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Oxidative stress is important in the pathogenesis of liver injury induced by sulindac and lipopolysaccharide cotreatment

Wei Zou,

Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI, Center for Integrative Toxicology, Michigan State University, East Lansing, MI

Robert A. Roth,

Center for Integrative Toxicology, Michigan State University, East Lansing, MI, Department of Pharmacology and Toxicology, Michigan State University, East Lansing, MI

Husam S. Younis,

Pfizer Global Research and Development, Drug Safety R&D, San Diego, CA

Lyle D. Burgoon, and

Center for Integrative Toxicology, Michigan State University, East Lansing, MI, Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI, Gene Expression in Development and Disease Initiative

Patricia E. Ganey

Center for Integrative Toxicology, Michigan State University, East Lansing, MI, Department of Pharmacology and Toxicology, Michigan State University, East Lansing, MI

Abstract

Among all the nonsteroidal anti-inflammatory drugs, sulindac (SLD) is associated with the greatest incidence of idiosyncratic hepatotoxicity in humans. Previously, an animal model of SLD-induced idiosyncratic hepatotoxicity was developed by cotreating rats with a nonhepatotoxic dose of LPS. Tumor necrosis factor- α (TNF) was found to be critically important to the pathogenesis. In this study, the mechanism of liver injury induced by SLD/LPS cotreatment was further explored. Protein carbonyls, products of oxidative stress, were elevated in liver mitochondria of SLD/LPS-cotreated rats. The results of analyzing gene expression in livers of rats before the onset of liver injury indicated that genes associated with oxidative stress were selectively regulated by SLD/LPS cotreatment. Antioxidant treatment with either ebselen or dimethyl sulfoxide attenuated SLD/LPS-induced liver injury. The role of oxidative stress was further investigated in vitro. SLD sulfide, the toxic metabolite of SLD, enhanced TNF-induced cytotoxicity and caspase 3/7 activity in HepG2 cells. SLD sulfide also increased dichlorofluorescein fluorescence, suggesting generation of reactive oxygen species (ROS). Hydrogen peroxide and TNF cotreatment of HepG2 cells caused greater cytotoxicity than either treatment alone. Either antioxidant tempol or a pancaspase inhibitor Z-VAD-FMK decreased cell death as well as caspase 3/7 activity induced by SLD sulfide/TNF coexposure. These results indicate that SLD/LPS treatment causes oxidative stress in livers of rats and suggest that ROS are important in SLD/LPS-induced liver injury in vivo. Furthermore, ROS contribute to the cytotoxic interaction of SLD and TNF by activating caspase 3/7.

Corresponding Author: Patricia E. Ganey, Department of Pharmacology and Toxicology, Michigan State University, Food Safety and Toxicology Building, Room 214, East Lansing, MI 48824, Tel: (517)-432-1761, Fax: (517)-432-2310, ganey@msu.edu.

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Keywords

Sulindac; Lipopolysaccharide; Idiosyncratic adverse drug reactions; Reactive oxygen species; Tumor necrosis factor-alpha

Introduction

Idiosyncratic adverse drug reactions (IADRs) occur at doses of drug that do not cause toxicity in most people and are generally not reproducible in animals. IADRs lead to not only a large number of withdrawals and restrictions on use of otherwise effective drugs on the market, but also cause permanent disability or death of patients. Thus, IADRs remain a challenge to public health and drug development. Liver is a common target of IADRs generally, and nonsteroidal anti-inflammatory drugs (NSAIDs), especially sulindac (SLD), are associated with idiosyncratic hepatotoxicity (Garcia Rodriguez et al., 1994).

Several hypotheses have been raised to explain the mechanism of IADRs. One is that inflammation might be a trigger for drug-induced idiosyncratic hepatotoxicity. An inflammatory episode that occurs at some time, unpredictably, during drug treatment could precipitate injury at an otherwise safe dose of drug. This scenario could explain the erratic temporal occurrence of these responses. Consistent with this hypothesis, several liver injury models of drug-inflammation interaction were developed in rodents. In previous studies, inflammatory stress induced by a nonhepatotoxic dose of lipopolysaccharide (LPS) interacted with SLD to cause severe hepatocellular and bile ductular injury in rats, which matches the pattern of liver injury from SLD in human patients (Tarazi et al., 1993; Zou et al., 2009b). The SLD/LPS hepatotoxic interaction in rats further supported the hypothesis that inflammation can trigger hepatic IADRs and provided an animal model to study the mechanism of SLD-induced idiosyncratic liver injury. Using this model, it was determined that tumor necrosis factor-alpha (TNF), hypoxia and neutrophils (PMNs) are important players in the pathogenesis of SLD/LPS-induced liver injury (Zou et al., 2009a; Zou et al., 2009b).

Reactive oxygen species (ROS) include oxygen free radicals and other nonradical but highly reactive molecules (e.g., hydrogen peroxide). Excessive generation of ROS tilts the balance between prooxidant and antioxidant influences and results in oxidative stress. Oxidative stress in liver can be induced under various conditions that include consumption of ethanol or other drugs and inflammatory stress (Galati et al., 2002; Choi and Ou, 2006; Cederbaum et al., 2009). ROS can directly oxidize proteins, DNA or membrane lipids in target cells, and such effects can result in cell death. One ROS-initiated pathway to cell death is through caspase-dependent, intracellular apoptotic signaling (Jones et al., 2000). In this study, gene expression was analyzed in livers from rats treated with SLD and/or LPS. The results of Ingenuity Pathway Analysis of SLD/LPS-specific gene expression profiles pointed to the occurrence of oxidative stress. We tested the hypothesis that oxidative stress plays a role in the pathogenesis of liver injury induced by SLD/LPS in vivo. In hepatocytes, we evaluated the ability of SLD and its metabolites to prompt ROS generation and explored the role of ROS in cytotoxicity.

Materials and Methods

Materials

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). The LPS (Lot 075K4038) used in animal experiments was derived from *Escherichia coli* serotype O55:B5 and had an activity of 3.3×10^6 endotoxin units (EU)/mg. HepG2/C3A cells were obtained from American Type Culture Collection (Manassas, VA).

Animals

Male, Sprague-Dawley rats (Crl:CD(SD)IGS BR; Charles River, Portage, MI) weighing 250 to 370 g were used in this study. They were allowed to acclimate for 1 week in a 12-hr light/dark cycle prior to use in experiments. Animals were fed standard chow (Rodent Chow/Tek 8640; Harlan Teklad, Madison, WI) and allowed access to spring water *ad libitum*. Experimental procedures complied with the “Guide for the Care and Use of Laboratory Animals” (National Academy of Sciences).

Design of experiments in vivo

As described in a previous study (Zou et al., 2009b), rats were given the first administration of SLD (50 mg/kg, p.o.) or its vehicle (0.5% methyl cellulose), and food was removed at this time. Sixteen hours later, SLD at the same dose or its vehicle was administered to rats. LPS (8.25×10^5 EU/kg, i.v.) or its vehicle (saline) was administered via a tail vein half an hour before the second administration of SLD. As reported previously, this protocol results in liver injury in the SLD/LPS-cotreated rats (Zou et al., 2009b). At various times (4, 8 or 12 hr) after the second administration of SLD, rats were anesthetized with isoflurane, and blood was drawn from the vena cava. Serum was prepared from clotted blood. A portion of the right medial lobe of the liver was flash-frozen in liquid nitrogen for RNA extraction. Another portion was collected and cooled in ice-cold isolation buffer for mitochondrial preparation. In experiments designed to evaluate the effect of antioxidants on liver injury, ebselen (50 mg/kg, p.o.) or its vehicle (0.5% methyl cellulose) was given to rats 1.5 hr before the second administration of SLD. Alternatively, dimethyl sulfoxide (DMSO; 0.3 mL/g, i.p.) was given to rats at the same time as SLD (i.e., 2 administrations of DMSO).

Evaluation of protein carbonyls in mitochondria

Mitochondria were isolated from livers of rats treated with SLD/LPS or vehicles at 8 hr using a mitochondrial isolation kit (Sigma, St. Louis, MO). Briefly, fresh livers (50 mg) were collected and homogenized in HEPES buffer containing 200 mM mannitol, 70 mM sucrose, and 1 mM EGTA. Liver homogenates were centrifuged at $600 \times g$ for 5 minutes. The supernatant fraction was transferred into a new tube and centrifuged at $11,000 \times g$ for 10 minutes. The pellets were washed and centrifuged at $11,000 \times g$ for 10 minutes again. The mitochondrial pellets were resuspended in HEPES for the evaluation of membrane potential or protein carbonyl concentration. Protein carbonyl concentration in liver mitochondria was measured using a commercially available kit purchased from Cayman Chemical (Ann Arbor, MI).

Gene expression analysis

Total RNA was extracted from frozen liver tissue collected at 4 hr using a kit purchased from Qiagen Inc. (Valencia, CA) as described previously (Younis et al., 2006). Microarray analysis was performed using the standard protocol provided by Affymetrix, Inc. (Santa Clara, CA). Total RNA (10 μ g) was reverse transcribed into cDNA in the presence of oligo dT primer using a Superscript II Double-Strand cDNA synthesis kit (Invitrogen, Carlsbad, CA). cDNA was purified, and biotin-labeled cDNA was synthesized using the Enzo RNA Transcript Labeling Kit (Affymetrix, Santa Clara, CA). After the labeled cDNA was purified and its quality was evaluated, cDNA was hybridized to a Rat Genome 230 2.0 Array (Affymetrix), which comprised more than 31,000 probe sets. The array was stained with streptavidin-phycoerythrin (Invitrogen, Carlsbad, CA) and scanned to generate signal intensity files.

Data normalization was performed using SAS version 9.1 (Eckel et al., 2005). Empirical Bayes analysis was used to calculate posteriori probabilities (P1(t) value) (Eckel et al., 2004). Ratios of gene expression were calculated by comparing SLD/Veh, Veh/LPS or SLD/LPS groups to the Veh/Veh group.

Evaluation of liver injury

Liver injury was assessed by measuring the activity of alanine aminotransferase (ALT) in serum using a diagnostic kit from Thermo Corp (Waltham, MA).

Evaluation of mitochondrial membrane potential

The effect of SLD and its metabolites on mitochondrial membrane potential was evaluated using JC-1 mitochondrial membrane potential assay kit from Cayman Chemical (Ann Arbor, MI). Mitochondria isolated from untreated rats (2 ug protein) were incubated with various concentrations of SLD or its metabolites at a final volume of 200 uL. After 30 min, the JC-1 dye solution (0.2 uL) was added. Fluorescence was read for JC-1 agglomerates (excitation/emission=560/595 nm) and monomers (excitation/emission= 485/535 nm), respectively. The ratio of JC-1 agglomerates to monomers in mitochondria was calculated, and the data were expressed as a percentage of vehicle control (0.5% dimethyl sulfoxide). A decrease in ratio of JC-1 agglomerates to monomers is associated with a decrease in membrane potential (Reers et al., 1995).

Evaluation of reactive oxygen species in HepG2 cells

ROS in HepG2 cells were assessed using 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein (DCF) diacetate, acetyl ester (CM-H2DCFDA) purchased from Invitrogen, Inc. (Carlsbad, CA). HepG2 cells (4×10^5 cells/mL) in suspension were incubated with 10uM CM-H2DCFDA in Dulbecco's Modified Eagle's medium (DMEM) for 45 min. The cells were washed twice with DMEM with 10% fetal bovine serum. Cells were plated in 96-well plates and treated with 250 uM SLD sulfide or its DMSO vehicle in the presence or absence of recombinant human TNF (200 ng/ml) dissolved in DMEM vehicle. DCF fluorescence intensity was read at 0, 0.5, 1 and 3 hr after treatment.

Evaluation of cytotoxicity and caspase 3 activity

HepG2 cells were plated in 96-well plates at a density of 4×10^5 cells/mL in DMEM with 10% fetal bovine serum. After overnight incubation, medium was renewed, and HepG2 cells were treated with tempol (0.2-1 mM) or with the pancaspase inhibitor, Z-VAD-FMK (Z-VAD, 10uM). Half an hour later, SLD sulfide (the active metabolite of SLD; 200- 250 uM), hydrogen peroxide (0.2- 2 mM), TNF (200 ng/mL) or appropriate vehicle was added depending on the purpose of the experiment. The percentage of LDH released was evaluated at 24 hr as described previously (Zou et al., 2009a). Caspase 3/7 activity was evaluated at 6 hr after treatment in HepG2 cells using a Caspase-Glo 3/7 assay kit (Promega, Madison, WI)

Statistical analyses

Results are expressed as means $\pm$ S.E.M. One-way or two-way analysis of variance was applied for data analysis, as appropriate, and Student-Newman-Keuls test was used as a *post hoc* test to compare means. For all studies, the criterion for statistical significance was $P < 0.05$. For gene array analysis, empirical Bayes analysis was used and posterior probability (P1(t)- value) > 0.9 was set as the criterion for significance.

Results

Oxidative stress in livers of SLD/LPS-cotreated rats

In previous studies, neither LPS nor SLD was hepatotoxic when given alone; however, cotreatment with SLD/LPS caused severe liver injury in rats (Zou et al., 2009b). Interestingly, protein carbonyl concentration was not affected in liver mitochondria of rats treated with SLD or LPS alone (Fig.1). In contrast, SLD/LPS cotreatment significantly increased protein

carbonyls in mitochondria of livers collected at 8 hr, suggesting that hepatic oxidative stress was associated with SLD/LPS cotreatment.

Gene expression changes regulated by treatment with SLD, LPS or SLD/LPS

SLD/LPS-induced liver injury occurred between 4-8 hr after the second administration of SLD (Zou et al., 2009b). Since genes regulated by SLD/LPS at the onset of liver injury (i.e., 4 hr) might be involved in the pathogenesis, livers were collected 4 hr after treatment, and gene expression was analyzed. To depict the number of gene expression changes (relative to Veh/Veh) caused by treatment with SLD/Veh, Veh/LPS or SLD/LPS, a Venn diagram was generated (Fig. 2). A large number of genes (1476) were changed by LPS administration. In contrast, SLD only caused a small number of genes expression changes (79). SLD/LPS cotreatment led to expression changes of 2040 genes. Not surprisingly, most of the gene expression changes caused by LPS (1309/1476) were represented in the genes regulated by SLD/LPS cotreatment. However, there were 721 genes regulated by SLD/LPS that were not affected by either SLD or LPS treatment alone.

Gene expression changes specifically regulated by SLD/LPS point to oxidative stress

SLD/LPS was the only treatment that resulted in liver injury (Zou et al., 2009b). Accordingly, those 721 genes selectively regulated by SLD/LPS are most likely to be involved in the pathogenesis of liver injury. These genes were further subjected to Ingenuity Pathway Analysis, which annotated 576 of the genes, including 83 that were upregulated and 493 that were downregulated by SLD/LPS. A list of toxicity pathways selectively affected by the 576 genes was determined (Table 1). Genes associated with pathways involved in fatty acid metabolism, LPS/IL-1-mediated inhibition of RXR function, NFkB signaling pathway and oxidative stress were highly impacted by SLD/LPS cotreatment.

Effect of antioxidants on liver injury

Ebselen and DMSO each have antioxidant properties. Pretreatment of rats with either ebselen or DMSO reduced liver injury (Fig. 3). That is, ALT activity at 12 hr was significantly increased by SLD/LPS, and either ebselen or DMSO markedly decreased serum ALT activity.

Effect of SLD sulfide on mitochondrial membrane potential

Mitochondria isolated from livers of untreated rats were incubated with SLD, SLD sulfide or SLD sulfone. Compared to vehicle treatment, SLD or SLD sulfone up to 1mM did not change the ratio of JC-1 aggregates and monomers, a marker of membrane potential (Fig. 4). SLD sulfide caused a concentration-related decrease in mitochondrial membrane potential.

Effect of SLD sulfide on the production of reactive oxygen species in HepG2 cells

We have previously shown that SLD sulfide is more toxic than SLD or its sulfone metabolite in both primary rat hepatocytes and human-derived HepG2 cells (Zou et al., 2009). In HepG2 cells SLD sulfide increased DCF fluorescence at 0.5, 1 and 3 hr after addition (Fig.5), indicating that SLD sulfide induced ROS production. TNF plays a critical role in the pathogenesis of SLD/LPS-induced liver injury by synergistically killing hepatocytes with SLD sulfide (Zou et al., 2009a). However, TNF alone had no effect on DCF fluorescence, and the effect of SLD sulfide on ROS generation was not influenced by TNF.

Cytotoxicity of hydrogen peroxide and TNF to HepG2 cells

Since TNF did not affect the production of ROS, the possibility exists that TNF interacts with ROS generated as a result of exposure to SLD sulfide, leading to increased cell killing. To test this, the effect of TNF on the cytotoxicity of hydrogen peroxide was evaluated. Neither TNF

(200ng/mL) nor hydrogen peroxide (up to 1 mM) was toxic to HepG2 cells (Fig. 6). At 2 mM, hydrogen peroxide caused very modest cytotoxicity. When HepG2 cells were treated with TNF (200ng/mL) and hydrogen peroxide (1-2 mM) together, significant cytotoxicity occurred, as marked by a pronounced increase in LDH release. This indicated that TNF and hydrogen peroxide interacted to cause cell death.

Effect of antioxidant treatment and caspase inhibition on cytotoxicity

SLD sulfide (250 uM) killed HepG2 cells within a 24 hr incubation (Fig. 7). Tempol, which is a superoxide dismutase mimetic, reduced the cell death caused by SLD sulfide. As shown previously (Zou et al., 2009a), a nontoxic concentration of TNF (see Fig. 6) significantly enhanced the cytotoxicity of SLD sulfide. Tempol decreased the cytotoxicity due to the interaction of SLD sulfide and TNF.

Effect of antioxidant treatment on caspase 3/7 activity induced by SLD sulfide and TNF cotreatment

SLD sulfide or TNF alone at the concentrations used did not increase caspase 3/7 activity in HepG2 cells (Fig. 8). In contrast, coadministration of SLD sulfide and TNF activated caspase 3/7 activity at 6 hr. Pretreatment of HepG2 cells with tempol or a pancaspase inhibitor, Z-VAD, abolished the increase in caspase 3/7 activity caused by SLD sulfide and TNF cotreatment. Z-VAD failed to protect HepG2 cells from the cytotoxic effect of SLD sulfide when it was given alone but reduced the cytotoxicity from SLD sulfide/TNF coadministration (Fig. 9).

Discussion

Oxidative stress has been associated with numerous models of liver injury. For example, ROS play a role in liver injury induced by alcohol or ischemia-reperfusion (Wiseman, 2006; Cederbaum et al., 2009). Hepatotoxic drugs such as acetaminophen can induce oxidative stress in mouse liver and in hepatocytes in vitro (Adamson and Harman, 1993; Lores Arnaiz et al., 1995). It has been suggested that ROS play a role in the idiosyncratic liver injury caused by NSAIDs (Boelsterli, 2002). SLD induces oxidative stress in cultured cell lines (Seo et al., 2007; Park et al., 2008). However, SLD has not been reported to cause oxidative stress in an animal model. In our experiments, treatment with SLD alone did not affect the concentration of protein carbonyls in liver mitochondria (Fig. 1). However, this marker of oxidative stress was increased in livers of rats cotreated with SLD and LPS. In previous studies, SLD/LPS cotreatment induced liver injury in rats, whereas the doses of either SLD or LPS employed were not hepatotoxic when given alone (Zou et al., 2009b). Therefore, oxidative stress in liver mitochondria is associated with SLD/LPS cotreatment.

To investigate further the hepatotoxic interaction between SLD and LPS, gene array analysis was performed on livers collected at 4 hr, a time before the onset of liver injury. Gene expression changes caused by SLD, LPS and SLD/LPS were compared. The results suggested that SLD alone had only a modest effect on gene expression (Fig. 2). As expected, LPS treatment caused changes in the expression of numerous genes. Interestingly, SLD interacted with LPS to change the expression of a large number of genes specifically after SLD/LPS cotreatment.

The pathway most impacted by SLD/LPS was fatty acid metabolism (Table 1). All the 12 genes in that category were downregulated, and half of them (ACSL1, AUH, ACAD9, ADHFE1, HSD17B4, GCDH) locate in the mitochondria, which might indicate impaired mitochondrial function. Several LPS-related pathways (LPS/IL-1-mediated inhibition of RXR function, NFkB signaling pathway) were highly impacted by SLD/LPS cotreatment. These results suggest that SLD has an effect on the signaling pathway driven by LPS. Interestingly, Ingenuity

Pathway Analysis indicated that genes related to oxidative stress were selectively influenced by cotreatment. Two important genes involved in detoxification of reactive oxygen species (glutathione peroxidase 3 and glutathione S-transferase mu 2) were significantly downregulated by SLD/LPS cotreatment. Glutathione peroxidase partially determines the susceptibility of cells to oxidative stress (Yang et al., 2006), and downregulation of glutathione S-transferase mu 2 has been associated with increased levels of superoxide anion (Zhou et al., 2008). Accordingly, this result suggests that the cellular defense system against oxidative stress was impaired before the onset of liver injury by SLD/LPS cotreatment, and this impairment might contribute to the increased protein carbonyl concentration in liver mitochondria (Fig. 1).

Ebselen is a glutathione peroxidase mimic, and DMSO is a scavenger of ROS. Both of these agents decreased ALT activity in sera of rats treated with SLD/LPS. It should be noted that DMSO can suppress conversion of the prodrug sulindac to its bioactive sulfide metabolite (Swanson et al., 1983). Nonetheless, the observation that two agents that reduce oxidative stress by different mechanisms attenuated liver injury suggests that oxidative stress contributes to the pathogenesis of liver damage in this model.

Injured mitochondria can be a major source of ROS arising from leakage of electrons from the electron transport chain (Zorov et al., 2006; Orrenius, 2007). There are numerous reports that NSAIDs lead to mitochondrial dysfunction by acting as mitochondrial uncouplers or causing mitochondrial membrane permeability transition pore opening (Moreno-Sanchez et al., 1999; Al-Nasser, 2000; Boelsterli, 2002). In isolated rat mitochondria, SLD sulfide decreased mitochondrial membrane potential, whereas SLD or SLD sulfone showed no effect (Fig. 4). Although a statistically significant effect of SLD sulfide on JC-1 fluorescence was seen only at a large concentration of the drug (1mM), a dose-responsive trend was evident at smaller concentrations that have been shown to produce cytotoxicity (Zou et al, 2009a). Consistent with this result, a previous study using the JC-1 assay also suggested that SLD sulfide may lead to dissipation of mitochondrial membrane potential in HepG2 cells (Leite et al., 2006). Reduced ATP synthesis can be a direct consequence of decreased mitochondrial membrane potential, which might explain why SLD sulfide is more toxic to liver cells compared to SLD or SLD sulfone (Zou et al., 2009a).

SLD sulfide can uncouple mitochondrial oxidative phosphorylation (Leite et al, 2006). Uncouplers can either decrease ROS production or increase ROS production (Han et al, 2009). In our hands, SLD sulfide alone induced ROS generation in HepG2 cells (Fig. 5). However, exposure to SLD by itself failed to increase liver protein carbonyl concentration in vivo; coexposure to LPS was necessary for this effect. A possible explanation for this difference is that the hepatic concentration of SLD sulfide in vivo was insufficient to cause oxidative stress in hepatocytes by itself. Inflammatory mediators induced by SLD/LPS cotreatment might contribute to ROS generation in SLD/LPS model. Hypoxia, which occurs in the livers of rats cotreated with SLD/LPS as a result of hemostasis (Zou et al., 2009b), is a potential contributor to oxidative stress in vivo (Arteel et al., 1999). Moreover, PMNs accumulate and become activated in the livers of rats cotreated with SLD/LPS and contribute to liver injury at least in part by releasing cytotoxic proteases (unpublished results). During PMN activation that results in protease release, NADPH oxidase assembles on the PMN plasma membrane, and ROS are generated (Dahlgren and Karlsson, 1999). Thus, activated PMNs might contribute to ROS generation and consequent oxidative stress in this model.

A previous study revealed that interference with TNF protected against SLD/LPS-induced liver injury in vivo and that TNF potentiated the cytotoxicity of SLD sulfide in HepG2 cells and primary hepatocytes in vitro (Zou et al., 2009a). However, TNF had no effect on ROS generation induced by SLD sulfide in HepG2 cells (Fig. 5), indicating that TNF does not

contribute to cell death by enhancing oxidative stress. On the other hand, the observation that TNF enhanced the cytotoxicity of hydrogen peroxide in HepG2 cells (Fig. 6) suggests the possibility that TNF interacts with ROS produced by SLD sulfide to enhance hepatocyte killing. This is consistent with previous observations that hydrogen peroxide and TNF synergistically kill primary hepatocytes in vitro (Imanishi et al., 1997; Han et al., 2006). These results might explain the synergistic killing by SLD sulfide and TNF; that is, although TNF does not enhance SLD sulfide-induced oxidative stress, it increases the sensitivity of cells to ROS-mediated killing. This is supported by the observation that antioxidant tempol decreased the cytotoxicity of SLD sulfide and significantly reduced the cytotoxic interaction between TNF and SLD (Fig. 7). In vivo, it seems unlikely that SLD sulfide concentrations become great enough to cause ROS-mediated liver injury; rather, TNF production caused by LPS coadministration renders the liver more sensitive to otherwise noninjurious ROS generation. The gene expression results discussed above suggest that compromised antioxidant protective mechanisms could play a role in this heightened sensitivity.

JNK is a common target of TNF and ROS and regulates apoptosis (Kanda and Miura, 2004; Schwabe and Brenner, 2006). TNF-induced JNK activation leads to caspase activation, which in turn leads to liver injury (Wang et al., 2006). Activation of caspase 3/7 was only observed in HepG2 cells cotreated with SLD sulfide and TNF (Fig. 8), whereas TNF or a toxic concentration of SLD sulfide alone had no effect. This indicates that the cytotoxicity of SLD sulfide alone in vitro depended on ROS but not on caspase activation. However, both ROS and caspase activation were critical in the synergistic killing induced by TNF and SLD sulfide cotreatment (Figs. 7 and 9). The observation that tempol reduced effector caspase activation (Fig. 8) suggests that ROS contribute to caspase activation induced by SLD sulfide/TNF interaction.

In summary, this study revealed mechanisms of SLD/LPS-induced liver injury in rats. SLD/LPS cotreatment induces oxidative stress in the livers of rats, whereas neither SLD or LPS alone has an effect. According to the comparison of gene expression in the livers of rats treated with SLD and/or LPS or their vehicles, genes associated with oxidative stress were selectively regulated by SLD/LPS. Antioxidants protected against liver injury induced by SLD/LPS cotreatment, which suggests that oxidative stress is involved in the hepatocellular injury. SLD sulfide exerts its cytotoxicity in vitro by decreasing mitochondrial membrane potential and increasing the production of ROS. The synergistic interaction of SLD sulfide and TNF to kill HepG2 cells is dependent on the oxidative stress induced by SLD sulfide. Under oxidative stress, TNF leads to the activation of caspase 3/7, which contributes to the cytotoxicity of the SLD sulfide/TNF interaction.

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Abbreviations

DMSO	dimethyl sulfoxide
IADRs	idiosyncratic adverse drug reactions
LPS	lipopolysaccharide
NSAID	nonsteroidal anti-inflammatory drug
PMN	polymorphonuclear neutrophil
ROS	reactive oxygen species
SLD	sulindac
TNF	tumor necrosis factor-alpha

Fig. 1. Evaluation of protein carbonyl concentration in liver mitochondria

Rats were treated with two administrations of SLD (50 mg/kg, p.o.) or its vehicle (0.5% methyl cellulose) with a 16 hr interval (n=5). Half an hour before the second administration of SLD, LPS (8.25×10^5 EU/kg, i.v.) or its saline vehicle was administered via a tail vein. Rats were euthanized at 8 hr, and the livers were collected. Liver mitochondria were isolated, and protein carbonyl concentration was determined. *Significantly different from SLD/Veh group. #Significantly different from Veh/LPS group. $P < 0.05$, n=4-8.

Fig. 2. Venn diagram of probe sets regulated by SLD/Veh, Veh/LPS or SLD/LPS

Rats were treated with SLD, LPS or their vehicles as described in Fig. 1. Livers were collected from rats euthanized at 4 hr after the second administration of SLD. RNA was isolated from liver, and gene expression was evaluated as described in Materials and Methods. The numbers of probe sets changed by SLD/Veh, Veh/LPS or SLD/LPS cotreatment were derived using Veh/Veh as baseline. S indicates SLD/Veh treatment, L indicates Veh/LPS treatment, and SL indicates SLD/LPS cotreatment. The intersection (^) of treatment groups represents the gene expression changed after both or all three of the indicated treatments.

Fig. 3. Effect of antioxidants on SLD/LPS-induced liver injury

(A) Ebselen, its vehicle (0.5% methylcellulose) or (B) DMSO was administered to rats treated with SLD/LPS as described in the Methods. Serum ALT activity was evaluated at 12 hr.

*Significantly different from Veh/Veh/Veh or Veh/Veh group. #Significantly different from Veh/SLD/LPS or SLD/LPS group. $P < 0.05$, $n = 3-9$.

Fig. 4. Effect of SLD and its metabolites on mitochondrial membrane potential

Mitochondria were isolated from the livers of untreated rats and incubated with SLD or its metabolites, SLD sulfone or SLD sulfide, for 30 min. Mitochondrial membrane potential was evaluated. *Significantly different from any other group at the same concentration. #Significantly different from vehicle (0 mM) group. $P < 0.05$, $n = 3$.

Fig. 5. Effect of SLD sulfide and TNF on production of reactive oxygen species in HepG2 cells SLD sulfide (250 μ M), TNF (200ng/mL) or vehicle was administered to HepG2 cells alone or in combination. ROS generation at various times (0, 0.5, 1 and 3 hr) after treatment was evaluated using DCF fluorescence. *Significantly different from Veh/Veh group at the same time. $P < 0.05$, $n = 4$.

N=4

Fig. 6. Cytotoxicity induced by hydrogen peroxide and TNF

Hydrogen peroxide was administered to HepG2 cells in the presence of TNF (200ng/mL) or its medium vehicle. After 24 hr incubation, the percentage of LDH released from cells was evaluated as a marker of cell injury. *Significantly different from 0 mM hydrogen peroxide group. #Significantly different from vehicle group at the same concentration of hydrogen peroxide. $P < 0.05$, $n = 4$.

Fig. 7. Effect of antioxidant on cytotoxicity induced by SLD sulfide and TNF

Tempol was administered to HepG2 cells half an hour before the administration of SLD sulfide (250 μ M), TNF (200ng/mL) or their vehicles. The percentage of LDH activity released into the medium was determined after 24 hr. *Significantly different from value for SLD sulfide alone. #Significantly different from value for SLD sulfide/TNF in the absence of tempol. $P < 0.05$, $n = 5$.

Fig. 8. Effect of antioxidant on caspase 3/7 activity

Tempol (200 μ M), Z-VAD (10 μ M) or their medium vehicle was administered to HepG2 cells. Half an hour later, SLD sulfide (250 μ M) and/or TNF (200 ng/mL) was administered. Activity of caspase 3/7 in HepG2 cells was determined after 6 hr incubation. *Significantly different from any other group. $P < 0.05$, $n = 4$.

Fig. 9. Effect of pan-caspase inhibitor on cytotoxicity induced by SLD sulfide and TNF
Z-VAD (10 uM), SLD sulfide (200 uM), TNF (200 ng/mL) or their vehicles were administered to HepG2 cells as described in Fig. 8. The percentage of LDH activity released from HepG2 cells was determined after 24 hr. *Significantly different from corresponding vehicle group. #Significantly different from SLD sulfide/TNF group in the absence of Z-VAD. $P < 0.05$, $n = 3$.

Table 1

Pathways associated with genes specifically changed by SLD/LPS cotreatment.

Toxicity Lists	-Log(P)	Ratio (%)	Genes
Fatty Acid Metabolism	3.27	11.0	ALDH1A1,PEC1,CYP4B1,AUH,ADH1C (includes EG:126),CYP2D6,CYP2J2,ACAD9,ADHFE1,HSD17B4,ACSL1,GCDH
PXR/RXR Activation	2.95	12.7	PRKACB,GSTM2,ALDH1A1,AKT1,PRKACA,IL6,HNF4A,RXR
LPS/IL-1 Mediated Inhibition of RXR Function	2.88	8.5	ALDH1L1,SLCO1A2,MAOB,GSTM2,ALDH1A1,MGST2,EG629219,XPO1,SULT1E1,I,BP,RXR,ACSL1,SULT1B1,MGST3,ALDH6A1
Xenobiotic Metabolism	2.55	9.1	GSTM2,MGST2,CYP4B1,ADH1C (includes EG:126),ALDH1L1,CYP2D6,CYP2J2,ADHFE1,MGST3
Aryl Hydrocarbon Receptor Signaling	2.43	8.6	IL1A,GSTM2,ALDH1A1,MGST2,MAPK1,CDK4,ALDH1L1,NFIB,IL6,RXR,ALDH6A1,MGST3
Hepatic Cholestasis	2.18	8.6	PRKACB,IL1A,NFKB1A,PRKACA,SLCO1A2,LBP,IL6,HNF4A,RXR,CYP8B1,IRAK2
Mechanism of Gene Regulation by Peroxisome Proliferators via PPAR α	1.66	8.60	PRKACB,IL1A,NFKB1A,MAPK1,PDGFA,ME1,RXR,HSD17B4
NFkB Signaling Pathway	1.53	7.6	PRKACB,TLR2,IL1A-AKT1,GHR,NFKB1A,PRKACA,MAP3K8
Cytochrome P450 Panel - Substrate is a Fatty Acid (Human)	1.43	20.0	CYP4B1,CYP2J2
Oxidative Stress	1.28	8.93	GPX3,VCAM1,GSTM2,ME1,IL6
PPAR α /RXR Activation	1.26	6.37	PRKACB,GHR,NFKB1A,MAPK1,PRKAA2,PRKACA,PLCG1,IL6,ADIPOR2,RXR

Expression of 721 genes was changed by SLD/LPS cotreatment but not by treatment with either LPS or SLD alone. These genes were imported into Ingenuity Pathway Analysis. A list of pathways was derived and ranked by p value, which indicates the deviation of observed number of genes for each pathway found in the imported list from the number expected to occur by chance. Ratio indicates the percentage of the number of genes affected in a particular pathway.