decreasing the turnover of the enzyme (Yang and Cederbaum, 1997). Indeed, using cycloheximide to inhibit synthesis of new CYP2E1, the degradation of CYP2E1 was decreased by salicylate, supporting the idea that salicylate increases CYP2E1 levels by decreasing turnover of the enzyme. Future pulse-chase experiments are proposed to more specifically evaluate this concept. The effect of salicylate on activity of the proteasome complex, the major proteolytic system responsible for CYP2E1 degradation (Roberts, 1997; Yang and Cederbaum, 1997; Goasduff and Cederbaum, 1999) also requires evaluation.

Although HepG2 cells maintain many liver-specific functions, it seemed important to extend the results on salicylate potentiation of AA toxicity to normal, nontransformed hepatocytes. As described previously, AA was more toxic to hepatocytes isolated from rats with high levels of CYP2E1 than control rats with lower levels of CYP2E1 (Wu and Ceder-

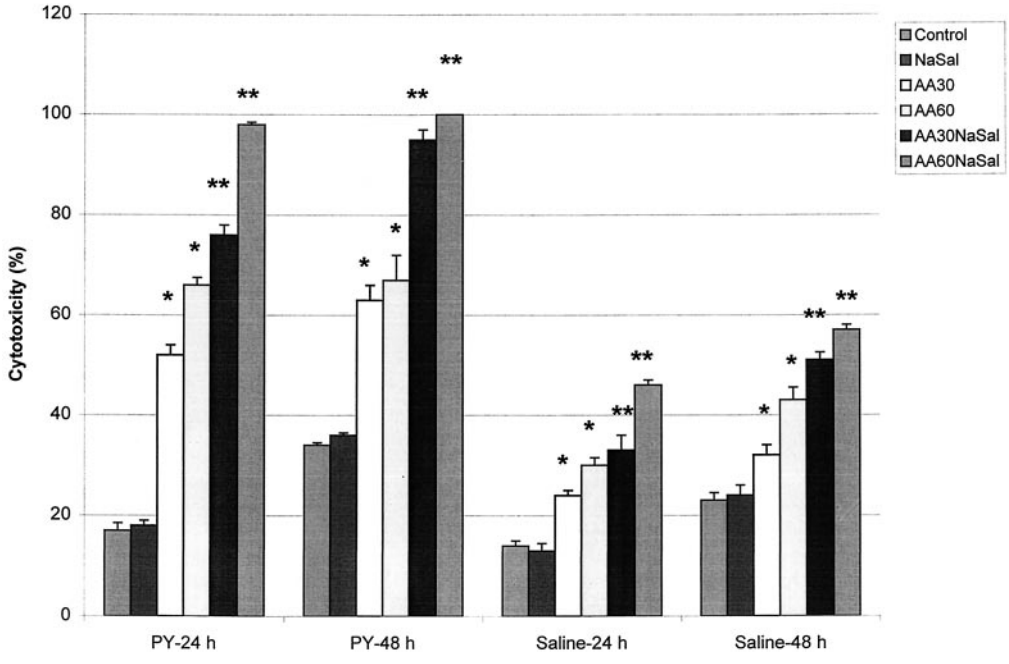


**Fig. 9.** Salicylate enhances AA-induced cytotoxicity in rat hepatocyte cultures. Rat hepatocytes were isolated from pyrazole-induced (PY) or saline control rats. The cells were placed into culture and treated with 30 or 60 $\mu$M AA in the presence or absence of 10 mM salicylate (NaSal) for 24 or 48 h. Cells were then harvested by trypsinization and cytotoxicity was determined by a Trypan Blue exclusion method. *Significant difference ($P < 0.05$) compared with control. **Significant difference ($P < 0.05$) compared with same arachidonic acid dose treated groups. $n = 4$.

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baum, 2000). Salicylate potentiated this AA induced toxicity in both preparations of hepatocytes. Studies are in progress to evaluate the effects of salicylate on CYP2E1 protein and mRNA levels, and degradation of CYP2E1 protein and mRNA in rat hepatocytes, to extend the results found with the E47 cells.

In view of the potential role of CYP2E1 in contributing to alcohol-induced oxidative stress and liver injury, the potentiation of CYP2E1-dependent AA toxicity by salicylate may be of clinical significance and merit caution in the use of salicylate and salicylate precursors such as acetylsalicylic acid by alcoholics. This precaution (e.g., with acetylsalicylic acid) may be somewhat analogous to the concerns over use of acetaminophen by active alcoholics. A similar concern may relate to other drugs that are metabolized by CYP2E1 to reactive intermediates (e.g., acetaminophen, halogenated compounds, benzene, nitrosamines) and relate to other metabolic conditions in which CYP2E1 may be induced (e.g., diabetes, starvation, obesity, non–alcohol-induced steatohepatis). Acetylsalicylic acid is rapidly metabolized to salicylate in vivo (e.g., a 73% conversion to salicylate was observed within 30 min after ingestion of aspirin; Flower et al., 1985). Salicylate concentrations of 0.5 mM are used for analgesic and antipyretic properties. Whereas most experiments in this report were carried out using high salicylate concentrations of 5 and 10 mM to observe potentiation of toxicity at relatively short time points (e.g., 24 h), salicylate concentrations of 1 mM did increase AA toxicity (Fig. 2) and increased CYP2E1 levels (Fig. 6) and slightly decreased CYP2E1 degradation (Fig. 8). Concentrations of salicylate of 1 to 4 mM have been found in plasma of patients undergoing salicylate treatment for inflammatory disease (Weissmann, 1991; Insel, 1996). These levels of salicylate increase the content of CYP2E1 and potentiated AA toxicity in cell lines and rat hepatocytes expressing CYP2E1. Although the possible potentiation of CYP2E1 toxicity by salicylate in vivo remains to be demonstrated, these results suggest that the use of salicylate under conditions in which CYP2E1 is in-

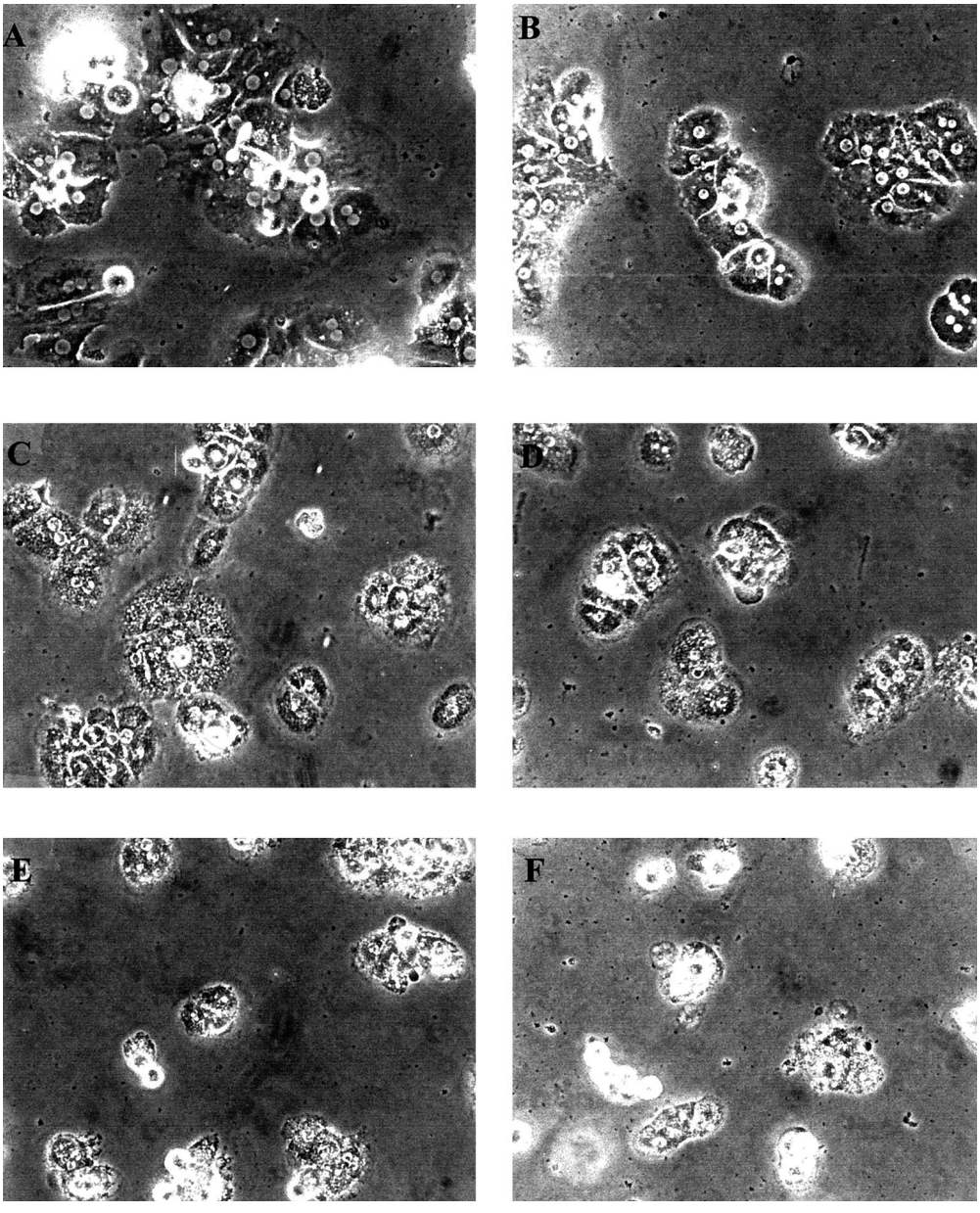


**Fig. 10.** Cytotoxic morphological changes in rat hepatocyte culture after AA or AA plus salicylate treatment. Hepatocytes from pyrazole-induced rats were placed into culture and treated with 30 or 60 $\mu$M AA or AA plus 10 mM salicylate for 24 h. Cytotoxic morphological changes were observed under the light microscope (10 $\times$ 20). A, control; B, 10 mM salicylate; C, 30 $\mu$M AA; D, 60 $\mu$M AA; E, 30 $\mu$M AA plus 10 mM salicylate; F, 60 $\mu$M AA plus 10 mM salicylate. AA plus salicylate induced significant cytotoxicity to the cells compared with AA treated alone. Most cells showed necrotic changes as shown in E and F versus C and D. Essentially similar results were observed after 48 h incubation, with more pronounced toxicity than at 24 h (data not shown).

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duced or in the presence of compounds metabolized by CYP2E1 to reactive intermediates may require further evaluation and consideration.

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