Opposite action of S-adenosyl methionine and its metabolites on CYP2E1-mediated toxicity in pyrazole-induced rat hepatocytes and HepG2 E47 cells

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Wu, Defeng, and Arthur I. Cederbaum. Opposite action of S-adenosyl methionine and its metabolites on CYP2E1-mediated toxicity in pyrazole-induced rat hepatocytes and HepG2 E47 cells. Am J Physiol Gastrointest Liver Physiol 290: G674–G684, 2006. First published November 23, 2005; doi:10.1152/ajpgi.00406.2005.—S-adenosyl-l-methionine (SAMe) is the principal biological methyl donor; the precursor of glutathione (GSH) through its conversion to cysteine, which elevate GSH, also protects against AA toxicity. 5'-Methylthioadenosine (MTA), which cannot produce GSH, did not protect. The toxicity of BSO was not prevented by SAMe and the analogs because GSH cannot be synthesized. In contrast, in E47 cells, SAMe and MTA protected against AA and BSO toxicity. Antioxidants such as trolox or oseltamivir prevented this synergistic toxicity of SAMe plus AA or SAMe plus BSO, respectively.

S-adenosyl-l-methionine; cytochrome P-450 2E1; hepatotoxicity; oxidative stress

S-ADENOSYL-L-METHIONINE (SAMe) is the principal biological methyl donor; the precursor of aminopropyl groups utilized in polyamine biosynthesis and in the liver, SAMe is also a precursor of glutathione (GSH) through its conversion to cysteine via the trans-sulfuration pathway (7). Given these critical reactions, any alteration in the availability of SAMe may have profound effects on cellular growth, differentiation, and function. Abnormalities in SAMe metabolism have been well recognized in liver diseases and in various neurological disorders (7, 36). Exogenous administration of SAMe can protect against injury induced by ethanol, CCl₄, acetaminophen, galactosamine, cytokines, thioacetamide, and ischemia-reperfusion (6, 11, 14, 16, 23, 29, 30, 36, 55). SAMe decreased a LPS or cytokine mix induction of inducible nitric oxide synthase in vivo, in cultured hepatocytes, and in HepG2 cells (3, 11, 34).

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whereas SAMe can prevent alcoholic liver injury and toxicity of hepatotoxins, it is of interest to study whether and how SAMe or its metabolites affect CYP2E1-mediated toxicity to liver cells. We (9) recently characterized the antioxidant properties of SAMe against Fe$^{2+}$-initiated oxidations; SAMe effectively prevented the interaction of Fe$^{2+}$ with molecular O$_2$ to produce ROS and Fe$^{2+}$ oxidation but was not very effective in blocking the interaction of Fe$^{2+}$ with H$_2$O$_2$, i.e., the Fenton reaction. In the present study, PY-induced rat hepatocytes, which contain high CYP2E1 activity, or CYP2E1-expressing HepG2 E47 cells were treated with AA or BSO in the presence or absence of SAMe, SAMe metabolites, S-adenosyl homocysteine (SAH), 5-methyltetrahydrofolate (MTA), or methionine (MET), respectively, and assays of cell viability and oxidant stress were carried out.

**MATERIALS AND METHODS**

**Hepatocyte isolation and cell culture.** Male Sprague-Dawley rats received humane care, and studies were carried out according to criteria outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals with Institutional Animal Care and Use Committee approval. Rats (150–200 g body wt) were injected intraperitoneally with PY at a dose of 200 mg/kg once a day for 2 days to induce CYP2E1 activity. After the second injection, rats were fasted (allowed drinking water) until hepatocytes were isolated. Rat hepatocytes were prepared by a two-step collagenase perfusion method as previously described (49). Hepatocyte viability, as evaluated by the trypsin blue exclusion method, was usually about 90%. CYP2E1 levels were validated by Western blot analysis and catalytic activity with p-nitrophenol. Isolated hepatocytes were seeded onto either 6-well plates at 1 × 10$^6$ cells/well or 24-well plates at 5 × 10$^3$ cells/well; wells were coated with basement membrane Matrigel (BD Biosciences). Cells were cultured in serum-free HepatoZYME-SFM (containing some plant protein to support cell growth, Invitrogen) containing 20 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate with 0.29 mg/ml L-glutamine solution (Invitrogen). Two hours after cells were seeded, the medium was changed, unattached cells were gently washed out, fresh medium was added, and experiments were initiated.

HepG2 E47 cells are HepG2 cells transfected with human CYP2E1 cDNA in the sense orientation and constitutively express CYP2E1. The cell line was cultured in minimal essential medium (MEM) supplemented with 10% FBS plus 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate plus 0.29 ng/ml L-glutamine in 5% CO$_2$ at 37°C. Every 3–4 wk, cells were cultured in MEM containing 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate with 0.29 mg/ml L-glutamine solution (Invitrogen). Mitochondrial membrane potential.

**Lipid peroxidation analysis.** PY-induced rat hepatocytes or E47 cells were treated with 60 μM AA or 1 mM BSO in the presence or absence of SAMe, SAH, MTA, or MET added at concentrations of 0.25 or 0.5 mM for 24 h. Cells were harvested by trypsinization with PBS containing 0.1 mM butylated hydroxytoluene (BHT) and sonicated for 10 s in an ice bath using a MICROSON Ultrasonic Cell Disruptor [output power 6 W (RMS)]. The cellular lysate was collected, and the cell extract containing 0.2 mg protein was incubated with 0.2 ml trichloroacetic acid-thiobarbituric acid (TBA)-HCl solution (containing 0.1 mM BHT) in a 100°C water bath for 1 h as previously described (8, 49). The formation of TBA-reactive substances (TBARS) was determined by measuring absorbance at 535 nm and using an extinction coefficient of 1.56 × 10$^5$ M/cm to calculate malondialdehyde equivalents. The presence of TBARS in the culture medium was about 10% of that of the cell extracts, so most of the TBARS was associated with cells.

**Mitochondrial membrane potential.** Changes in the mitochondrial membrane potential (MMP) were studied by monitoring cells after a double staining with 5 μg/ml rhodamine-123 (Rh123) and 25 μg/ml propidium iodide (PI). The intensity of fluorescence from Rh123 and PI was analyzed by flow cytometry (49–51).

**Statistical analysis.** Statistical analysis was performed using one-way ANOVA with subsequent post hoc comparisons by Scheffé. Values reflect means ± SE, and numbers of experiments are given in the figures.

**RESULTS**

**Effect of SAMe on AA or BSO toxicity in E47 cells and PY hepatocytes.** To study the effect of SAMe on CYP2E1-dependent toxicity, HepG2 E47 cells were treated with 30 or 60 μM AA in the presence or absence of 0.1, 0.25, 0.5, or 1 mM SAMe for 24 h. SAMe alone at concentrations ranging from 0.1 to 1 mM had no toxicity or a slight toxicity effect on E47 cells. Treatment of E47 cells with 30 μM AA for 24 h produced 27 ± 5% LDH leakage. This concentration of AA had no effect on the viability of HepG2 cells not expressing CYP2E1 (C34 cells). SAMe significantly potentiated the toxicity of 30 μM AA (Fig. 1A). BSO induces cell toxicity in E47 cells by inhibiting glutamate cysteine ligase and thereby reducing the synthesis of GSH (12, 50). E47 cells were treated with 1 mM BSO in the absence or presence of SAMe for 24 h, and LDH leakage was determined. BSO alone induced a 49 ± 6% increase in cell toxicity in E47 cells. SAMe significantly potentiated the toxicity of BSO in a concentration-dependent manner (Fig. 1A). Similar results were observed when a MTT assay was used to assess cell viability; SAMe potentiated the...
toxicity of AA and BSO in a concentration-dependent manner (Fig. 1B). These data show that, in contrast to the expected/anticipated SAMe protection effect, SAMe potentiated AA or BSO toxicity in E47 cell cultures.

To study the effect of SAMe and its metabolites on primary rat hepatocytes with high levels of expression of CYP2E1, hepatocytes isolated from a PY-induced rat were treated with 60 μM AA or 1 mM BSO in the absence or presence of 0.25 mM SAMe for 24 h, respectively. Cell viability was determined by the MTT reduction method. In contrast to E47 cells, SAMe prevented AA-induced toxicity in rat primary hepatocytes (Fig. 1C). SAMe, however, had no protective effect against BSO-induced toxicity (Fig. 1C). Thus opposite effects of SAMe on CYP2E1 toxicity were observed in E47 cells compared with PY hepatocytes.

Because the E47 cells were cultured in MEM containing 2% FCS, whereas the hepatocytes were incubated in serum-free HepatoZYME-SFM, a reviewer asked whether the different media could play a role in the opposite effects of SAMe on AA or BSO toxicity in E47 cells or PY hepatocytes. To evaluate this, we compared the effect of SAMe on AA or BSO toxicity when E47 cells were cultured in either MEM plus 2% FCS or HepatoZYME-SFM. AA produced a 22% loss of cell viability in MEM and a 30% loss in HepatoZYME-SFM. This loss of cell viability was potentiated by 0.1, 0.25, and 1 mM SAMe to levels of 59%, 68%, and 80%, respectively, in MEM and to levels of 55%, 61%, and 70%, respectively, in HepatoZYME-SFM. BSO produced a 43% loss of cell viability in the absence of SAMe and an 88% decline in the presence of 1 mM SAMe in MEM; the loss of viability in HepatoZYME-SFM was 40% and 88% for BSO alone and BSO plus SAMe, respectively. Thus SAMe potentiated AA and BSO toxicity similarly in both media.

Antioxidants prevent SAMe potentiation of toxicity. To study the possible mechanisms by which SAMe potentiates AA or BSO toxicity in E47 cells, E47 cells were treated with 30 μM AA or 1 mM BSO plus either 5 mM NAC, a GSH precursor and an antioxidant, or with 0.1 mM trolox, a vitamin E analog that inhibits lipid peroxidation. After treatment for 24 h, 30 μM AA induced 29 ± 5% toxicity in E47 cells, and 1 mM SAMe potentiated toxicity to 84 ± 6%. Trolox but not NAC completely prevented this toxicity (P < 0.01 compared with the AA + SAMe group; Fig. 2A). E47 cells treated with 1 mM BSO for 24 h induced 44 ± 6% toxicity, and 1 mM SAMe enhanced toxicity to 77 ± 6%. In the presence of 5 mM NAC but not trolox, the toxicity was prevented (P < 0.01 compared with the BSO + SAMe group; Fig. 2A). Similar results were obtained when cell viability was assayed by the MTT reduction assay as AA toxicity, and SAMe potentiation of AA toxicity was prevented by trolox but not NAC, whereas BSO toxicity and SAMe potentiation of BSO toxicity was prevented by NAC but not trolox (Fig. 2B). These results suggest that SAMe potentiation of either AA or BSO toxicity to E47 cells may involve enhanced oxidant stress to the cells, although the}

Fig. 1. Effect of S-adenosyl-l-methionine (SAMe) on arachidonic acid (AA) and l-buthionine sulfoximine (BSO) toxicity in E47 cells and pyrazole (PY)-treated hepatocytes. A: E47 cells were treated with 30 μM AA or 1 mM BSO in the presence or absence of 0, 0.1, 0.25, 0.5, or 1 mM SAMe for 24 h. A lactate dehydrogenase (LDH) leakage method was used to determine cell toxicity. *Significantly different (P < 0.05) compared with 30 μM AA alone; **significantly different (P < 0.05) compared with 1 mM BSO alone. Data are from 3 experiments with duplicate samples. B: E47 cells were treated with varying concentration of SAMe in the presence or absence of either 30 μM AA or 1 mM BSO for 24 h. After treatment, cell viability was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results are from 3 experiments with duplicate samples in each group. *P < 0.01 compared with AA alone; **P < 0.01 compared with BSO alone. C: hepatocytes were isolated from PY-treated rats as described in MATERIALS AND METHODS and were treated with either 60 μM AA or 1 mM BSO in the presence or absence of 0.25 mM SAMe. S-adenosyl homocysteine (SAH), 5’-methylthioadenosine (MTA), or methionine (MET) for 24 h, respectively. At the end of treatment, cell viability was determined by a MTT method. *Significantly different (P < 0.05) compared with the AA-treated group. Data are from 3 independent experiments with duplicate samples in each group.
sensitivity to antioxidants was toxin specific, i.e., trolox blocks AA toxicity, whereas NAC blocks BSO toxicity. We believe that these differences reflect the fact that trolox is a hydrophilic antioxidant, effectively blocking lipid peroxidation of cellular membranes, whereas NAC is effective as an aqueous phase antioxidant, not penetrating cellular membranes and therefore not effective in blocking lipid peroxidation but perhaps effective in removing ROS in the cytosol compartment, thereby preventing BSO toxicity.

In PY hepatocytes, trolox but not NAC prevented AA toxicity, analogous to the results with AA toxicity in E47 cells (Fig. 2C). SAMe, as shown above, was also protective against AA toxicity, although somewhat less than was trolox (Fig. 2C).

**SAMe does not potentiate toxicity of TNF-α in E47 cells.** TNF-α toxicity to liver cells requires cycloheximide (CHX) or actinomycin to prevent the synthesis of protective factors and is CYP2E1 independent. To expand the study of whether SAMe potentiates toxicity to E47 cells to this agent, E47 cells were treated with 2 μg/ml TNF-α plus 40 μM CHX in the presence or absence of 0.5 or 1 mM SAMe, and the toxicity was evaluated by LDH leakage. SAMe had no effect on TNF-α + CHX-induced toxicity in E47 cells under conditions in which it potentiated AA- or BSO-induced toxicity (data not shown). These results suggest that SAMe selectively potentiates the toxicity of some oxidants or protoxins in E47 cells.

**SAMe and lipid peroxidation.** Previous studies have shown that the AA-induced increase in lipid peroxidation is a major cause of cell toxicity in E47 cells and PY hepatocytes. It seemed reasonable to evaluate whether the potentiation of toxicity in E47 cells by SAMe is via a further increase in lipid peroxidation and whether the prevention of AA toxicity in PY hepatocytes by SAMe is associated with a decrease in lipid peroxidation. Indeed, AA increased the formation of TBARS, and TBARS formation was further elevated in the presence of AA plus SAMe (Fig. 3A). NAC did not block the AA + SAMe-induced lipid peroxidation, consistent with the inability of NAC to prevent the AA + SAMe-induced toxicity. However, trolox did prevent the SAMe-enhanced AA lipid peroxidation (as well as the AA-induced lipid peroxidation), consistent with the ability of trolox to prevent the AA + SAMe-induced toxicity (Fig. 3A). BSO only slightly increased lipid peroxidation in E47 cells, consistent with the inability of trolox to prevent the BSO-induced toxicity (Fig. 3A). SAMe did not significantly potentiate BSO-induced lipid peroxidation (Fig. 3A).

Treatment of the PY hepatocytes with 60 μM AA alone caused a twofold increase in lipid peroxidation. This increase was prevented in the presence of 0.25 mM SAMe (P < 0.05; Fig. 3B). Treatment with 1 mM BSO instead of AA did not result in a significant increase in lipid peroxidation, and SAMe had no effect on the production of TBARS (Fig. 3B).

**Effect of SAMe metabolites on AA or BSO toxicity.** SAMe is synthesized from MET through the MET adenosyltransferase reaction. After trans-methylation reactions, SAMe produces SAH. Alternatively, SAMe is decarboxylated in the polyamine synthesis pathway and converted to MTA. SAMe can also undergo nonenzymatic hydrolysis into MTA and homoserine. To study the effect of these SAMe metabolites, E47 cells were treated with 30 μM AA plus 0.5 or 1 mM SAMe, SAH, MTA, or MET for 24 h, respectively, and the cell toxicity was determined. SAMe, as discussed above, potentiated AA toxic-
Effect of SAMe, SAMe metabolites, and NAC on GSH levels. BSO inhibits γ-glutamate cysteine ligase, which results in the lowering of cellular GSH levels. As shown in Fig. 5A, a time-course study was carried out to evaluate the effect of SAMe and NAC on GSH levels. Incubation with BSO caused a striking decrease in cellular GSH levels. Treatment of the E47 cells with 1 mM BSO and SAMe did not affect this decline in GSH levels (Fig. 5A). SAMe did not elevate GSH levels in the absence of BSO (Fig. 5A); this is likely due to the absence of the trans-sulfuration pathway in HepG2 cells (32). NAC, however, did slow the rate of decrease of GSH induced by BSO plus SAMe (Fig. 5A). This preservation of cellular GSH may contribute to the protection by NAC against BSO + SAMe-potentiated toxicity. The effect of NAC is likely to reflect its general antioxidant actions because GSH is not synthesized in the presence of BSO, i.e., antioxidant effects of NAC spare GSH, thereby helping to maintain cellular viability. GSH levels were determined to evaluate whether SAMe and its metabolites affect GSH levels after hepatocytes were treated with AA (or BSO). Treatment of the hepatocytes with 60 μM AA for 24 h lowered GSH levels from 32 ± 5 to 17 ± 2 nmol/mg protein. In the presence of 0.25 mM SAMe, SAH, or MET, GSH levels were restored to 33 ± 4 or 29 ± 3 nmol/mg or elevated (MET) to 60 ± 3 nmol/mg, respectively (P < 0.05 compared with the AA-treated group; Fig. 5B). Thus SAMe, SAH, and MET prevented the decline in GSH produced by AA. MTA (0.25 mM) did not significantly increase GSH levels compared with the AA-treated group (P > 0.05; Fig. 5B). BSO (1 mM) treatment reduced GSH levels to 3 ± 0.5 nmol/mg protein, and, as expected, SAMe and its metabolites could not restore GSH levels because BSO blocks GSH synthesis (Fig. 5B). In the absence of AA or BSO (control), SAMe, SAH, and especially MET elevated hepatocyte GSH levels, whereas MTA had no effect (Fig. 5B).

SAMe and ROS production. To study whether SAMe alters ROS production after BSO treatment, E47 cells were treated with 1 mM BSO or 1 mM BSO plus 1 mM SAMe, and DCF fluorescence was determined. BSO treatment increased ROS
generativity (Fig. 4). MTA also potentiated AA toxicity and was more reactive than SAMe in promoting AA toxicity. SAH had no effect on AA toxicity. MET, however, reduced AA toxicity (Fig. 4). In the absence of AA, SAMe, MET, and SAH had no effect on cell viability, whereas MTA was toxic to the cells, e.g., 0.5 or 1 mM MTA treatment for 24 h increased cell toxicity from 3 ± 1% to 13 ± 2% or 29 ± 4%, respectively (Fig. 4). These results suggest that MTA may play a role in the potentiation of AA toxicity by SAMe.

In PY hepatocytes, AA toxicity was partially prevented by SAMe and SAH and strongly prevented by MET (Fig. 1C). MTA was slightly but not significantly protective against AA toxicity. None of these compounds protected against BSO-induced toxicity (Fig. 1C). Importantly, SAMe, SAH, and MET also decreased the AA-induced lipid peroxidation in PY hepatocytes (Fig. 3B), suggesting that their ability to protect against AA-induced toxicity may be associated with their ability to prevent lipid peroxidation.
production fourfold; however, SAMe did not further increase this production of ROS (Fig. 6A). NAC reduced ROS production in both the absence and presence of SAMe, consistent with its preservation of GSH levels and protection against BSO plus SAMe toxicity. Trolox did not prevent the increase in ROS production produced by BSO, consistent with its inability to protect against BSO plus SAMe toxicity. AA also increased ROS production by E47 cells, and this increase was further elevated by SAMe (Fig. 6A), as was AA-induced lipid peroxidation. The increase in ROS production produced by AA and the further elevation in the presence of AA plus SAMe were lowered by trolox but not NAC (Fig. 6A). Thus trolox, which prevents against AA and AA plus SAMe toxicity, prevents AA and AA plus SAMe elevations of lipid peroxidation and ROS production, whereas NAC, which is not protective, does not prevent these elevations in lipid peroxidation and ROS production.

In PY hepatocytes, 1 mM BSO treatment significantly increased ROS production about 50% (Fig. 6B). SAMe and its metabolites did not lower ROS production when they were added in the medium with BSO (Fig. 6B), consistent with their inability to prevent BSO toxicity. AA increased ROS production by about 80%, and this increase was strongly prevented by SAMe, SAH, and MET and partially prevented by MTA (Fig. 6B).

CHX does not prevent SAMe potentiation of AA or BSO toxicity in E47 cells. To determine whether the potentiation of AA or BSO toxicity by SAMe requires protein synthesis, the effect of CHX on this potentiation was studied. The toxicity by AA (30 μM) alone (25% loss of cell viability) or by BSO (0.3 mM) alone (15% loss of cell viability) was not affected by CHX does not prevent SAMe potentiation of AA or BSO toxicity in E47 cells. To determine whether the potentiation of AA or BSO toxicity by SAMe requires protein synthesis, the effect of CHX on this potentiation was studied. The toxicity by AA (30 μM) alone (25% loss of cell viability) or by BSO (0.3 mM) alone (15% loss of cell viability) was not affected by CHX but was prevented by SAMe, SAH, and MET and partially prevented by MTA (Fig. 6B).

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CHX (25% loss of cell viability AA + CHX or BSO + CHX). SAMe potentiated AA toxicity (57% loss of cell viability) in the absence of CHX; CHX only partially prevented this potentiated toxicity as there was a 42% loss of cell viability in the presence of AA plus SAMe plus CHX. SAMe potentiated BSO toxicity to a 70% loss of cell viability in the absence of CHX and a 60% loss of cell viability in the presence of CHX. Thus CHX did not effectively prevent the potentiation of AA or BSO toxicity by SAMe, suggesting that this potentiation did not require stimulation of the synthesis of, e.g., death proteins/factors.

**Different effects of SAMe on MMP in PY hepatocytes and E47 cells.** Changes in MMP may finally trigger cells to undergo either apoptosis or necrosis. We (8, 49, 51, 52) have previously reported that a decline in MMP occurs in AA-induced toxicity in PY hepatocytes and E47 cells and that damage to mitochondria played a role in CYP2E1-mediated toxicity. MMP was determined by the Rh123 method in cells double stained with Rh123 and PI. In control PY hepatocytes, most cells were found in the high-Rh123, low-PI quadrant (bottom right quadrant), which reflects viable cells with high MMP (Fig. 7). The same pattern was observed after SAMe treatment. The addition of AA caused 37% of cells to shift to a lower Rh123 fluorescence (bottom left quadrant), indicating a decline in MMP (Fig. 7). Many of the cells with a lower MMP still excluded PI, i.e., were viable, indicating that the decline in MMP occurred before overt toxicity. Treatment with SAMe partially prevented the AA-induced decline in MMP to 21% cells in the bottom left quadrant (Fig. 7). An opposite effect by SAMe was observed with E47 cells; 9% of cells were found with low Rh123 fluorescence after AA treatment in the absence of SAMe, and this was elevated to 17% of cells in the presence of SAMe (Fig. 7). Thus SAMe protected against the AA-induced decline in MMP in PY hepatocytes but potentiated this decline in E47 cells.

**Opposite effects of SAMe on Nrf2 expression in hepatocytes and E47 cells.** Nrf2 is a key transcription factor that regulates important antioxidant and phase II detoxification genes such as glutathione-S-transferases, NADPH-quinone oxidoreductase, heme oxygenase I, and glutamate cysteine ligase (1, 20, 39, 41). These genes are upregulated in E47 cells as an adaptive response to CYP2E1 oxidant stress (24, 35). The effect of SAMe on Nrf2 protein levels in PY hepatocytes and E47 cells was evaluated. In hepatocytes, SAMe produced a 30% decrease in total cell Nrf2 levels (Fig. 8). Treatment with AA or BSO for 24 h produced a 50% decrease in Nrf2 levels; SAMe did not significantly prevent the BSO-induced decline but did prevent the AA-induced decline in Nrf2, restoring levels to 60% or 80% of control values (Fig. 8). Opposite effects were observed with E47 cells. Whereas SAMe or AA or BSO caused small decreases in Nrf2 levels (about 20%), the combination of SAMe plus AA or SAMe plus BSO produced striking decreases (70% or 80%) in Nrf2 levels (Fig. 8). Further studies are in progress to evaluate the levels of Nrf2-dependent antioxidant proteins and nuclear Nrf2 levels under these conditions. Thus SAMe helped to partially maintain Nrf2 levels in hepatocytes but potentiated the AA- or BSO-induced decrease in Nrf2 levels in E47 cells.

*p38 and ERK activation do not play a role in the different effects of SAMe on AA or BSO toxicity in liver cells.* Previous studies (53, 54) have shown that p38 MAPK plays a role in AA or BSO toxicity to PY hepatocytes and E47 cells. The role of MAPK in SAMe potentiation of AA and BSO toxicity was evaluated. In PY hepatocytes (Table 1), SAMe prevented AA-induced toxicity. SB-203580, a p38 MAPK inhibitor, also prevented AA toxicity, whereas U-0126, a MEK kinase inhibitor, was not significantly protective (Table 1), as previously reported (53, 54). The combinations of SAMe plus SB-203580 or SAMe plus U-0126 were as protective as SAMe alone was against AA toxicity (Table 1). BSO toxicity was prevented by either SB-203580 or U-0126 (PY hepatocytes) but not by SAMe. In E47 cells, the toxicity of AA was prevented by p38 MAPK and MEK kinase inhibitors (Table 1) but not by a JNK inhibitor (data not shown). SAMe potentiated AA toxicity, and this potentiation was not modified by SB-203580 or U-0126 (Table 1) or the JNK inhibitor (not shown). Similarly, BSO toxicity in E47 cells was potentiated by SAMe to the same extent in the absence or presence of the various MAPK inhibitors (Table 1). These results suggest that p38 MAPK,
MEK kinase, or JNK do not play a role in the potentiation of AA or BSO toxicity by SAMe in E47 cells.

**CYP2E1 levels.** Treatment of PY hepatocytes or E47 cells for 24 or 48 h with SAMe alone, SAMe plus AA, or SAMe plus BSO had no effect on levels of CYP2E1 (data not shown).

Table 1. Role of p38 MAPK and ERK on the effect of SAMe on PY hepatocyte and HepG2 E47 cell viability

<table>
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<th>PY Hepatocytes</th>
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<tr>
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Values are means ± SE; n = 3 independent experiments. Data are percent viability of cells, with viability of nontreated control hepatocytes and HepG2 E47 cell taken as the 100% value. Pyrazole (PY) hepatocytes or HepG2 E47 cells were treated with 30 μM arachidonic acid (AA) or 0.3 mM L-β-homothione sulfoximine (BSO) in the presence or absence of 0.25 mM L-adenosyl-L-methionine (SAMe) (0.5 mM in HepG2 (E47 cells) or SAMe plus either 5 μM SB-203580 or 2.5 μM U-0126 for 24 h, respectively. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. ND, not determined. *Significantly different compared with the AA group alone (P < 0.05); †Significantly different (P < 0.05) compared with BSO group alone.

**DISCUSSION**

In the MET adenosyl transferase (MAT) 1A knockout mouse, one association with the lower levels of SAMe is an increase in CYP2E1 expression and activity and an increase in lipid peroxidation and oxidant stress (31). This increase in CYP2E1 predisposes MAT 1A knockout mice to liver injury, e.g., by CCl₄, because the injury can be blocked by the CYP2E1 inhibitor diallyldisulfide (37). Thus there appears to be interesting interactions between SAMe and CYP2E1.

The objective of this study was to evaluate whether SAMe can protect against CYP2E1-dependent toxicity. Two models were utilized: cultures of hepatocytes isolated from PY-treated rats, which exhibit enhanced levels of CYP2E1, and the HepG2 E47 cell line, which was developed to constitutively express CYP2E1. Previous studies have shown that AA, a representative polyunsaturated fatty acid, and treatment with BSO to deplete GSH caused toxicity to PY hepatocytes and E47 cells under conditions in which saline hepatocytes with lower levels of CYP2E1 or control HepG2 cells (which do not express CYP2E1) were not affected (8, 13, 49, 50, 51). AA was studied in view of the necessity for polyunsaturated fatty acids in the diet to promote the development of alcoholic liver injury in the intragastric infusion model. Depletion of GSH by BSO treatment was studied because GSH, likely the most critical antioxidant in cells, is lowered after ethanol treatment and the finding that depletion of mitochondrial GSH after ethanol treatment appears to sensitize the liver to injury by various hepatotoxins (19, 45).

In PY hepatocytes, SAMe significantly protected against the toxicity by AA treatment but not the toxicity by BSO treatment. Concentrations of SAMe that were protective, 0.25–1 mM, were similar to effective concentrations used by others, e.g., okadaic acid-induced apoptosis in cultured rat hepatocytes.
was prevented by 0.5–4 mM SAMe (2). The induction of inducible nitric oxide synthase by a cytokine mixture added to rat hepatocytes was prevented by 0.5–2 mM SAMe (34). MAT 1A protein levels in rat hepatocytes were elevated by 1–4 mM SAMe (22).

SAMe elevated GSH levels by about 30% in hepatocytes from PY-treated rats. Treatment with AA lowered GSH levels, whereas in the presence of AA plus SAMe, GSH levels were normalized (Fig. 5B). The addition of AA caused an increase in lipid peroxidation, which plays a central role in the CYP2E1 plus AA toxicity because antioxidants such as trolox and vitamin E prevent AA toxicity. SAMe completely prevented the AA-induced lipid peroxidation and AA-induced increase in ROS production. These results suggest that the ability of SAMe to prevent the decline in GSH produced by AA prevents the AA initiation of lipid peroxidation and ROS production and the subsequent loss of hepatocyte viability. Consistent with this suggestion are the following. SAMe did not protect against BSO toxicity; in the presence of BSO, GSH is not synthesized, and hence SAMe cannot elevate GSH levels. Indeed, the dramatic decline in hepatocyte GSH caused by BSO was not prevented by SAMe (Fig. 5B), and the increase in ROS production (DCF fluorescence) caused by BSO could not be prevented by SAMe (Fig. 6B). In addition, SAH and MET, which also elevated hepatocyte GSH levels, prevented the AA-induced fall in GSH, prevented the AA-induced lipid peroxidation and ROS production, and, ultimately, prevented the loss of cell viability caused by AA. MET, which was more effective than SAH and SAMe in elevating GSH levels, was also more effective than SAMe and SAH in protecting against the AA toxicity. Similar to SAMe, SAH and MET failed to protect against the BSO toxicity, most likely because GSH cannot be synthesized. These agents did not prevent the BSO-mediated decline in GSH (Fig. 5B) and the increase in ROS generation (Fig. 6B). Finally, MTA, which cannot produce GSH, could not prevent the AA-induced decline in GSH levels and thus could not protect against AA toxicity (or BSO toxicity). Although other actions of SAMe, e.g., methylation, or membrane fluidization may contribute to the protection against AA toxicity in PY hepatocytes, the elevation of and prevention of the decline of GSH appears to be the major mechanism for SAMe protection against AA toxicity. Interestingly, SAMe prevented the decline in Nrf2 levels produced by AA in PY hepatocytes. Several important antioxidants are transcriptionally regulated by Nrf2, including glutamate cysteine ligase, the rate-limiting enzyme in GSH synthesis.

SAMe did not protect but rather potentiated the toxicity of AA and BSO in E47 cells. On the basis of the hepatocyte results implicating GSH as a major contributor to SAMe protective actions, the lack of protection by SAMe against AA toxicity in E47 cells likely reflects the absence of the trans-sulfuration pathway in HepG2 cells (32). Indeed, SAMe failed to prevent the decline in GSH caused by BSO, nor did SAMe elevate GSH levels in E47 cells. SAMe has been shown to reduce the induction of inducible nitric oxide synthase by a cytokine mixture in HepG2 cells (34) and could reduce the toxicity by hydrophobic bile acids in HepG2 cells (15). In the latter study, SAMe at up to 1 mM was not toxic to HepG2 cells, an observation confirmed with E47 cells. Ansorena et al. (2) reported that 0.5–4 mM SAMe caused some toxicity to several transformed cell lines, including HepG2 cells. We did not observe toxicity by up to 1 mM SAMe in E47 cells or in C34 HepG2 cells, which do not express CYP2E1 (data not shown). Interestingly, whereas SAMe (and MTA) protected rat hepatocytes against okadaic acid toxicity, SAMe (and MTA) did not protect in HuH7 and HepG2 cells, because of their own toxicity (2). Thus SAMe and MTA were antiapoptotic in cultured rat hepatocytes but proapoptotic in human hepatoma cells. In these latter studies, SAMe and MTA were found to affect the mitochondrial membrane, blocking cytochrome c release and the subsequent activation of caspase 3 and apoptosis in primary hepatocyte culture. However, SAMe and MTA promoted cytochrome c release and caspase 3 activation to promote apoptosis in HepG2 cells (2). A recent advance by the Lu group (58) was the report that treatment with SAMe or MTA induced Bcl-X expression in HepG2 cells, specifically, the proapoptotic Bcl-Xs form. They concluded that the trigger for the development of apoptosis by SAMe and MTA in HepG2 cells is formation of the Bcl-Xs variant, a reaction that involves the activation of protein phosphatase-1 levels and activity. SAMe and MTA did not induce PPI and Bcl-Xs in primary mouse hepatocytes, suggesting that this may be responsible for the antiapoptotic versus proapoptotic effects of SAMe and MTA in hepatocytes and HepG2 cells (58). Future experiments are planned to extend these observations by Lu and co-workers on the effects of SAM and MTA on protein phosphatase-1 and Bcl-Xs levels to their possible role in the potentiation of AA or BSO toxicity by SAMe or MTA.

The failure of CHX to afford significant protection against the potentiation of toxicity by SAMe suggests that new protein synthesis is not required for this potentiation. Damage to mitochondria and changes in MMP may play a role in the contrasting actions of SAMe in hepatocytes compared with E47 cells. Whereas SAMe protected against the decline in MMP produced by AA in hepatocytes, SAMe potentiated this decline in E47 cells. To try to understand why SAMe potentiated AA and BSO toxicity in E47 cells, we evaluated indexes of oxidant stress. Trolox, which decreases AA toxicity, also completely prevented the AA plus SAMe synergistic toxicity. AA-induced lipid peroxidation and ROS production were elevated in the presence of SAMe, and these increases were prevented by trolox. It appears that SAMe potentiation of AA toxicity is associated with an increase in lipid peroxidation and ROS production in the combined presence of SAMe plus AA. NAC, a general antioxidant and GSH precursor, failed to protect against AA plus SAMe synergistic toxicity and also failed to prevent the elevated lipid peroxidation and ROS production. With respect to BSO toxicity, an opposite protection was observed. The synergistic BSO plus SAMe toxicity was prevented by NAC but not by trolox. Unlike AA, lipid peroxidation was only slightly increased by BSO treatment, and this was not altered by SAMe, explaining the lack of effect of trolox. However, ROS production was increased by BSO treatment or BSO plus SAMe; NAC lowered this ROS production (Fig. 6A). NAC also spared GSH levels from their rapid decline after BSO treatment (Fig. 5A). This likely reflects the ROS scavenging activity of NAC lessening the requirement for GSH utilization as a ROS scavenger. Thus the protective effects of NAC against the BSO plus SAMe synergistic toxicity may reflect the maintenance of cellular GSH levels and lowering of ROS production.
The trolox and NAC results suggest that the potentiation of AA or BSO toxicity by SAMe is due to an increase in oxidative stress. Why is SAMe potentiating oxidant stress in E47 cells? The marked decrease in Nrf2 levels produced by the AA or BSO addition to E47 cells was further lowered by SAMe. This is likely to lower the overall antioxidant defense of cells and to increase the production of ROS or fail to remove various oxidants produced by the treatment with AA or BSO. MTA was also found to potentiate AA toxicity and was even somewhat more effective than SAMe. MTA itself at 0.5 and 1 mM was toxic in E47 cells, as has already been reported for HepG2 cells (2). SAH had no effect on AA toxicity, whereas MET was partially protective. The mechanism for the MET protection is not clear because HepG2 cells cannot synthesize GSH from MET because of the lack of MAT 1A (32). In view of the potentiation of AA toxicity by MTA and SAMe, and the lack of effect of SAH, it is interesting to speculate that the actions of SAMe may be mediated, in part, via its conversion to MTA. MTA is produced from SAMe by the polyamine pathway and can also be generated by the spontaneous splitting of SAMe into MTA plus homoserine at neutral pH (43, 56). A recent study (43) showed that in vivo, MTA was more effective than SAMe in preventing lipid peroxidation and fibrogenesis induced by CCl4. MTA (25–500 μM) in vitro prevented the activation of stellate cells by transforming growth factor and PDGF (43). MTA was also more effective than SAMe in protecting rat hepatocytes from okadaic acid-induced apoptosis (2) and was more effective than SAMe in preventing endotoxin stimulation of TNF-α expression in hepatic Kupffer cells (46). However, as mentioned above, in transformed cell lines, MTA is toxic (Fig. 4) (2). Unfortunately, because SAMe can nonenzymatically be decomposed to MTA, there are no inhibitors available to specifically address the role of MTA in the actions of SAMe.

In summary, these experiments show that SAMe can protect against AA toxicity in PY hepatocytes by a mechanism that involves, at least in part, increased GSH synthesis. In contrast, BSO toxicity, in which GSH cannot be synthesized, is not protected by SAMe. SAH and MET, but not MTA, also protect against AA toxicity and also fail to protect against BSO toxicity. The former two, but not the latter, increase GSH levels, as do SAMe. Thus SAMe can protect against CYP2E1 toxicity by a GSH-dependent mechanism. In contrast, SAMe potentiates AA and BSO toxicity in E47 cells. The potentiation is mimicked by MTA but not SAH or MET, suggesting that MTA may play a role in the synergistic action of SAMe. The potentiation of toxicity by SAMe can be prevented by either trolox or NAC with AA or BSO treatment, respectively, suggesting that increased oxidant stress plays a role in the synergistic toxicity by SAMe. Further studies are necessary to define how SAMe and MTA potentiate AA or BSO toxicity in E47 cells.

GRANTS

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REFERENCES


CONTRASTING ACTION OF S-ADENOSYL METHIONINE IN LIVER CELLS EXPRESSING CYP2E1


