S-adenosyl methionine protects ob/ob mice from CYP2E1-mediated liver injury

Aparajita Dey, Andres A. Caro, and Arthur I. Cederbaum

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

Submitted 4 January 2007; accepted in final form 16 April 2007

Obesity is rapidly emerging as an epidemic disease (15, 45). Obese individuals have a predisposition to develop nonalcoholic fatty liver disease (NAFLD), which may progress to the successive stages of steatohepatitis and cirrhosis (6, 28). The obese mouse liver (7) is a useful model of liver injury in obesity. The aim of the present study was to examine the protective effects of SAM on CYP2E1-mediated toxicity to the mouse liver (7). The model of obesity is well characterized and has been used extensively as animal models to study human obesity (8, 11, 16, 44).

Cytochrome P-450 2E1 (CYP2E1) metabolizes various xenobiotics into toxic metabolites (3, 14). The CYP2E1-mediated metabolism of substrates results in the generation of reactive oxygen species, which further contributes to the development of alcoholic liver injury, which includes steatohepatitis (3, 13). Induction of CYP2E1 with ethanol, pyrazole, or other chemicals has been shown to promote oxidative stress (3, 13). The ob/ob mouse, a genetic model for obesity, and pyrazole as an inducing agent to elevate CYP2E1 levels (31, 40) were used in the present study. We have recently observed that pyrazole-induced CYP2E1 promotes liver injury in ob/ob mice, compared with saline-treated ob/ob mice or with lean controls treated with pyrazole (7).

S-adenosyl methionine (SAM) is a major methyl donor and also a precursor to glutathione (GSH) (24). Decreased SAM synthesis in the liver leads to decreased hepatic GSH levels. Impaired levels of SAM have been shown to affect cellular processes adversely (20, 25, 27). Liver homocysteine and S-adenosyl-homocysteine (SAH) levels are elevated, along with decreased SAM and SAM-to-SAH ratio in alcoholic liver disease (1, 5, 25). Exogenous administration of SAM has been shown to confer protection against injury induced by toxic agents, therefore suggesting the role of SAM in prevention of liver injury (1, 27, 37, 39).

Since CYP2E1 potentiates liver injury in various animal models and SAM has been shown to prevent toxicity in various models of liver injury (20, 24, 27, 37, 39), we investigated whether SAM can prevent CYP2E1-mediated toxicity to the obese mouse liver (7). The aim of the present study was to examine the protective effects of SAM on CYP2E1 promotion of oxidative and nitrosative stress and liver injury in obesity.

MATERIALS AND METHODS

Animals and treatment. Male 8-wk-old homozygous obese C57Bl/6J ob/ob mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The animals were housed in a facility approved by the American Association for Accreditation of Laboratory Animal Care and divided into eight groups, each of which consisted of four to six animals. Groups 1–3 were designed to evaluate whether SAM can prevent pyrazole-induced liver injury in obese mice. Group 1 consisted of obese mice that were injected intraperitoneally with pyrazole (Sigma, St. Louis, MO), 150 mg/kg body wt, once per day for 2 days. Group 2 consisted of obese mice injected with SAM (Sigma) at a dosage of 50 mg/kg body wt every 12 h for 3 days and treated with pyrazole on days 2 and 3 as per group 1. Group 3 consisted of obese mice that were injected intraperitoneally with 0.9% saline for 2 days and served as control. Groups 4–6 were designed to evaluate whether SAM can reverse or correct pyrazole-induced liver injury in obese mice. Group 4 consisted of obese mice injected intraperitoneally with pyrazole, 150 mg/kg body wt, once per day for 1 day and with SAM at a dosage of 50 mg/kg body wt every 12 h on the next day. Group 5 consisted of obese mice injected intraperitoneally with pyrazole, 150 mg/kg body wt, once per day for 1 day and with SAM at a dosage of 50 mg/kg body wt every 12 h on the next day. Group 6 consisted of obese mice treated with 0.9% saline for 2 days and served as control. Groups 7 and 8 were designed to investigate the effects of SAM on the CYP2E1-dependent liver injury in ob/ob mice but not in lean mice. The present study investigated the effects of S-adenosyl-L-methionine (SAM) on the CYP2E1-dependent liver injury in ob/ob mice. CYP2E1 treatment increased lipid peroxidation, 4-hydroxyxeno- and 3-nitrotyrosine protein adducts, and protein carbonyls. These increases in oxidative and nitrosative stress were prevented by SAM. Treatment of ob/ob mice with pyrazole lowered the endogenous SAM levels, and these were elevated after SAM administration. Mitochondrial GSH levels were very low after pyrazole treatment of the ob/ob mice; this was associated with elevated levels of malondialdehyde and 4-hydroxynonaldehyde. In the present study, we have recently observed that pyrazole-induced CYP2E1 promotes liver injury in ob/ob mice, compared with saline-treated ob/ob mice or with lean controls treated with pyrazole (7).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Liver samples for histology were fixed in 10% formalin and paraffin water and standard chow ad libitum. Liver homogenates in ice-cold mitochondria isolation medium containing Mitochondria were prepared by differential centrifugation of liver homogenates or mitochondria were determined by Western blot analysis of protein expression. Levels of CYP2E1, inducible nitric oxide synthase (iNOS), and other proteins in 30–50 μg of protein samples from freshly prepared liver homogenates or mitochondria were determined by Western blot analysis with anti-human CYP2E1 polyclonal antibody (1:3,000) (kindly provided by Dr. J. M. Lasker, Hackensack Biomedical Research Institute, Hackensack, NJ), anti-iNOS antibody (1:1,000), anti-4-hydroxy-2-nonenal (HNE) Michael adducts (1:1,000), anti-uncoupling protein 2 (UCP-2) (1:100) (Santa Cruz Biotechnology), and results were expressed as the protein/ • VOL 293 • JULY 2007 • www.ajpgi.org GSH, lipid peroxidation, protein carbonyl adducts, and immuno-histochemical staining for HNE adducts. Lipid peroxidation was measured by a previously described method and results were expressed as malondialdehyde equivalents (41). Protein carbonyl adducts were assayed in liver homogenates by using 20 μg of protein samples and the OxyBlot protein oxidation detection kit (Chemicon). The 2,4-dinitrophenylhydrazine–derivatized protein samples were separated by polyacrylamide gel electrophoresis followed by Western blotting and the difference in the signal intensity of the bands was compared. Paraffin-embedded liver sections were immunostained for 4-hydroxynonenal (HNE) adducts by use of the ImmunoCruz Rabbit ABC staining system kit (Santa Cruz Biotechnology, Santa Cruz, CA). The liver samples were incubated with rabbit anti-HNE-Michael adduct antibody (1:100 dilution; Calbiochem, San Diego, CA) for 2 h and, after subsequent steps, visualized with 3,3-diaminobenzidine, and counterstained with Mayer’s hematoxylin (Sigma). The positive staining was detected by dark brown color and was evaluated as negative (−), weakly positive (+), moderately positive (++) and strongly positive (+++). Liver and mitochondrial GSH were determined by the enzymatic method of Tietze (38).

Immunohistochemistry and slot blot for nitrotyrosine residues. Immunohistochemical staining was performed with the ImmunoCruz Rabbit ABC staining system kit for 3-nitrotyrosine (3-NT) protein adducts. Slides containing liver tissue sections were incubated with polyclonal rabbit anti-3-NT antibody (1:100) (Upstate USA, Lake Placid, NY) and after subsequent steps were visualized with 3,3-diaminobenzidine. Positive staining was detected by a brownish-yellow color and was graded as negative (−), weakly positive (+), moderately positive (++) and strongly positive (+++). For immunohistochemical detection of protein nitrotyrosine residues (3-NT) in liver mitochondria, a slot-blot technique with 0.5 μg of homogenate protein plus polyclonal rabbit anti-3-NT antibody was used.

SAM or SAH concentration assay by HPLC. The concentration of SAM or SAH in obese mice liver homogenates or mitochondria was quantified by HPLC (35), by using a Shimadzu SPD-10A UV-C visis detector (Shimadzu, Kyoto, Japan) operating at 254 nm. Homogenates were mixed 1:2 with 0.4 M HClO4 and filtered, and 100 μl was applied directly for HPLC analysis. A TSK gel octadecylsilyl column (15-cm × 4.6-mm ID; Tosoh, Tokyo, Japan) was used, with a mobile phase that consisted of 40 mM NH4H2PO4, 8 mM 1-heptanesulfonic acid, and 18% (vol/vol) 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.

MAT activity. Methionine adenosyltransferase (MAT) activity was measured in obese mice liver cytosols (18). Livers were homogenized in 4 vol of 10 mM Tris·HCl (pH 7.5) containing 0.3 mol/l sucrose, 0.1% β-mercaptoethanol, 1 mM/l benzamidine, and 0.1 mM/l phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 12,000 g for 15 min, and the supernatant was subsequently centrifuged at 100,000 g for 60 min to obtain liver cytosol. The reaction mixture contained 80 mM/l N-[Tris(hydroxymethyl)methyl]-2-aminoethane sulfonic acid (pH 7.4), 50 mM/l KCl, 40 mM/l MgCl2, 5 mM/l adenosine triphosphate, 10 mM/l dithiothreitol, 0.5 mM/l ethylenediaminetetraacetic acid, 50 μmol/l (MAT2A activity) or 5 mM/l (MAT1A activity) methionine, and 0.3 to 0.5 μCi [1-14C]-methionine. Liver cytosol containing 250–400 μg of protein was then added to the above reaction mixture (final volume, 100 μl) for 30 min at 37°C. At the end of the incubation, the mixture was applied to a phosphocellulose paper square (Millipore) and placed on a filtering system for washing with cold distilled water. The square was added to 4 ml of Hydroluor solution for scintillation counting. A 4°C blank was included for each condition and subtracted from the 37°C values. MAT activity is reported in units of nanomoles of SAM formed per milligram of protein per 30 min.

Statistical analysis. ANOVA followed by Student-Newman-Keuls post hoc test was employed to calculate the statistical significance between the different groups of treated and untreated mice. Data are presented as means ± SE. P < 0.05 was considered statistically significant.

RESULTS

SAM prevents pyrazole induced pathological changes in obese mice. Previous experiments showed that pyrazole treatment had no effect on promoting liver injury in lean mice (7). Hence, the effects of SAM were only studied in control or pyrazole-treated obese mice. Hematoxylin and eosin staining of liver sections showed that pyrazole induced massive steatosis (75–90%) characterized by the presence of large lipid droplets and extensive necrosis (>75%) in the livers of obese mice which was accompanied with infiltrating inflammatory
cells compared with the saline-treated control obese mice (50% steatosis and <5% necrosis) (Fig. 1A, left and middle). In contrast, SAM- plus pyrazole-treated obese mice displayed steatotic (50–65%) and necrotic changes (<15%) similar to that found with the saline-treated obese mice (Fig. 1A, right). The pyrazole-treated obese mice had almost threefold higher ALT and AST levels than the control obese mice, and treatment with SAM caused a decrease in the serum transaminase levels almost to the control levels (Fig. 1B). Pyrazole treatment of obese mice resulted in a twofold increase in hepatic triglyceride levels (Fig. 1C). However, SAM- plus pyrazole-treated obese mice had triglyceride levels similar to the control obese mice.

**SAM decreases pyrazole-induced DNA fragmentation.** Pyrazole-treated obese mice exhibited numerous apoptotic cells in their liver compared with control obese mice (Fig. 2A, left and middle; quantification in Fig. 2B). Treatment of obese mice with SAM caused a significant decrease in the number of apoptotic liver cells (Fig. 2A, right, and B). Caspase 3 activity was threefold higher in pyrazole-treated obese mice compared with control obese mice. SAM partially, but not completely, lowered the pyrazole-induced increase in the caspase 3 activity (Fig. 2C).

**SAM lowers the pyrazole-mediated increase in CYP2E1 catalytic activity.** Pyrazole treatment resulted in a twofold increase in CYP2E1 protein in the obese mice (Fig. 3A). SAM had no effect on this increase in CYP2E1 protein. The CYP2E1 catalytic activity was 2.5-fold higher in pyrazole-treated obese mice compared with the untreated group (Fig. 3B). SAM decreased the activity of CYP2E1 in the pyrazole-treated obese mice, to a net increase of ~50% over the control obese values.

**SAM prevents the pyrazole induced increases in oxidative stress.** The intensity of the protein carbonyl bands in pyrazole-treated obese mice was threefold higher than the control obese mice (Fig. 4A). Treatment with SAM decreased the elevated levels of the protein carbonyl adducts almost back to the control obese levels (Fig. 4A). Control obese mice showed negligible or very little brown staining (-/+ ) in sections of liver, immunohistochemically stained for HNE-adducts (Fig. 4B). The pyrazole-treated obese mice displayed more intense staining and extensive distribution for HNE adducts

![Figure 1](http://ajpgi.physiology.org/)

**Fig. 1.** A: effect of S-adenosyl-L-methionine (SAM) on liver morphology. Liver section from control obese mouse (left) shows steatosis as depicted by the presence of scattered lipid droplets; liver section from pyrazole-treated obese mouse (middle) shows pronounced steatosis and presence of inflammatory cells and necrosis. Liver section from SAM- plus pyrazole-treated obese mouse (right) shows almost normal morphology with characteristic fatty droplets and minimal necrosis. B: effect of SAM on serum transaminases. ALT, alanine aminotransferase; AST, aspartate aminotransferase. C: effect of SAM on triglyceride levels. †P < 0.05 for pyrazole-treated obese mice vs. control obese mice. *P < 0.05 for SAM- plus pyrazole-treated obese mice vs. pyrazole-treated obese mice. Data represent means ± SE of 3–5 animals/group.
SAM AND CYP2E1 TOXICITY IN ob/ob MICE

(+ + +), which was highly pronounced in the regions near the central vein, a region containing the highest levels of CYP2E1 (13). Treatment with SAM greatly decreased the formation of HNE-adducts in the pyrazole-treated obese mice (−) (Fig. 4B). Pyrazole-treated obese mice had increased lipid peroxidation as reflected from the threefold higher malondialdehyde (MDA) product formation compared with the control obese mice (Fig. 4C). However, MDA formation in SAM- plus
Effects of SAM on pyrazole induced increases in nitrosative stress.

The expression of iNOS in control, pyrazole and SAM-plus pyrazole-treated obese mice was examined by Western

Fig. 4. Effect of SAM on protein carbonyl, 4-hydroxy-2-nonenal (HNE) adduct formation, and lipid peroxidation. A: protein carbonyl bands and their densitometric values (du) in liver homogenates from obese mice were assayed by detecting the formation of 2,4-dinitrophenylhydrazone. Data represent means ± SE of 3 independent experiments. †P < 0.05 for pyrazole-treated obese mice vs. control obese mice. *P < 0.05 for SAM- plus pyrazole-treated obese mice vs. pyrazole-treated obese mice. B: immunohistochemical detection of pyrazole-induced HNE-protein adducts in liver. Liver sections from control obese mice show minimal HNE adduct formation, whereas pyrazole-treated obese mice show positive staining for HNE adducts. This positive staining is blunted by SAM. C: lipid peroxidation was evaluated by measuring the formation of thiobarbituric acid-reactive components as determined by the malondialdehyde equivalents. †P < 0.05 for pyrazole-treated obese mice vs. control obese mice. *P < 0.05 for SAM- plus pyrazole-treated obese mice vs. pyrazole-treated obese mice. D: GSH content was measured as described in MATERIALS AND METHODS. †P < 0.05 for pyrazole-treated obese mice vs. control obese mice. *P < 0.05 for SAM- plus pyrazole-treated obese mice vs. pyrazole-treated obese mice. ‡P < 0.05 for SAM- plus pyrazole-treated obese mice vs. control obese mice. Data represent means ± SE of 3–5 animals/group.

SAM and CYP2E1 toxicity in ob/ob mice.
blot analysis. Pyrazole treatment caused a threefold induction of iNOS expression in the obese mice (Fig. 5A). Treatment with SAM did not decrease the pyrazole-elevated iNOS levels significantly. Immunohistochemical analysis was performed to examine the formation of 3-NT adducts. 3-NT-positive staining was not detected in the control obese mice (−) (Fig. 5B). However, the pyrazole-treated obese mice exhibited strong positive staining for 3-NT (+++) and this intense staining was prevented by SAM (−) (Fig. 5B).

**SAM prevents the pyrazole-induced increases in TNF-α levels in obese mice.** TNF-α is considered to be an important factor contributing to the etiology of obesity (17), and obese mice have been shown to exhibit high levels of TNF-α (42). Pyrazole caused a 3.5-fold increase in liver TNF-α content in the obese mice and SAM partially decreased the elevated TNF-α levels to a twofold increase over the obese controls (data not shown).

**Effects of pyrazole on endogenous SAM and MAT activities.** The SAM levels in control obese mice were similar to those observed in obese mice treated with SAM for 1 day before pyrazole treatment for the next 2 days (0.21 nmol/mg protein vs. 0.18 nmol/mg protein). Treatment of obese mice with pyrazole caused a 30% decrease in hepatic SAM levels; SAM levels were increased almost twofold in SAM- plus pyrazole-treated obese mice compared with the obese mice treated with pyrazole alone (Fig. 6A). SAH, a metabolite of SAM produced after methylation reactions, was decreased 40% after pyrazole treatment of obese mice, and the addition of exogenous SAM did not affect these lowered levels (Fig. 6B). In view of the comparable decline in SAM and SAH levels, the SAM-to-SAH ratio was not altered significantly in the pyrazole-treated obese mice; however, it was almost 1.5-fold higher in the SAM- plus pyrazole-treated groups (Fig. 6C).

**MAT is an enzyme that catalyzes the formation of SAM in the cell (23).** Pyrazole treatment of obese mice caused almost threefold decreases in MAT1A and MAT2A activities; these low activities were unchanged in SAM- plus pyrazole-treated obese mice (Fig. 6, D and E). It is important to note that administration of SAM elevated hepatic SAM levels (Fig. 6A) despite the decline in MAT1A and 2A activity (Fig. 6, D and E). Obviously, the elevated SAM is coming from the administered SAM and not newly synthesized SAM and indicates that sufficient transport of SAM into the liver occurs to elevate SAM levels.

**Effects of SAM on mitochondrial UCP-2, GSH, and SAM levels in obese mice.** Subsequent experiments were designed to evaluate whether mitochondria were targets of the pyrazole-induced increase in oxidative and nitrosative stress in the obese mice. UCP-2 is a mitochondrial protein that increases mitochondrial electron transport chain activity and decreases the formation of superoxide during mitochondrial respiration (43). UCP-2 protein levels normalized to porin, a mitochondrial loading control, were increased sevenfold in pyrazole-treated obese mice and treatment with SAM nearly completely blocked this increase (Fig. 7A). In contrast to the total liver GSH levels, the mitochondrial GSH content was strongly decreased with pyrazole treatment of obese mice (3.5-fold) and SAM increased the low mitochondrial GSH levels about two-
fold (Fig. 7B). Mitochondrial SAM was present in mitochondria from the control obese mouse but could not be detected in the pyrazole-treated obese mouse; however, SAM- plus pyrazole-treated obese mice had 3.7-fold higher mitochondrial SAM levels compared with mitochondria from the control obese mice (Fig. 7C). Similarly, the pyrazole-treated obese mice did not have detectable SAH levels in their mitochondria, but SAH was present in mitochondria from the control obese mice and in the SAM- plus pyrazole-treated mice (Fig. 7D).

**Effects of SAM on mitochondrial oxidative stress.** A twofold increase in MDA formation was observed in liver mitochondria isolated from pyrazole-treated obese mice and SAM treatment caused a significant reduction in MDA levels (Fig. 8A). HNE adducts as identified by immunoblotting were low in control Fig. 6. Effect of SAM on endogenous SAM and S-adenosyl-homocysteine (SAH) levels, SAM-to-SAHA ratio, and MAT1A and 2A enzyme activities. Assays were carried out as described in MATERIALS AND METHODS. †P < 0.05 for pyrazole-treated obese mice vs. control obese mice. *P < 0.05 for SAM- plus pyrazole-treated obese mice vs. pyrazole-treated obese mice. ‡P < 0.05 for SAM- plus pyrazole-treated obese mice vs. control obese mice. Data represent means ± SE of 3–5 animals/group.

Fig. 7. Effect of SAM on mitochondrial uncoupling protein-2 (UCP-2), GSH, SAM, and SAH levels. A: Western blot and densitometric values (du) showing the protein expression of UCP-2 in liver mitochondria from obese mice. †P < 0.05 for pyrazole-treated obese mouse vs. control obese mouse. *P < 0.05 for SAM- plus pyrazole-treated obese mouse vs. pyrazole-treated obese mouse. Data represent means ± SE of 3 independent experiments. B: mitochondrial GSH content was measured as described in MATERIALS AND METHODS. †P < 0.05 for pyrazole-treated obese mouse vs. control obese mouse. *P < 0.05 for SAM- plus pyrazole-treated obese mouse vs. control obese mouse. Data represent means ± SE of 3–5 animals/group. C and D: mitochondrial SAM and SAH levels were measured as described in MATERIALS AND METHODS. *P < 0.05 for SAM- plus pyrazole-treated obese mouse vs. control obese mouse. ‡P < 0.05 for SAM- plus pyrazole-treated obese mouse vs. pyrazole-treated obese mouse. Data represent means ± SE of 3–5 animals/group.
obese mice liver mitochondria whereas a highly significant 17-fold increase in mitochondrial HNE adduct formation was observed in the pyrazole-treated obese mice (Fig. 8B). In SAM- plus pyrazole-treated obese mice, the mitochondrial HNE adduct formation was lowered to that observed in control obese mice. Similarly, the mitochondrial 3-NT protein adduct levels were very low in control obese mice (Fig. 8, C and D). A 15-fold increase in 3-NT adduct levels was observed in pyrazole-treated obese mice liver mitochondria, and SAM treatment completely prevented this increase in 3-NT levels.

**SAM can reverse pyrazole induced liver injury in obese mice.** The above experiments showed that SAM, when administered before and along with pyrazole, can protect against the liver injury in obese mice. To evaluate whether administration of SAM after pyrazole treatment can reverse pyrazole induced injury in obese mice, these animals were injected with a single dose of pyrazole for 1 day, then treated with either SAM or saline, which was injected twice on the second day, and the animals were killed on the third day. Pyrazole treatment for 1 day increased steatosis (65–70%) and induced necrotic lesions that were almost similar to that seen in obese mice treated with pyrazole for 2 days (>65%) (Fig. 9A, left and middle). In contrast, when the pyrazole-treated obese mice were treated with two doses of SAM on the day after the pyrazole treatment, the steatosis was similar to that found with the obese mice treated with saline for 2 days (50%) and livers were devoid of necrotic changes (<5%) (Fig. 9A, left and right). Pyrazole treatment for 1 day caused a 1.5-twofold increase in the serum transaminase levels and the subsequent administration of two doses of SAM decreased the ALT and AST levels to almost control levels (Fig. 9B). The obese mice treated with pyrazole for 1 day exhibited 1.6-fold higher hepatic triglyceride level, which was decreased to the control obese levels after SAM addition on the day after the pyrazole treatment (Fig. 9C). The 1-day pyrazole treatment elevated CYP2E1 protein expression almost 1.5-fold on day 3 when the mice were killed, and when SAM was given on day 2 after the pyrazole treatment and mice killed on day 3 the CYP2E1 protein content remained 1.5-fold elevated (data not shown). One-day pyrazole treatment increased the CYP2E1 catalytic activity 1.3-fold and SAM slightly lowered this modest increase (Fig. 9D). The obese mice treated with a single dose of pyrazole exhibited 1.6-fold higher caspase-3 activity, and this increase was lowered to almost control levels by the postpyrazole SAM treatment (data not shown). Lipid peroxidation (formation of MDA) was 2.5-fold elevated in obese mice treated with pyrazole for 1 day. Administration of SAM for 1 day after pyrazole treatment lowered the MDA level to almost the control obese levels (data not shown).

**Effect of SAM in the absence of pyrazole in obese liver.** The control obese mice exhibit almost 45–50% steatosis whereas control lean mice did not exhibit any fatty droplets in their livers (7). ALT and AST levels, triglycerides, caspase-3 activity, and oxidative stress as reflected by increased MDA were two- to threefold higher in obese livers than the lean mice (7). Thus modest liver injury occurs in obese mice compared with lean mice. We studied whether SAM could also be protective
against this injury in obese livers. When the control obese mice were treated with SAM for 3 days, steatosis was unchanged in these animals compared with the obese mice treated with saline for 3 days (Fig. 10A). The ALT and AST levels were decreased almost 1.5-fold in SAM-treated obese mice compared with the control obese mice (Fig. 10B). SAM treatment for 3 days...
caused an almost 1.5-fold decrease in triglyceride level in obese mice (Fig. 10C); however, triglycerides were still elevated compared with values of lean controls (0.3 arbitrary absorbance units) (7). A decrease was observed in caspase-3 activity in the SAM-treated obese mice compared with the saline-treated obese mice (Fig. 10D). Lipid peroxidation, as indicated by MDA levels was also decreased 1.5-fold in obese mice treated with SAM for 3 days compared with the obese mice treated with saline for the same duration (Fig. 10E). Thus SAM provided partial but not complete protection against the liver injury but not the steatosis in obese mice.

**DISCUSSION**

CYP2E1 induction by treatment with either acetone or pyrazole was shown to potentiate liver injury in obese mice but not lean mice through its ability to generate oxidative stress (7). SAM has been shown to alleviate liver injury in several studies (1, 20, 24, 37, 39). In the present study, we investigated whether SAM can prevent CYP2E1 induced toxicity in obese mice. The relevance of SAM in the context of liver injury is also due to the fact that rodents fed a methionine choline-deficient diet develop fatty liver (19, 36). Human beings having
cirrhotic liver have also been shown to have impaired metabolism of methionine and reduced synthesis of SAM in the liver (9, 21).

Administration of SAM to pyrazole-treated obese mice prevented the necrotic symptoms, steatosis, elevated transaminases, and increased triglycerides observed in these mice. Thus SAM can block the toxic effects of CYP2E1 in obesity and prevent the increase in fat accumulation produced by pyrazole in the liver of obese mice. The cleavage of cellular proteins with caspases leads to apoptosis and the consequent degradation of chromosomal DNA is a critical feature in apoptosis (29). Pyrazole induced apoptosis in obese mice, as evidenced by increased TUNEL staining and caspase 3 activity, and pretreatment with SAM lowered these changes. Thus SAM prevents the increases in both necrosis and apoptosis that occur in the pyrazole-treated obese mice.

Several mechanisms may play a role in the ability of SAM to blunt the toxicity found in the pyrazole-treated obese mice. Since CYP2E1 is critical to this increased toxicity produced by pyrazole, as evident from the ability of the CYP2E1 inhibitor chloromethiazole to prevent the toxicity (7), the decrease in catalytic activity of CYP2E1 by SAM (50 mg/kg) may be important. A lower concentration of SAM (5 mg/kg) was reported not to inhibit CYP2E1 activity (37). The lack of effect of SAM on CYP2E1 levels in the pyrazole-treated obese mice is consistent with the findings of a recent study that SAM did not affect the induced CYP2E1 protein expression significantly in livers of rats exposed to chronic ethanol (1). TNF-α is considered to be an important factor in the development of inflammatory response and liver injury in NAFLD. SAM lowers TNF-α production by macrophages (4), hence it is possible that effects on TNF-α levels may play a role in the pyrazole toxicity and the protection by SAM. Indeed, TNF-α levels were elevated in the pyrazole-treated obese mice, and these elevated TNF-α levels were decreased in the SAM- plus pyrazole-treated obese mice. Further studies are required to evaluate the role of TNF-α in the CYP2E1 potentiated toxicity in the obese mice and in the mechanism of protection by SAM. SAM has antioxidant actions that may be important in its protective actions. Oxidative stress occurs in the pyrazole-treated obese mice as reflected by the elevations in protein carbonyls, MDA, and HNE adducts. SAM decreased this consequent oxidative stress as exhibited by lower protein carbonyl, MDA, and HNE adduct formation.

Antioxidants such as GSH remove reactive oxygen species and help in maintaining the proper thiol redox environment of the cell (22). Pyrazole increased the cytosolic GSH levels in obese mice, perhaps an important metabolic adaptation to protect against oxidant stress. Induction of CYP2E1 by pyrazole treatment in vivo elevated GSH levels (30), and this antioxidant was also elevated in HepG2 cells overexpressing CYP2E1 (12, 26). Interestingly, if true, this adaptation is not sufficient to protect the obese liver cells from the enhanced oxidant stress generated by CYP2E1. Injection of pyrazole-treated obese mice with SAM caused a small but insignificant increase in GSH over the already elevated GSH level, suggesting that SAM is not hepatoprotective in this model by elevating cytosolic GSH levels.

iNOS has been associated with the development of insulin resistance and impaired insulin signaling in the liver of obese mice (10). Peroxynitrite formation due to the association of superoxide and nitric oxide is a powerful oxidizing agent, causing 3-NT adduct formation and subsequent cellular damage. Pyrazole treatment of obese mice elevated iNOS levels and promoted 3-NT protein adduct formation, suggestive of nitrosative stress. SAM did not affect the iNOS protein expression in pyrazole-treated obese mice but decreased the 3-NT staining. Inhibition of 3-NT protein adduct formation by SAM in the absence of any effect on iNOS is likely due to the inhibition of superoxide formation, e.g., decline in CYP2E1 catalytic activity or TNF-α levels by SAM.

In view of the hepatoprotective actions of SAM, we evaluated whether the pyrazole treatment of obese mice altered endogenous SAM levels, and the possibility that exogenous SAM protected in this model by restoring SAM levels. Indeed, the pyrazole treatment decreased SAM and SAH levels, and SAM treatment elevated the hepatic SAM levels even to higher concentrations than in the obese controls. The decrease in hepatic SAM levels may be due to the decrease in MAT1A and MAT2A activities. MAT1A is known to be sensitive to oxidant stress (34), and it is possible that the induction of CYP2E1 and subsequent enhanced oxidant stress lowers MAT1A activity. However, exogenous SAM, which decreases CYP2E1 activity and the oxidative/nitrosative stress, did not protect against the loss in MAT1A activity.

Mitochondrial dysfunction is an important event in cellular injury. The mitochondrial protein UCP-2, which is upregulated in response to oxidative stress as an adaptive response (43), showed a distinct increase in pyrazole-treated obese mice. The significance of this increase is not clear; the increase in electron transfer activity would decrease mitochondrial superoxide production and should be protective; however, the uncoupling and decline in ATP would be detrimental. The latter might be important in the overall toxicity in the pyrazole-treated obese mice because treatment with SAM decreased the UCP-2 levels to levels of the control obese mice; this decrease may play a role in the protection afforded by SAM, helping to maintain cellular ATP levels. In contrast to the increase in GSH content observed in the liver cytosol, pyrazole caused a highly significant decrease in GSH levels in the liver mitochondria. This suggests that CYP2E1-generated oxidative stress can have effects in mitochondria of obese mice; i.e., pyrazole treatment ultimately induces oxidative stress in the mitochondrial compartment. Mitochondrial SAM was not detectable in the pyrazole-treated obese mice, indicating that a decreased SAM level is associated with a decreased GSH level in mitochondria. Exogenous administration of SAM resulted in the presence of SAM in the mitochondrial compartment of the pyrazole-treated obese mice. Importantly, mitochondrial GSH levels were elevated after exogenous administration of SAM. Besides the decline in mitochondrial GSH, additional evidence for CYP2E1-mediated oxidative stress in liver mitochondria of obese mice was evident from the increased formation of MDA and HNE-protein adducts in the mitochondria. The SAM-treated animals exhibited low MDA levels and no HNE adduct formation. Pyrazole also increased nitrosative stress in the mitochondria of obese mice as shown by the strong 3-NT bands; band intensity was decreased in the SAM-treated obese mice.

The preceding observations indicate that SAM prevents pyrazole-induced CYP2E1 toxicity in obese mice as SAM was
administered 1 day before pyrazole treatment and on the 2 days when pyrazole was injected. To assess whether SAM reverses pyrazole-induced CYP2E1-mediated injury, we designed an experimental protocol to treat the obese mice with pyrazole for 2 days followed by treatment with SAM for the next 3 days. However, the pyrazole treatment was too toxic because the mice died 48 h after the second pyrazole injection. The experimental protocol was redesigned to treat the obese mice with a single dose of pyrazole and administer SAM or saline on the next day, with death on day 3. Pyrazole treatment for 1 day caused necrotic changes in the liver of obese mice and elevated transaminases levels, effects that were not observed in pyrazole- plus SAM-treated animals. The single dose of pyrazole increased the CYP2E1 catalytic activity, although to a much lesser extent than that observed with 2 days pyrazole treatment. Pyrazole- plus 1-day SAM-treated obese mice also exhibited decreased triglyceride levels, caspase-3 activity, and MDA levels compared with obese mice treated with pyrazole plus saline for 1 day. The decrease in toxicity with SAM administration after the pyrazole-induced increase in CYP2E1 catalytic activity suggests that SAM may also reverse liver injury in the obese mice through mechanisms other than its ability to decrease the activity of CYP2E1. These mechanisms may be varied and may include the decrease in TNF-α levels, increase in GSH levels due to SAM, and antioxidant actions of SAM.

In summary, pyrazole induced necrotic changes and apoptosis in livers of obese mice, and SAM pretreatment abrogated these pathological changes. Important mechanisms involved in the protective actions of SAM are the blunting of the increased catalytic activity of CYP2E1, possibly the decline in TNF-α levels, and the lowering of the elevated oxidative/nitrosative stress produced by SAM treatment. Endogenous SAM levels were lowered after pyrazole treatment and SAM treatment increased the endogenous SAM levels. SAM also protected pyrazole-treated obese mice from CYP2E1-generated oxidative and nitrosative stress in the mitochondria, especially the depletion of mitochondrial GSH. Two doses of SAM treatment after pyrazole treatment for 1 day also decreased the pyrazole-induced liver injury and lipid peroxidation, suggesting that SAM can reverse the CYP2E1-promoted liver injury in obesity. Therefore, SAM is effective in protecting against pyrazole-induced oxidative and nitrosative stress and liver injury in obese mice by lowering CYP2E1-generated oxidative/nitrosative stress in the liver and in the mitochondrial compartment.

GRANTS

These studies were supported by National Institute on Alcohol Abuse and Alcoholism Grant AA-14132.

REFERENCES


