S-Adenosyl-L-methionine Attenuates Hepatotoxicity Induced by Agonistic Jo2 Fas Antibody following CYP2E1 Induction in Mice

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ABSTRACT
S-Adenosyl-L-methionine (SAM) has been shown to be hepatoprotective against many toxic agents. Its possible effectiveness in protecting against CYP2E1-dependent toxicity is not known. We recently reported that treatment of mice with pyrazole to induce CYP2E1 increased hepatotoxicity produced by Fas agonistic Jo2 antibody. The current study was designed to investigate the effect of exogenous administration of SAM on the synergistic hepatotoxicity produced by Fas agonistic Jo2 antibody plus CYP2E1 following pyrazole pretreatment in C57BL/6 mice. Suboptimal administration of Jo2 Fas antibody combined with pyrazole pretreatment caused severe hepatotoxicity as determined by elevations in serum transaminase levels and histopathology. Exogenous administration of SAM (50 mg i.p./kg body weight every 12 h for 3 days) significantly decreased serum transaminases and ameliorated morphological changes of the liver. Addition of SAM elevated hepatic SAM and total reduced glutathione levels and inhibited CYP2E1 activity. SAM also lowered the elevated oxidative stress (lipid peroxidation, protein carbonyls, and superoxide production) and nitrosative stress (induction of inducible nitric-oxide synthase and 3-nitrotyrosine adducts) and increases in caspase-8 and -3 activation produced by the pyrazole plus Jo2 treatment. SAM did not prevent the increase in serum TNF-α levels or the decrease in catalase activity in this model. These results indicate that SAM can have an important hepatoprotective role as an effective reagent against Fas plus CYP2E1-induced hepatotoxicity by lowering oxidative and nitrosative stress.

S-Adenosyl-L-methionine (SAM) is the principal biological methyl donor and also a precursor of reduced glutathione (GSH), through its sequential conversion from S-adenosylhomocysteine (SAH) to cysteine. Methylthioadenosine (MTA) is produced as an end-product in the biosynthesis of polyamines. Methionine adenosyltransferase is the enzyme responsible for the synthesis of SAM using methionine and ATP (Pegg, 1988; Finkelstein, 1990). In liver, there are three pathways that metabolize homocysteine. One is the transsulfuration pathway, which converts homocysteine to cysteine. Cysteine is often rate-limiting for GSH synthesis; hence, methionine metabolism via SAM and transsulfuration is very important in regulating GSH levels in the liver. The other two pathways that metabolize homocysteine resynthesize methionine from homocysteine, methionine synthase, and betaine-homocysteine methyltransferase (Cantoni, 1975). Under physiological conditions, abnormalities of SAM may have profound effects on cellular growth, differentiation, and function. Interestingly, pathophysiological changes in SAM metabolism have been well recognized in liver diseases because the liver plays the central role in the homeostasis of SAM as the major site of its synthesis and degeneration (Mato et al., 1997).

Many studies have found elevations of homocysteine and SAH in serum or liver, accompanied by reductions of SAM and the SAM/SAH ratio in clinical and animal experiments involving alcoholic liver diseases (Barak et al., 1987; Cravo and Camilo, 2000; Lu, 2000). A decrease in SAM synthesis in the liver results in a decrease in hepatic GSH levels. Liver injury causes a decrease in SAM concentration largely because of decreased methionine adenosyltransferase LA activity (Avila et al., 2000). Impairment of SAM synthesis is...
believed to play an important role in liver injury since exogenous administration of SAM can protect against injury induced by CCl₄, acetaminophen, galactosamine, cytokines, thioacetamide, and ischemia-reperfusion (Corrales et al., 1991; Bray et al., 1993; Wu et al., 1996; Mato et al., 1997; Chawla et al., 1998). SAM decreased lipopolysaccharide or a cytokine mix induction of inducible nitric-oxide synthase in vivo, in cultured hepatocytes, and in HepG2 cells (Arias-Diaz et al., 1996; Chawla et al., 1998; Majano et al., 2001). The decrease in liver GSH produced by various hepatotoxins was prevented by SAM (Lieber et al., 1990). Induction of CYP2E1 by ethanol appears to be one of the central pathways by which ethanol generates a state of oxidative stress (Castillo et al., 1992; Morimoto et al., 1994; Nanji et al., 1994; Gouillon et al., 2000). CYP2E1 also activates many toxicologically important substrates (Guengerich et al., 1991; Koop, 1992). Since CYP2E1-mediated toxicity may play a role in alcoholic liver injury and toxicity of many hepatotoxins, whereas SAM can prevent alcoholic liver injury and toxicity of hepatotoxins, it is of interest to study whether and how SAM can affect CYP2E1-mediated toxicity to the liver.

We recently reported that induction of CYP2E1 in mice by treatment with pyrazole increased the hepatotoxicity caused by Fas agonistic Jo2 antibody (Wang et al., 2005). Increased hepatotoxicity in the pyrazole/Jo2-treated mice was associated with increased oxidative and nitrosative stress in association with decreased GSH and other antioxidant levels. Chloroethiazole, an inhibitor of CYP2E1, prevented the synergistic toxicity of Jo2 antibody in pyrazole-treated mice. The current study was designed to investigate the effect of SAM on agonistic Jo2 FAS-induced hepatotoxicity following induction of CYP2E1 by pyrazole pretreatment in vivo and explore a possible relationship among oxidative stress, hepatic apoptosis, and homeostasis of SAM metabolism.

### Materials and Methods

#### Animal Experiments and Sample Collections

Male C57BL/6 mice, weighing 20 to 22 g at 6 to 8 weeks of age, were purchased from Charles River Breeding Laboratory (Boston, MA) and housed in a facility approved by the American Association for Accreditation of Laboratory Animal Care. All mice were divided into groups that received Saline (Sal), Jo2 following pyrazole (Sigma, St. Louis, MO) pretreatment (Pyr/Jo2), and Jo2 following pyrazole pretreatment plus S-adenosylmethionine (SAM) (Sigma) (Pyr/Jo2/SAM). Mice were injected i.p. with pyrazole, 120 mg/kg body weight, once a day for 2 days to induce CYP2E1; some of these mice were also given SAM pretreatment, 50 mg/kg body weight, injected i.p. twice a day for 3 days. Mice were fasted for 16 h after the last dose of pyrazole and then were administered i.p. either with saline or with agonistic Jo2 hamster anti-mouse Fas monoclonal antibody (BD Pharmingen, San Diego, CA), 0.2 μg/kg body weight.

At 8 h after administration of Jo2 or saline, mice were bled from the retro-orbital venous sinus for measurement of serum transaminases. The liver was rapidly excised, and specimens were immediately cut into small pieces and placed in fixative for histopathological and immunohistochemical assessment. The remaining liver samples were immediately frozen in liquid nitrogen and stored at −70°C in aliquots for preparation of homogenates and further use.

#### Serum Transaminases and TNF-α Assay

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured using a diagnostic kit (ThermoDMA, Louisville, CO) and kinetically following changes in absorbance at 340 nm. The serum TNF-α level was assayed using a mouse TNF-α ELISA kit (Pierce Biotechnology, Rockford, IL). A total of 50 μl of standards (standard curve concentration: 0, 50, 350, and 2450 pg/ml) or serum was added into each well in duplicate. The absorbance was measured at 450 minus 550 nm, and the results were calculated using the standard curve.

#### SAM Concentration Assay by HPLC

The concentration of SAM in liver homogenates was quantified by HPLC as described by She et al. (1994), using a Shimadzu SPD-10A UV-C visible detector (Shimadzu, Kyoto, Japan) operating at 254 nm. Homogenates were mixed 1:2 with 0.4 M HClO₄, filtered, and 100 μl was applied directly for HPLC analysis. A TSKgel ODS column (15-cm × 4.6-mm i.d.; Tosoh Co., Tokyo, Japan) was used, with a mobile phase that consisted of 40 mM NH₄H₂PO₄, 10 mM 1-hexanesulfonic acid, and 18% (v/v) methanol, pH adjusted to 3.0 with HCl. HPLC analyses were conducted at a flow rate of 1 ml/min. A calibration curve was carried out with authentic SAM or SAH standard. The concentrations of SAM related linearly to the areas under the HPLC chromatogram. Calculation for concentration (nanomoles per milligram of protein) was [Conc] (nanomoles per milligram of protein) = area under the curve (arbitrary units) × 0.1 × 1000/1000 × slope of the concentration curve (micromolar) × 0.5 volumes (milliliters) × protein (milligrams per milliliter).

#### Liver Pathology and Immunohistochemistry

Small liver pieces were fixed in 10% buffered formalin and processed into paraffin sections for H&E staining and histopathological observation. The morphological changes of liver tissues were observed by two pathologists who were blinded from the experimental information. All changes of degeneration, apoptosis, and necrosis were graded as none (0), mild (<25%), moderate (25–50%), and severe (>75%).

Immunohistochemical staining was performed by using the ImmunoCruz Rabbit ABC Staining System Kit (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for CYP2E1 with polyclonal rabbit-anti-CYP2E1 antibody (1:200) (gift from Dr. Jerome Lasker, Hackensack Biomedical Research Institute, Hackensack, NJ), for 3-nitrotyrosine (3-NT) protein adducts with polyclonal rabbit-anti-3-NT antibody (1:100) (Upstate USA Inc., Lake Placid, NY), and for inducible nitric-oxide synthase (iNOS) with polyclonal rabbit-anti-iNOS antibody (1:200) (Chemicon, Temecula, CA). The procedures were performed according to the kit instructions. In each case, a negative control (nonimmune serum) was used. The evaluation of specific positive reaction was marked as negative (−), weakly positive (+), moderately positive (++), and strongly positive (+++).

#### CYP2E1 Activity

Liver tissue homogenates were freshly prepared in 5 to 10 volumes of ice-cold 150 mM KCl. Microsomes, mitochondria, and the cytosol fractions were prepared using differential centrifugation. The protein concentration of the different fractions was determined using a protein assay kit based on the Lowry assay (Bio-Rad, Hercules, CA). CYP2E1 activity was measured in liver microsome fractions by the spectrophotometric analysis at 546 nm of the oxidation of p-nitrophenol to p-nitrocatechol in the presence of NADPH and oxygen (Reinke and Moyer, 1985). The reaction mixture consisted of 500 μg of microsomal protein in 200 μl of solution volume containing 100 mM potassium-phosphate buffer, pH 7.4, 0.2 mM NPN, and 1 mM NADPH; reactions were carried out at 37°C for 30 min, and the activity of CYP2E1 (picomoles per minute per milligram of protein) was calculated using the formula of activity = OD₄₅₀/9.530.5/30/57 × 10⁶.
extinction coefficient of $1.56 \times 10^5$ M$^{-1}$ cm$^{-1}$ and expressed as picomoles per milligram of protein.

**Superoxide Determination in Situ.** In situ production of superoxide was determined as described by Minamiyama et al. (2004). Liver tissues were placed into 50 mM sodium phosphate containing 18% sucrose at 4°C for overnight, and samples were frozen and cut into 10-μm frozen sections. The oxidation-dependent fluorescent dye dihydroethidium (DHE; 40 μM; Molecular Probes, Eugene, OR) and NADPH (1 mM) were added into the incubation solution, and tissue sections were incubated in a light-protected humid chamber at 37°C for 30 min. After being rinsed, the sections were observed using a fluorescence microscope. Control tissue sections were incubated with 40 μM DHE without NADPH.

**Western Blot Analysis.** Liver homogenates were prepared in 5 to 10 volumes of ice-cold 150 mM KCl. Mitochondria were isolated after centrifugation at 10,000 rpm for 30 min. The postmitochondrial supernatant was centrifuged at 40,000 rpm for 60 min to obtain the microsomal pellets and the cytosolic supernatant fraction. The levels of CYP2E1, Bcl-2, Bcl-X$_L$, and the precursor and cleaved form of caspase-8, or caspase-3 in 50 μg of protein samples from freshly prepared microsome or cytosol fractions, were determined by Western blot analysis with anti-CYP2E1 (1:10,000), anti-Bcl-2 (1:1000), anti-Bcl-X$_L$ (1:2000), and anti-caspase-8 and anti-caspase-3 antibody (1:2000) (Santa Cruz Biotechnology Inc.), respectively, followed by incubation with horseradish peroxidase conjugated to either goat anti-rabbit IgG (1:5000) or goat anti-mouse IgG (1:4000) (Biomed, Foster City, CA) or sheep anti-goat IgG (1:2000) (Sigma). Chemiluminescence reaction using the ECL kit (Amersham Biosciences, England, UK) was carried out for 1 min followed by exposure to Kodak BioMax film (Eastman Kodak, Rochester, NY). All specific bands of proteins detected by Western blot were quantitated by densitometry with the Automated Digitizing System (ImageJ gel programs, version 1.34S; National Institutes of Health, Bethesda, MD). Ratios of these proteins/β-actin are shown below the specific blots.

**Protein Carbonyls Assay.** The protein carbonyl adducts were assayed in liver homogenates using 20 μg of protein samples and the OxyBlot Protein Oxidation Detection Kit (Chemicon). The assay is based on the analysis of the protein carbonyl groups derivatized to 2,4-dinitrophenylhydrazine (DNPH). The DNP-derivatized protein samples were separated by SDS-polyacrylamide gel electrophoresis followed by Western blot as described above. In each case, a negative control (derivatization-control solution instead of the DNPH solution) and a positive control (dinitrophenylated standard protein) were used. The sample loading was controlled by addition of equal concentration of protein samples.

**Caspase-8 and -3 Activity.** Caspase-8 and -3 activity was determined in liver tissue homogenates by measuring proteolytic cleavage of the added substrate. Briefly, the promluenesine substrates Z-IETD-AFC or AC-DEVD-AMC (Calbiochem, La Jolla, CA) can be cleaved by caspase-8 or -3, respectively. Samples were diluted at 1:100 in reaction buffer containing 100 mM HEPES (pH 7.5), 10% sucrose, 10 mM dithiothreitol, 0.5 mM EDTA, and 0.02 mM of the caspase substrate and incubated at 37°C in a shaking water bath overnight. The fluorescence was determined with a spectrofluorometer (PerkinElmer, Wellesley, MA) based on the amount of released AFC (caspase-8, $\lambda_{em}$ = 400, $\lambda_{ex}$ = 550) or AMC (caspase-3, $\lambda_{em}$ = 380, $\lambda_{ex}$ = 460). The results were expressed as arbitrary units of fluorescence per milligram of protein.

**Antioxidant Assay.** GSH was analyzed in liver tissue homogenates by a fluorescence assay with the promluenesine substrate o-phthalaldehyde. After mixing 200 μl of samples with 200 μl of 10% trichloroacetic acid and centrifugation, 50 μl of supernatant was incubated with 1 ml of o-phthalaldehyde (1 mg dissolved in 1 ml of 50 mM sodium phosphate) at 37°C in the dark for 15 min. The fluorescence was determined with a spectrofluorometer (PerkinElmer) ($\lambda_{ex}$ = 350, $\lambda_{em}$ = 420). The concentration of GSH in the samples was calculated from a GSH standard curve, and results were expressed as nanomoles per milligram of protein. Catalase activity was assayed by measuring the decomposition of H$_2$O$_2$ at 240 nm (Claiborne and Fridovich, 1979). The activity of catalase was calculated using a formula of activity (units per milligram of protein) = OD$_{240}$/43.6/mg protein $\times 10^5$.

**Statistical Analysis.** Values reflect means ± S.E.M. The number of experiments is indicated in the figure legends. One-way analysis of variance (subsequent post hoc comparisons) was performed by Excel 2000 data analysis toolpac. $p < 0.05$ was considered statistically significant.

Fig. 1. Timeline of the experimental protocol and levels of serum ALT and AST after treatment with Jo2 plus pyrazole with or without SAM. A, mice were injected i.p. with saline or with pyrazole, 120 mg/kg body weight, once a day for 2 days to induce CYP2E1. Some mice were also treated with SAM, 50 mg/kg body weight, twice a day for 3 days. The mice were fasted for 16 h and then treated with Jo2 anti-Fas antibody (0.2 μg/g body weight). Serum ALT (B) and AST (C) were measured at 8 h after the treatment with Jo2 anti-Fas antibody. Data are the mean ± S.E.M. for 8 to 12 mice. ** or ##, significantly different from the Pyr/Jo2 or Sal group, $p < 0.01$. ΔΔΔ, significantly different from the Sal group, $p < 0.001$.
Results

Serum Transaminases, TNF-α, and Liver Pathological Changes. A timeline describing the protocol used in these experiments is shown in Fig. 1A. Liver toxicity was evaluated by assays of transaminase activity and light microscopy. In the Pyr/Jo2 group, serum ALT and AST activities were significantly lower than that in the Sal group (p < 0.001). In the Pyr/Jo2/SAM group, serum ALT and AST activities were significantly lower than that in the Pyr/Jo2 group (p < 0.01) although still elevated compared with the Sal control (p < 0.01) (Fig. 1, B and C). The content of TNF-α in serum was markedly higher in the Pyr/Jo2 or Pyr/Jo2/SAM group compared with the Sal group (p < 0.01). There was no significant change in levels of TNF-α in the Pyr/Jo2/SAM group compared with the Pyr/Jo2 group after applying SAM administration (p > 0.05) (Fig. 2A). Evaluation of the gross specimen of liver showed that the liver was dramatically swollen and its envelop was hyperemic or hemorrhagic with dark-red color in the Pyr/Jo2 group, whereas in the Pyr/Jo2/SAM group, only moderate liver swelling and no obvious hemorrhage in the hepatic envelop were observed (data not shown). After observation by light microscopy, severe pathological changes were detected in the Pyr/Jo2 group, in which many hepatocytes appeared to display extensive acidophilic necrosis (apoptosis) and focal hemorrhages in the hepatic lobule (Fig. 2C); however, there were only moderate or mild pathological changes including limited focal necrosis and acidophilic degeneration in the Pyr/Jo2/SAM group (Fig. 2D). In the Sal control group, there were no obvious pathological changes (Fig. 2B).

SAM Concentration Changes. The concentration of SAM and SAH in liver homogenates was measured by using isocratic HPLC with ultraviolet detection. Under the standard conditions, the retention times for SAH and SAM were 14.8 and 18.1 min, respectively (data not shown). Results summarized in Table 1 show that the level of SAM was significantly higher in the Pyr/Jo2/SAM group (p < 0.05) compared with the Pyr/Jo2 group. The ratio of SAM/SAH was elevated in the Pyr/Jo2/SAM group compared with the Pyr/Jo2 group.

CYP2E1 Protein Expression and Catalytic Activity. There was an approximately 1.8-fold increase in PNP hydroxylase activity by microsomes isolated from the Pyr/Jo2 group compared with the Sal control (p < 0.01); this increase was blunted in the Pyr/Jo2/SAM group (Fig. 3A). Small increases of CYP2E1 protein expression were detected by Western blot analysis in the Pyr/Jo2 group compared with the Sal control, likely due to liver toxicity at this point of analysis, whereas the level in the Pyr/Jo2/SAM group declined to the level in the Sal group (Fig. 3B). Similarly, immunohistochemistry confirmed that expression of CYP2E1 in situ was higher in the Pyr/Jo2 group (+ + + + + + + +) compared with the Sal control (− − −); CYP2E1 levels were lower in the Pyr/Jo2/SAM group (+ + + + + + +) compared with the Pyr/Jo2 group (Fig. 3C). The positive expression of CYP2E1 was mainly in the centrilobular zone of the hepatic acinus, the area with the higher liver toxicity.

Fig. 2. Serum TNF-α levels and histopathological changes observed after H&E staining. A, serum TNF-α level was assayed using a mouse TNF-α ELISA kit. A total of 50 μl of standards (standard curve concentration: 0, 50, 350, and 2450 pg/ml) or serum was added into each well in duplicate as described under Materials and Methods. The absorbance was measured at 450 minus 550 nm, and TNF-α concentration was calculated using the standard curve. Results are from six to eight mice in each group. ** or ##, significantly different from the Sal group, p < 0.01. C, liver from the Pyr/Jo2 group showed extensive acidophilic necrosis (apoptosis), severe hemorrhages, but no marked infiltration of inflammatory cells in hepatic centrilobular zone or intermediate lobular zone (arrows, H&E ×200). D, liver from the Pyr/Jo2/SAM group showed slightly sinusoid dilation and congestion, limited acido­philic degeneration, and necrosis of hepatocytes in centrilobular zone (arrows, H&E ×200). B, liver from the Sal controls showed normal morphology (H&E ×200).

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<td>The concentration of SAM in liver homogenates.</td>
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<td>Data are presented as means ± S.E.M. for four to six mice in each group.</td>
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<td>[SAM] (nmol/mg protein)</td>
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* Significant difference at p < 0.05 for Pyr/Jo2/SAM vs. Pyr/Jo2 group.
Caspase-8 and -3 Activity and Cleavage Analysis. The activity of caspase-8 or -3 was significantly higher in the Pyr/Jo2 group compared with the Sal control group (p < 0.001) (Fig. 4, A and B). Caspase activities were markedly lower in the Pyr/Jo2/SAM group compared with the Pyr/Jo2 group (Fig. 4, A and B). Similarly, expression of the precursor form of caspase-8 and of -3 significantly decreased, and their cleavage forms were increased in the Pyr/Jo2 group compared with the Sal group as detected via Western blot analysis. However, changes of caspase-8 or -3 precursor form were not significant, and their cleavage forms were not detected in the Pyr/Jo2/SAM group (Fig. 4C).

Lipid Peroxidation and Protein Carbonyl Generation. The amount of MDA, an end product of LPO, was significantly higher in the mitochondrial fractions of liver in the Pyr/Jo2 group than that in the Sal control group (p < 0.001) (Fig. 5A). Treatment with SAM partially lowered mitochondrial MDA levels in the Pyr/Jo2 group (p < 0.05). The amount of MDA in whole liver homogenates was not significantly different in the Pyr/Jo2 group compared with the Sal control group but was dramatically lower in the Pyr/Jo2/SAM group (p < 0.01) (Fig. 5B). The level of protein carbonyl adducts was approximately 5-fold higher in the Pyr/Jo2 group compared with the Sal group. Treatment with SAM partially lowered protein carbonyl adducts to a 3-fold higher level in the Pyr/Jo2/SAM group compared with the Sal group (Fig. 5C).

3-NT Protein Adducts, iNOS Expression, and Superoxide Generation. Immunohistochemical observation showed that 3-NT protein adducts were mainly expressed in situ in hepatocytes in the central lobular zone of the liver, interestingly, the area subjected to injury (Fig. 6A2). The positive expression of 3-NT adducts was significantly stronger in the Pyr/Jo2 group (+++++) compared with that in the Sal group (---) (Fig. 6A1), whereas relatively weaker in the Pyr/Jo2/SAM group (+++) (Fig. 6A3). Expression of iNOS was mainly found in the centrilobular zone and nearby areas showing liver injury (Fig. 6B2). iNOS levels were higher in the Pyr/Jo2 group (Fig. 6B2) (+++++) compared with that in the Sal group (-) (Fig. 6B1), whereas weaker in the Pyr/Jo2/SAM group (+++) (Fig. 6B3). In situ observation of superoxide using the oxidation-dependent fluorescent dye DHE showed that the red fluorescence was significantly stronger around the central veins in the Pyr/Jo2 group (++++) compared with the Sal group (---) (Fig. 6C1). Fluorescence was weaker in the Pyr/Jo2/SAM group (+) compared with the Pyr/Jo2 group (Fig. 6C3). The negative control sections incubated without NADPH had no obvious fluorescence.

Antioxidant and Antiapoptotic Protein Changes. The total GSH levels decreased in the Pyr/Jo2 group compared with the Sal group (p < 0.001) (Fig. 7A). After applying SAM administration, the level of GSH was significantly higher in
Fig. 4. Activation of caspase-8 and -3 in liver tissue. The fluorescence associated with cleavage of the proluminescent substrates Z-IETD-AFC and AC-DEVD-AMC was determined with a spectrofluorometer based on the amount of released AFC (caspase-8, \( \lambda_{\text{ex}} = 400; \lambda_{\text{em}} = 505 \)) or AMC (caspase-3, \( \lambda_{\text{ex}} = 380; \lambda_{\text{em}} = 460 \)). The results were expressed as arbitrary units of fluorescence per milligram of protein. The levels of the precursor and cleaved form of caspase-8 and -3 in 50 μg of protein samples from freshly prepared cytosol fractions were determined by Western blot analysis with anti-caspase-8 or anti-caspase-3 (1:2000), conjugated to goat anti-rabbit IgG (1:5000) and sheep anti-goat IgG (1:2000), respectively. A, caspase-3 activity in liver tissue. ΔΔΔ, significantly different from the Sal group, \( p < 0.001 \). *, significantly different from the Sal group, \( p < 0.05 \). #, different from the Pyr/Jo2 group, \( p = 0.065 \) (N.S.). B, caspase-8 activity in liver tissue. ΔΔΔ, significantly different from the Sal group, \( p < 0.001 \). *, or #, significantly different from the Pyr/Jo2 or Sal group, \( p < 0.05 \). Results are from six to eight mice in each group. C, levels of the precursor and cleaved form of caspase-8 or -3. Typical blots are shown, and the caspase-8 50-kDa precursor/β-actin ratio or the caspase-3 37-kDa precursor/β-actin ratio from four Sal, four Pyr/Jo2-treated, and eight Pyr/Jo2/SAM-treated mice are shown below the blots.

Fig. 5. Levels of lipid peroxidation and protein carbonyl formation. The production of thiobarbituric acid-reactive substances, expressed as MDA equivalents, was assayed in liver mitochondria (A) and liver homogenate fractions (B) by the spectrophotometric analysis at 535 nm of the formation of thiobarbituric acid-reactive components. ΔΔΔ, significantly different from the Sal group, \( p < 0.001 \). *, significantly different from the Pyr/Jo2 group, \( p < 0.05 \). **, significantly different from the Pyr/Jo2 group, \( p < 0.01 \). ###, significantly different from the Sal group, \( p < 0.001 \). C, protein carbonyl level was assayed in liver homogenates using 20 μg of protein samples and the OxyBlot Protein Oxidation Detection Kit as described under Materials and Methods. In each case, a negative control (derivatization-control Solution instead of the DNPH solution) and a positive control (dinitrophenylated standard protein) were used.
Pyr/Jo2/SAM compared with the Pyr/Jo2 group (p < 0.001) (Fig. 7A). Catalase activity decreased both in the Pyr/Jo2 group and the Pyr/Jo2/SAM group compared with the Sal group, and there was no significant difference between the two former groups (p > 0.05) (Fig. 7B). The antiapoptotic proteins, Bcl-2 and Bcl-XL, were analyzed by Western blot; expression levels of Bcl-2 and Bcl-XL were lowered by approximately 40 and 20%, respectively, in the Pyr/Jo2 group compared with the Sal group, respectively (Fig. 7C). Expression levels of Bel-2 and Bel-XL were increased in the Pyr/Jo2/SAM almost to the saline control values (Fig. 7C).

Discussion

A mouse model, using pyrazole treatment to overexpress CYP2E1, has been established to observe the synergistic toxicity and potential susceptibility to Fas antibody-induced liver damage (Wang et al., 2005). The current study confirms that a minimal dose of Jo2 Fas antibody, which by itself causes little liver injury, caused a synergic hepatotoxicity and apoptosis following pyrazole pretreatment. The administration of SAM in this model significantly attenuated the elevated liver injury. The administration of SAM, every 12 h for 3 days, with the last dose administered 1 h before Jo2 injection, decreased the elevated serum ALT/AST and liver tissue necrosis produced by the Pyr/Jo2 treatment. These results indicate that SAM has a protective effect on the elevated hepatotoxicity caused by Jo2 antibody challenge following pyrazole pretreatment in mice. The SAM pretreatment was partially but not completely protective; whether other concentrations of SAM or longer pretreatment could provide further protection was not evaluated.

Exogenous administration of SAM can increase the intracellular levels of SAM, influence transmethylation and transulfuration, and raise the levels of endogenous GSH (Lu et al., 2000). To evaluate the changes of SAM levels in liver, we determined the concentration of SAM, SAH, and the SAM/SAH ratio in liver. The concentrations of SAM and the ratio of SAM/SAH were significantly higher in the Pyr/Jo2/SAM group compared with the Pyr/Jo2 group. The increase in the intracellular levels of SAM and elevation of the ratio of SAM/SAH after exogenous administration of SAM may be important in the protection against Jo2 Fas antibody plus pyrazole-induced hepatotoxicity and hepatic apoptosis. Although

Fig. 6. Levels of 3-NT protein adducts (A), iNOS (B), and production of superoxide (C). Immunohistochemical staining was performed by using the immunoCruz ABC Kit in paraffin section for 3-NT protein adducts with rabbit-anti-3-NT antibody and for iNOS with rabbit-anti-iNOS antibody. Slides were visualized with 3,3-diaminobenzidine, and positive staining was reflected by the brownish-yellow color in centrolobular or surrounding vessel area. In each case, a negative control (nonimmune serum) was used. A1, Sal control; A2, Pyr/Jo2; A3, Pyr/Jo2/SAM for 3-NT immunohistochemical observation (arrows, IHC ×200). B1, Sal control; B2, Pyr/Jo2; B3, Pyr/Jo2/SAM for iNOS immunohistochemical observation (arrows, IHC ×200). C, oxidation-dependent fluorescent dye DHE (40 μM) and NADPH (1 mM) were added into the incubation solution, and liver frozen sections were incubated in a light-protected humid chamber at 37°C for 30 min. C1, weak red-fluorescence in the Sal group; C2, strong red fluorescence around the central veins in the Pyr/Jo2 group; C3, weak red fluorescence in the Pyr/Jo2/SAM group (arrow, ×200).

Fig. 7. Activity of catalase and levels of GSH, Bcl-2, and Bcl-XL. A, GSH was analyzed in liver tissue homogenates by measuring fluorescence (λex = 350, λem = 420), as described under Materials and Methods. The concentration of GSH in the samples was calculated from a GSH standard curve. The results are expressed as nanomoles per milligram of protein and are from six to eight mice in each group. ***, significantly different from the Pyr/Jo2 group, p < 0.001; ###, significantly different from the Sal group, p < 0.001. B, catalase activity was assayed by measuring the decomposition of H2O2 at 240 nm as described under Materials and Methods. Results are from six to eight mice in each group. ##, significantly different from the Sal group, p < 0.01; *, significantly different from the Sal group, p < 0.05. C, levels of Bcl-2 and Bcl-XL in 50 μg of protein samples from freshly prepared cytosol fractions were determined by Western blot analysis with anti-Bcl-2 (1:1000) and anti-Bcl-XL (1:2000), respectively, followed by incubation with horseradish peroxidase conjugated to either goat anti-rabbit IgG (1:5000) or sheep anti-goat IgG (1:2000). Typical blots are shown, and the Bcl-2/B-β-actin ratio or the Bcl-XL/β-actin ratio from four Sal, four Pyr/Jo2-treated, and eight Pyr/Jo2/SAM-treated mice are shown below the blots.
SAM's beneficial effects appear well accepted, the exact mechanism of the protection or therapeutic effects remain unclear. The main speculations are that SAM plays a protective effect as an antioxidant against oxidative stress or a regulator of GSH levels.

In our experiments, we found that SAM not only elevated GSH levels but also caused a decrease of CYP2E1 activity. These results suggest that the administration of SAM could be protective by lowering the CYP2E1-dependent oxidative stress in this model. We previously found that chloromethiazole, a CYP2E1-specific inhibitor, can reduce hepatotoxicity and CYP2E1-dependent oxidative stress in the Pyr/Jo2 injury model (Wang et al., 2005). However, in vivo treatment of mice with SAM plus ethanol was shown to not decrease CYP2E1 catalytic activity (PNP oxidation) compared with mice treated with ethanol only (Song et al., 2003). The concentration of SAM employed in that study, 5 mg/kg body weight, given i.p. once a day for 3 days, was less than that used in our study, 50 mg/kg body weight, twice a day for 3 days, which may account for the different effects on CYP2E1 activity. This requires further evaluation. In addition, CYP2E1 levels were already declining because of the developing severe injury in the Pyr/Jo2-treated mice. The administration of SAM decreased the lipid peroxidation, protein carbonyl formation, superoxide generation, and nitrosative stress produced by the Pyr/Jo2 treatment. These findings indicate that SAM plays a protective role as an effective antioxidant. The increase in 3-NT protein adducts in the Pyr/Jo2 group suggests that peroxynitrite, formed by the rapid reaction between NO and superoxide radical, may play a role in the overall liver injury. We speculate that the increase in iNOS, which produces NO, and the increase in CYP2E1, which produces superoxide, sets the stage for the generation of peroxynitrite and subsequently for formation of 3-NT protein adducts. The increase in 3-NT adducts, in iNOS, and in superoxide production by the Pyr/Jo2 treatment was greatest in the centrilobular zone of the liver acinus, the zone where liver injury was the most pronounced. Protection by SAM was associated with decreases in 3-NT protein adducts, in association with down-regulation of iNOS, CYP2E1, and superoxide production. The critical role of peroxynitrite and iNOS in the Pyr/Jo2 toxicity model and in the protection by SAM is under further evaluation.

To assess whether other antioxidants besides GSH were activated in this model, we assayed the activity of catalase in liver in the Pyr/Jo2 group with SAM or without SAM. Pyrazole is known to decrease hepatic catalase activity (Feytmans et al., 1974), and we previously discussed the possible role of this decreased catalase activity in the toxicity produced by pyrazole pretreatment (Lu et al., 2005). Although catalase levels were lowered in the Pyr/Jo2 group compared with the saline-treated, treatment with SAM failed to significantly elevate catalase despite providing hepatoprotection. This result suggests that SAM plays a protective role independent of the decline of the antioxidant catalase and low catalase levels do not play a significant role in the synergistic toxicity of Pyr/Jo2 treatment. Besides elevating GSH and decreasing CYP2E1 in this model, SAM can act as a direct antioxidant that reduces oxidative reactions and scavenge various radicals (Evans et al., 1997).

Accumulating evidence shows that SAM can moderately reduce the number of apoptotic cells (Benz et al., 1998; Ansorena et al., 2002). Exogenous administration of SAM is known to help maintain the levels of mitochondrial GSH and the content of cellular ATP so as to inhibit the transition from apoptosis to necrosis (Spivey et al., 1993; Gasso et al., 1996). The activities of caspase-8 and -3 were elevated by the Pyr/Jo2 treatment, and these elevated activities were subjected to inhibition after administration of SAM. Moreover, caspase cleavage detection showed a decreased precursor form and an increased cleaved form of caspase-8 and -3 in the Pyr/Jo2 group, respectively, whereas no reduction of the precursor form and no production of the cleaved form were detected in the SAM protective group. In addition, the antiapoptotic proteins, bcl-2 and bcl-XL, which were down-regulated by Pyr/Jo2 treatment, could be increased by SAM pretreatment. These results indicate that SAM exerted a protection against hepatotoxicity via an antiapoptotic effect, largely decreasing activation of caspases. Further evaluation of the antioxidant and antiapoptotic properties of SAM and perhaps of SAM metabolites such as MTA would be important.

In experimental models of liver injury, including alcoholic liver damage, the biochemical mechanisms of TNF-α up-regulation are not fully understood. It is also not clear whether SAM affects the biosynthesis of TNF or its serum content. Some studies suggest that exogenous SAM can decrease serum TNF levels, lower transaminases levels, and attenuate hepatoxicity in a lipopolysaccharide-induced model of liver injury. SAM may also play an important role in modulating levels of the anti-inflammatory cytokine IL-10 (Eastin et al., 1997; Chawla et al., 1998; McClain et al., 2002). Our previous study showed that TNF-α levels were elevated in the Jo2-alone-mediated hepatic toxicity model but did not appear to exert an important role in the synergic injury by Jo2-addition following pyrazole pretreatment because TNF-α levels were not elevated beyond those found in the Jo2-alone group (Wang et al., 2005). We speculate that the limited TNF-α response in the Pyr/Jo2 group may be due to an acute phase reaction in vivo, which is not associated with infiltration of inflammatory cells in the extensive apoptosis of the synergistic Pyr/Jo2 injury. The current study demonstrated that there was no significant difference in the TNF-α serum levels in the Pyr/Jo2 group compared with the Pyr/Jo2/SAM group. However, the exact role of TNF-α in the synergistic injury produced by pyrazole plus Jo2 requires further study.

In conclusion, suboptimal Jo2 Fas antibody administration combined with pyrazole pretreatment synergically caused severe hepatotoxicity and apoptotic necrosis. Exogenous administration of SAM significantly attenuated the liver injury. SAM plays an important role in hepatoprotection as an antioxidant by preventing the depletion of hepatic SAM and GSH and perhaps inhibiting CYP2E1-dependent-oxidative stress (including lipid peroxidation and nitrosative stress) and apoptosis in liver. The current study suggests that SAM is an effective protective reagent against Fas-induced hepatic apoptosis combined with CYP2E1-dependent hepatotoxicity.
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References


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