

# Sulindac Metabolism and Synergy with Tumor Necrosis Factor- $\alpha$ in a Drug-Inflammation Interaction Model of Idiosyncratic Liver Injury

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## ABSTRACT

Sulindac (SLD) is a nonsteroidal anti-inflammatory drug (NSAID) that has been associated with a greater incidence of idiosyncratic hepatotoxicity in human patients than other NSAIDs. In previous studies, cotreatment of rats with SLD and a modestly inflammatory dose of lipopolysaccharide (LPS) led to liver injury, whereas neither SLD nor LPS alone caused liver damage. In studies presented here, further investigation of this animal model revealed that the concentration of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in plasma was significantly increased by LPS at 1 h, and SLD enhanced this response. Etanercept, a soluble TNF- $\alpha$  receptor, reduced SLD/LPS-induced liver injury, suggesting a role for TNF- $\alpha$ . SLD metabolites in plasma and liver

were determined by LC/MS/MS. Cotreatment with LPS did not increase the concentrations of SLD or its metabolites, excluding the possibility that LPS contributed to liver injury through enhanced exposure to SLD or its metabolites. The cytotoxicities of SLD and its sulfide and sulfone metabolites were compared in primary rat hepatocytes and HepG2 cells; SLD sulfide was more toxic in both types of cells than SLD or SLD sulfone. TNF- $\alpha$  augmented the cytotoxicity of SLD sulfide in primary hepatocytes and HepG2 cells. These results suggest that TNF- $\alpha$  can enhance SLD sulfide-induced hepatotoxicity, thereby contributing to liver injury in SLD/LPS-cotreated rats.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used as analgesics and antipyretics in the United States. However, NSAIDs are notorious for causing gastrointestinal mucosal injury and idiosyncratic hepatotoxicity (O'Connor et al., 2003). Although all NSAIDs have been associated with idiosyncratic liver injury in people, hepatotoxicity is most common in patients taking sulindac (SLD). SLD is metabolized to its active form, SLD sulfide (Duggan et al., 1977), which acts pharmacologically through nonselective inhibition of cyclooxygenases 1 and 2.

Idiosyncratic adverse drug reactions (IDRs) are toxic reactions that occur in a minority of patients during drug therapy. IDRs occur at doses that do not cause toxicity in most people and typically have an inconsistent temporal relationship to exposure. The liver is a common target of IDRs. Several hypotheses have been put forward to explain the basis for IDRs; however, the modes of action are still unclear, in part, because of the lack of animal models. One hypothesis is that inflammatory stress precipitates hepatic IDRs in humans (Roth et al., 2003; Ganey et al., 2004). In concert with this hypothesis, cotreatment of rats with lipopolysaccharide (LPS), which induces modest inflammation, and SLD resulted in liver necrosis, whereas neither LPS nor SLD was hepatotoxic alone (Zou et al., 2009).

In this study, we examined factors that could contribute to the pathogenesis of liver injury in rats cotreated with LPS and SLD. In vivo, SLD can be metabolized either irreversibly

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**ABBREVIATIONS:** NSAID, nonsteroidal anti-inflammatory drug; ALT, alanine aminotransferase; DMSO, dimethyl sulfoxide; IDR, idiosyncratic adverse drug reaction; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; SLD, sulindac; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; UPLC, ultra performance liquid chromatography; LC/MS/MS, liquid chromatography-tandem mass spectrometry; GI, gastrointestinal; NF- $\kappa$ B, nuclear factor  $\kappa$ B; ROS, reactive oxygen species; EU, endotoxin units.

to SLD sulfone or reversibly to SLD sulfide, which is more cytotoxic than SLD itself. Because LPS can regulate drug metabolism (Renton, 2001), we tested whether LPS coexposure enhances bioactivation of SLD. Moreover, we determined the effect of SLD on LPS-induced tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) production and its role in the development of liver injury.

## Materials and Methods

**Materials.** Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). LPS (Lot 075K4038) derived from *Escherichia coli* serotype O55:B5 with an activity of  $3.3 \times 10^6$  endotoxin units (EU)/mg was used in experiments. Etanercept was purchased from Amgen (Thousand Oaks, CA). HepG2/C3A cells for in vitro studies were obtained from American Type Culture Collection (Manassas, VA).

**Animals.** Male, Sprague-Dawley rats (CrI:CD(SD)IGS BR; Charles River Breeding Laboratories, Portage, MI) weighing 250 to 370 g were used for studies in vivo (rats weighing 290 to 300g were used to evaluate SLD and its metabolites in the gastrointestinal (GI) tract and feces), and rats weighing 150 to 200 g were used for primary hepatocyte isolation. Animals were fed standard chow (Rodent Chow/Tek 8640; Harlan Teklad, Madison, WI) and allowed access to spring water. They were allowed to acclimate for 1 week in a 12-h light/dark cycle before use in experiments.

**Experimental Protocol.** As described in previous studies (Zou et al., 2009), rats were given two administrations of SLD (50 mg/kg p.o.) or its vehicle (0.5% methyl cellulose) with a 16-h interval, and food was removed after the first administration. One-half hour before the second administration of SLD, LPS ( $8.25 \times 10^5$  EU/kg i.v.) or its vehicle (saline) was administered via a tail vein. Depending on the purpose of experiments, rats were anesthetized with isoflurane and euthanized at various times (0, 1, 2, 4, 8, and 12 h) after the second administration of SLD. For the collection of plasma, a portion of blood drawn from anesthetized rats was transferred into Vacutainer tubes (BD, Franklin Lakes, NJ) containing sodium citrate (final concentration, 0.38%). The rest of the blood was allowed to clot at room temperature for preparation of serum. Collected plasma and serum were stored at  $-80^\circ\text{C}$  until use. Three slices (3–4 mm thick) of the left lateral liver lobe were collected and fixed in 10% buffered formalin for histological analysis. A portion of the right medial lobe of the liver was flash-frozen in liquid nitrogen for pharmacokinetic study of SLD and its metabolites. For determining drug concentration in the GI tract and feces, each rat was housed in a separate cage after LPS or vehicle injection and euthanized at 2 h. The entire GI tract and its contents were collected. Feces were retrieved from the cages and were homogenized with the GI tract and its contents for each rat. In the TNF- $\alpha$  inhibition study, rats were given etanercept (8 mg/kg) or vehicle (sterile water) subcutaneously an hour before LPS ( $8.25 \times 10^5$  EU/kg i.v.) or its saline vehicle. We have demonstrated that etanercept inactivates TNF- $\alpha$  activity induced by LPS administration in rats using this treatment protocol (Tukov et al., 2007).

**Evaluation of Liver Injury.** The activity of alanine aminotransferase (ALT), a marker of hepatic parenchymal cell injury, was assessed in serum by use of a diagnostic kit from Thermo Fisher Scientific (Waltham, MA). Liver slices fixed in 10% buffered formalin were embedded in paraffin, cut into 6- $\mu\text{m}$  sections, and stained with hematoxylin and eosin for histological evaluation.

**Determination of TNF- $\alpha$  Concentration in Serum.** The concentration of TNF- $\alpha$  in serum collected at 0, 1, 2, 4, and 8 h after the second administration of SLD was measured by enzyme-linked immunosorbent assay (BD Biosciences; San Diego, CA).

**LC/MS/MS Analysis.** Plasma samples, liver homogenates, GI and fecal homogenates or HepG2 cells in culture medium were mixed with acetonitrile containing diclofenac as internal standard. After

vortexing and centrifugation, protein was removed, and the supernatant was diluted and transferred to ultra performance liquid chromatography (UPLC) sample vials for LC/MS/MS analysis.

LC/MS/MS analysis was performed by use of an Acquity UPLC System coupled to a Quattro Premier XE tandem quadrupole mass spectrometer (Waters, Milford, MA). An extract volume of 2  $\mu\text{l}$  was injected into the UPLC system and eluted with a gradient mixture (0–99%) of formic acid and acetonitrile. Electrospray ionization in positive ion mode was performed for analyses of plasma samples, and the collision and source cone voltages were optimized independently for each analyte. Multiple reaction monitoring of the following  $m/z$  transitions was used for the quantitative analysis of diclofenac (296 $\rightarrow$ 214), SLD (357 $\rightarrow$ 333), SLD sulfone (373 $\rightarrow$ 233), SLD sulfide (341 $\rightarrow$ 234), SLD acyl glucuronide (533 $\rightarrow$ 339), SLD sulfone acyl glucuronide (549 $\rightarrow$ 355), and SLD sulfide acyl glucuronide (517 $\rightarrow$ 323).

For samples other than plasma, electrospray ionization was performed in negative ion mode, and metabolite concentrations were determined by the Multiple reaction monitoring of transition of diclofenac (294 $\rightarrow$ 250), SLD (311 $\rightarrow$ 296), SLD sulfone (327 $\rightarrow$ 264), SLD sulfide (295 $\rightarrow$ 280), SLD acyl glucuronide (531 $\rightarrow$ 355), SLD sulfone acyl glucuronide (547 $\rightarrow$ 371), and SLD sulfide acyl glucuronide (515 $\rightarrow$ 339).

The LC/MS/MS method achieved lower limits of quantification of 30 ng/ml or less for all three forms of sulindac (sulfoxide, sulfide, and sulfone) using both positive and negative ion modes. Analytical reproducibility was judged to be  $\pm 12\%$  in the middle of the calibrated range of concentrations.

**Evaluation of Cytotoxicity of SLD and Its Metabolites in Vitro.** HepG2 cells were plated at a density of  $4 \times 10^4$  cells/well in 96-well plates. After overnight incubation in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, medium was renewed and SLD, SLD sulfone, SLD sulfide (0–500  $\mu\text{M}$ ) or vehicle (0.5% dimethyl sulfoxide, DMSO) was added to the wells. After a 24-h incubation, lactate dehydrogenase (LDH) released into the medium and total cellular LDH were evaluated by use of a kit from Promega (Madison, WI). Cytotoxicity was assessed as the percentage of LDH released into the medium relative to the total LDH in the well (medium plus lysed cells).

For primary rat hepatocytes, isolation was performed as described previously (Tukov et al., 2006). In brief, rat liver was first perfused in situ through the portal vein and then digested with Liver Digest Medium (Invitrogen, Carlsbad, CA). The digested liver was combed gently, and hepatocytes were obtained after centrifugation (100g, 30 s).

Hepatocytes were suspended in Williams' Medium E (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum, and the cell viability was always above 80%. Hepatocytes were plated at a density of  $2.5 \times 10^5$  cells/well in 12-well plates and incubated for 3 h to attach to the plate. Serum-containing medium was replaced by serum-free medium, and SLD, SLD sulfone, SLD sulfide (0–120  $\mu\text{M}$ ) or vehicle (0.1% DMSO) was added to the culture wells. After an 8-h incubation, cytotoxicity was assessed by calculating the ALT activity in the medium plus unattached cells as a percentage of the total ALT activity in the well as described previously (Zou et al., 2009).

**Cytotoxicity from TNF- $\alpha$  and SLD Metabolites.** SLD sulfide or its vehicle (0.5% DMSO) was administered to HepG2 cells with recombinant human TNF- $\alpha$  (200 ng/ml) or its vehicle (medium). After 24 h of incubation, the percentage of LDH released was evaluated. To determine the remaining concentration of SLD sulfide in each well, HepG2 cells were scraped, and acetonitrile was added to precipitate protein. After centrifugation, the concentration of SLD sulfide in supernatant was determined by use of LC/MS/MS.

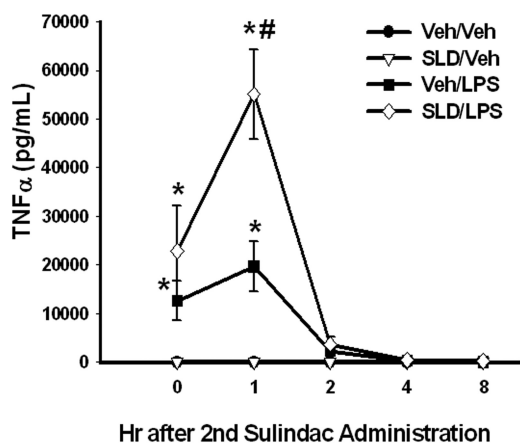
To assess further whether TNF- $\alpha$  can affect the cytotoxicity of SLD metabolites, isolated primary rat hepatocytes were treated with SLD sulfide (60  $\mu\text{M}$ ) in the presence or absence of recombinant rat TNF- $\alpha$  (2  $\mu\text{g}/\text{ml}$ ), and the percentage of ALT released was evaluated 8 h later.

**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 Tukey's test was used as a post hoc test. Student's *t* test was performed when only two groups were compared. For all studies,  $P < 0.05$  was considered as the criterion for statistical significance.

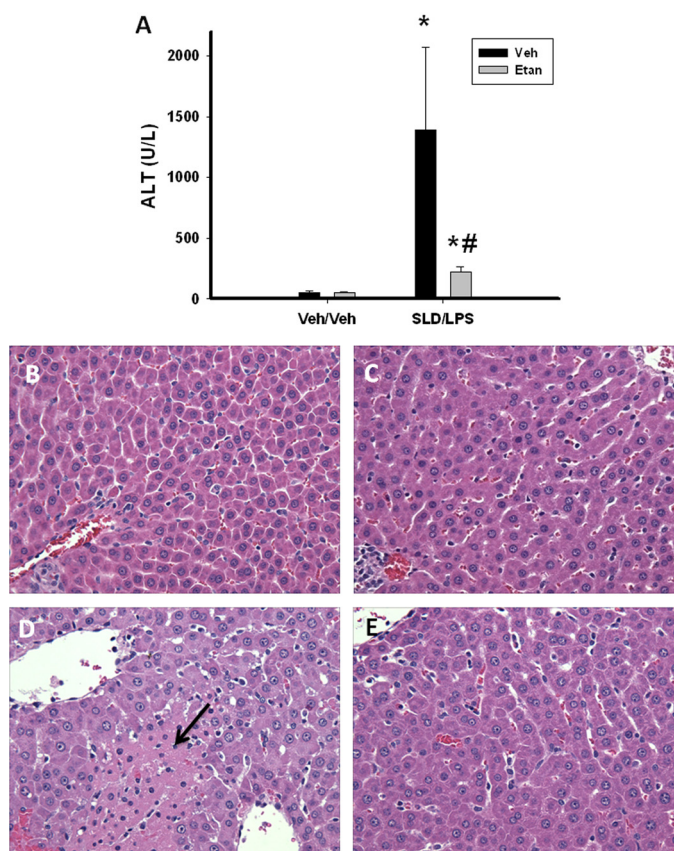
## Results

**Time Course of TNF- $\alpha$  Concentration in Plasma.** Rats were treated with LPS and two administrations of SLD or their vehicles as described in *Materials and Methods*, and TNF- $\alpha$  concentration in serum was evaluated at various times up to 8 h after the second administration of SLD. SLD had no effect on serum TNF- $\alpha$  concentration in rats. LPS alone led to a significant increase in TNF- $\alpha$  serum concentration at 0 and 1 h (i.e., 0.5 and 1.5 h after LPS). The elevation of TNF- $\alpha$  concentration induced by LPS was significantly increased by SLD at 1 h after the second administration of the drug (Fig. 1).

**Effect of TNF- $\alpha$  Inhibition on Liver Injury.** Etanercept is a soluble TNF- $\alpha$  receptor that neutralizes the biological activity of TNF- $\alpha$ . To investigate the role of TNF- $\alpha$  in liver injury, rats were treated with etanercept 1 h before LPS administration. This treatment protocol inhibits the activity of TNF- $\alpha$  in rats (Geier et al., 2003). We have reported previously that neither LPS nor SLD produces liver injury when given alone at the doses used in these studies (Zou et al., 2009). Also consistent with our previous report, SLD/LPS cotreatment increased serum ALT activity significantly (Fig. 2). Etanercept significantly attenuated this increase, whereas etanercept alone had no effect on serum ALT activity. Histological examination of hematoxylin and eosin-stained livers of rats revealed a pattern consistent with the ALT activity. That is, midzonal necrotic foci were present in livers of rats treated with SLD/LPS but were found infrequently in livers of rats treated with etanercept/SLD/LPS.



**Fig. 1.** Time course of TNF- $\alpha$  concentration in rat serum. Rats were treated with two administrations of SLD (50 mg/kg p.o.) or its vehicle (0.5% methyl cellulose) with a 16-h interval. One-half hour before the second administration of SLD, LPS ( $8.25 \times 10^5$  EU/kg i.v.) or its saline vehicle was administered via a tail vein. TNF- $\alpha$  was evaluated by enzyme-linked immunosorbent assay in serum samples obtained from rats at 0, 1, 2, 4, or 8 h after the second administration of SLD. \*, significantly different from Veh/Veh group at the same time. #, significantly different from Veh/LPS group at the same time.  $P < 0.05$ ,  $n = 4$ –5 for all points except the 8-h group ( $n = 3$ ), Veh/LPS and SLD/LPS at 1 h ( $n = 8$  and 9, respectively), and SLD/LPS at 0 h ( $n = 8$ ). Veh, vehicle.



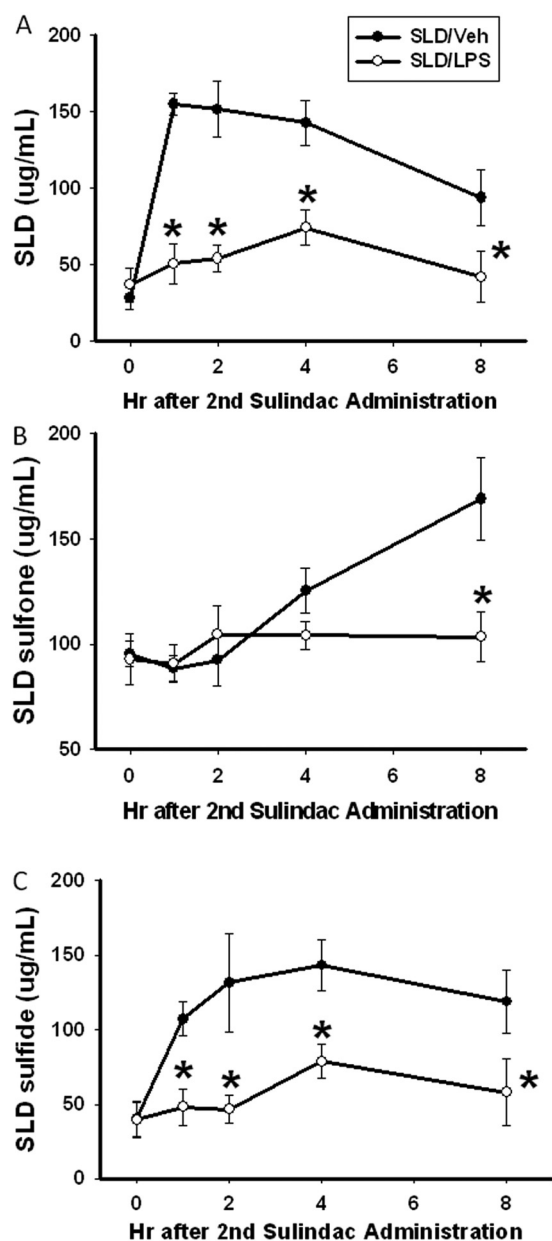
**Fig. 2.** Effect of TNF- $\alpha$  inhibition on liver injury induced by SLD/LPS. Rats were treated with etanercept (8 mg/kg s.c.) or its vehicle 1 h before LPS. SLD and LPS or their vehicles were administered to rats as described in *Materials and Methods*. ALT activity was determined at 12 h (A). \*, significantly different from respective Veh/Veh group. #, significantly different from Veh/SLD/LPS group.  $P < 0.05$ ,  $n = 4$  for all groups except SLD/LPS/Etan ( $n = 6$ ). Liver sections from rats treated with Veh/Veh/Veh (B), Etan/Veh/Veh (C), Veh/SLD/LPS (D), and Etan/SLD/LPS (E) were examined. An arrow indicates a necrotic area. Veh, vehicle; Etan, etanercept.

**Effect of LPS on SLD Metabolism in Rats.** SLD and its sulfone and sulfide metabolites were determined in rat plasma at various times after the second administration of SLD. Plasma SLD concentration reached a peak 1 h after administration and decreased gradually over 8 h (Fig. 3A). In LPS-treated rats, plasma SLD concentration was significantly smaller. SLD treatment increased SLD sulfone concentration in plasma steadily between 2 and 8 h (Fig. 3B). This increase was not observed after SLD/LPS cotreatment, so that the plasma concentration of SLD sulfone was significantly less in SLD/LPS-cotreated rats by 8 h. Plasma SLD sulfide concentration reached a peak within 4 h in both groups, and LPS administration decreased the SLD sulfide concentration in plasma significantly at 1, 2, 4, and 8 h compared with that of SLD/vehicle-treated rats (Fig. 3C).

In livers of rats treated with SLD alone, the concentrations of SLD and its metabolites showed trends similar to those in plasma. LPS cotreatment decreased SLD and SLD sulfide concentrations, but SLD sulfone concentration was unaffected (Fig. 4). LPS selectively lowered the SLD concentration in liver at 1 and 2 h, and decreased SLD sulfide concentration in liver at 2 and 4 h.

To investigate further the effect of LPS on SLD metabolism, rats were euthanized at 2 h, and SLD metabolite

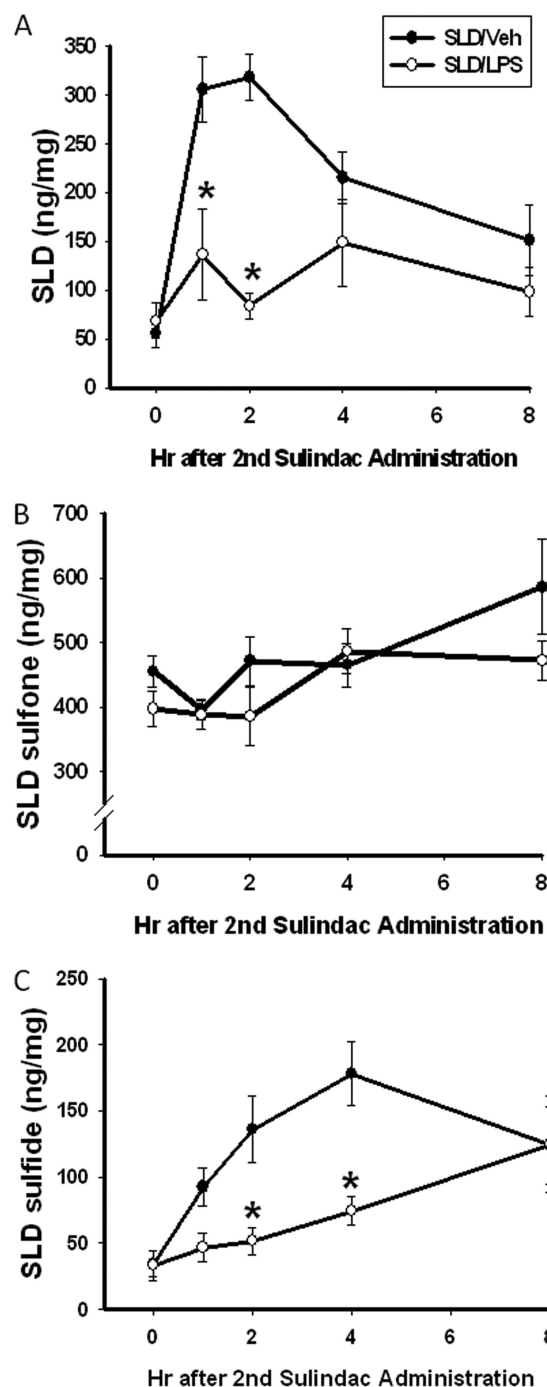




**Fig. 3.** Effect of LPS on plasma concentrations of SLD, SLD sulfone, and SLD sulfide. Rats were treated with SLD and with LPS or its saline vehicle as described in Fig. 1. They were euthanized, and plasma was collected at 0, 1, 2, 4, and 8 h after the second administration of SLD. The plasma concentrations of SLD, SLD sulfone, or SLD sulfide were determined as described in *Materials and Methods*. \*, significantly different from SLD/Veh group at the same time.  $P < 0.05$ ,  $n = 5$  for all groups except SLD/LPS at 4 h ( $n = 7$ ). Veh, vehicle.

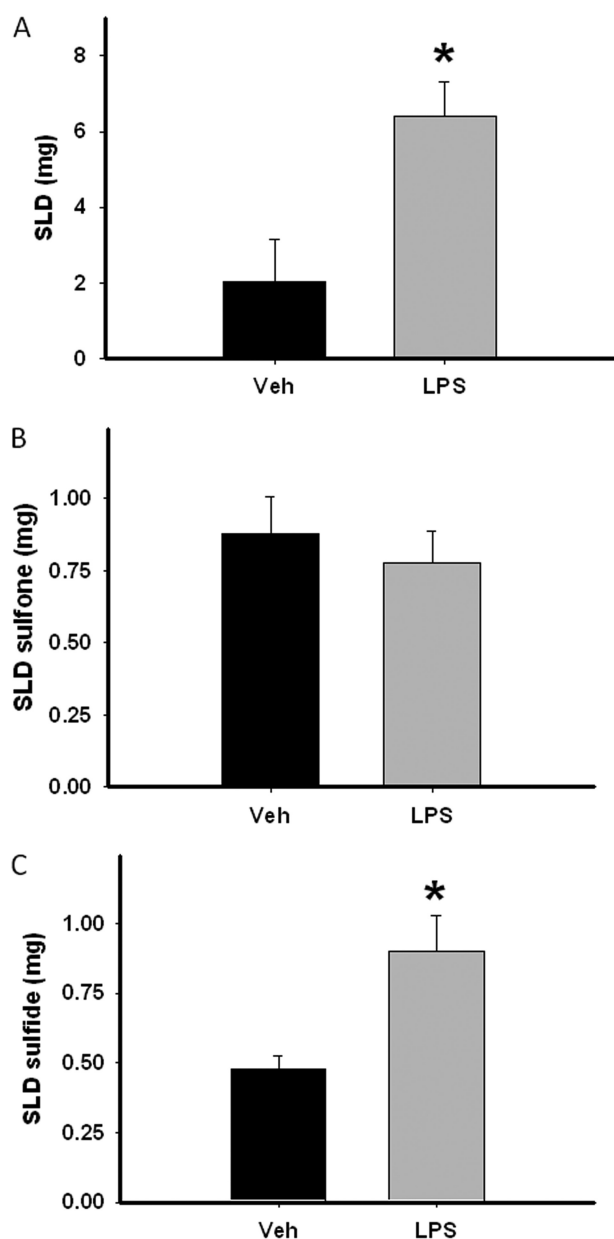
amounts were determined in the GI tract and feces collected between  $-0.5$  and  $2$  h. The amounts of SLD and SLD sulfide in the GI tract and feces were significantly increased by LPS (Fig. 5). However, the amount of SLD sulfone was not affected by LPS. The concentrations of acyl glucuronide conjugates of SLD, SLD sulfone, and SLD sulfide were below the limit of detection in all of the samples measured.

**Effect of Etanercept on SLD Metabolism in Rats.** SLD and its sulfone and sulfide metabolites were determined in rat plasma at 8 h after the second administration of SLD. Etanercept had no effect on SLD metabolite concentrations in plasma of rats cotreated with SLD/LPS (Table 1).



**Fig. 4.** Effect of LPS on liver concentrations of SLD, SLD sulfone, and SLD sulfide. Rats were treated with SLD and with either LPS or its saline vehicle as described in Fig. 1. Liver concentrations of SLD, SLD sulfone, and SLD sulfide were determined at 0, 1, 2, 4, and 8 h after the second administration of SLD. \*, significantly different from SLD/Veh group at the same time.  $P < 0.05$ ,  $n = 5$  for all groups except SLD/LPS at 4 h ( $n = 8$ ). Veh, vehicle.

**Cytotoxicity of SLD and Its Metabolites in HepG2 cells and Rat Primary Hepatocytes.** Neither SLD nor SLD sulfone at concentrations up to  $500 \mu\text{M}$  led to an increase in released LDH when applied to HepG2 cells (Fig. 6A). In contrast, SLD sulfide induced significant LDH release at concentrations greater than  $125 \mu\text{M}$ . In rat primary hepatocytes, SLD and SLD sulfone also produced no cytotoxicity at the concentrations examined (Fig. 6B), but SLD sulfide



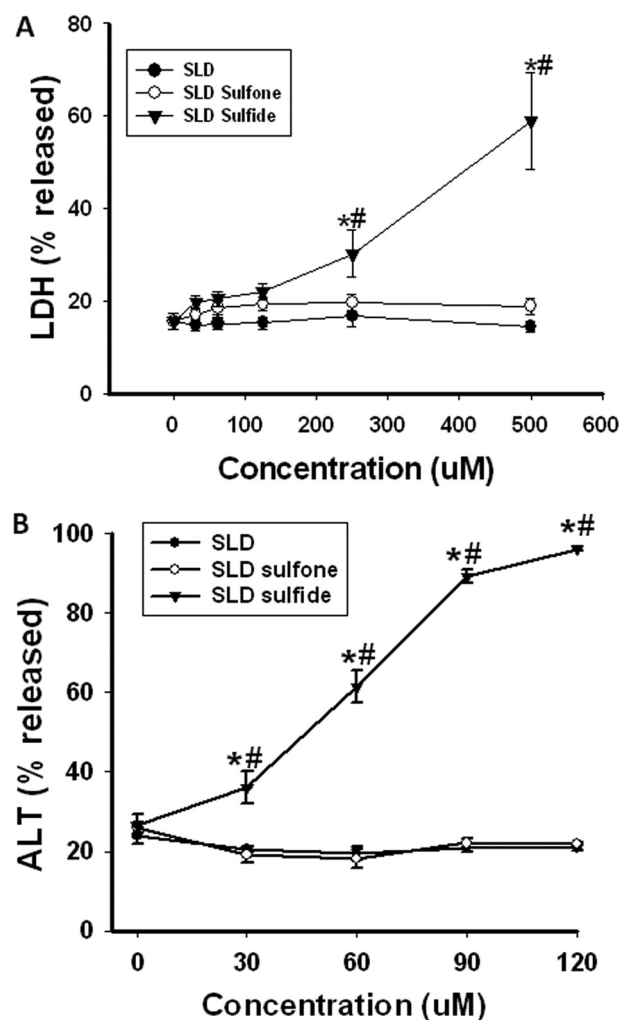
**Fig. 5.** The amounts of SLD, SLD sulfone, and SLD sulfide in GI tract and feces. Rats were treated with SLD and with either LPS or its saline vehicle as described in Fig. 1. Each rat was housed in a different cage after the LPS injection. Two hours after the second administration of SLD, feces in the cage and the whole GI tract and its contents were collected for each rat. The mixture was homogenized with acetonitrile, and the amounts of SLD, SLD sulfone, and SLD sulfide were determined by LC/MS/MS. \*, significantly different from SLD/Veh group.  $P < 0.05$ ,  $n = 4$ . Veh, vehicle.

**TABLE 1**  
Effect of etanercept on SLD metabolism

Rats were killed and plasma was collected at 8 h after the second SLD administration. Metabolite concentrations were determined as described under *Materials and Methods*.  $n = 4-5$ .

Treatment	Concentration in Plasma		
	SLD	SLD Sulfone	SLD Sulfide
	$\mu\text{g/ml}$		
Veh/SLD/LPS	$59.6 \pm 12.2$	$139.3 \pm 16.7$	$62.2 \pm 12.1$
Etan/SLD/LPS	$52.8 \pm 10.8$	$153.8 \pm 13.8$	$39.8 \pm 13.3$

Veh, vehicle; Etan, etanercept.

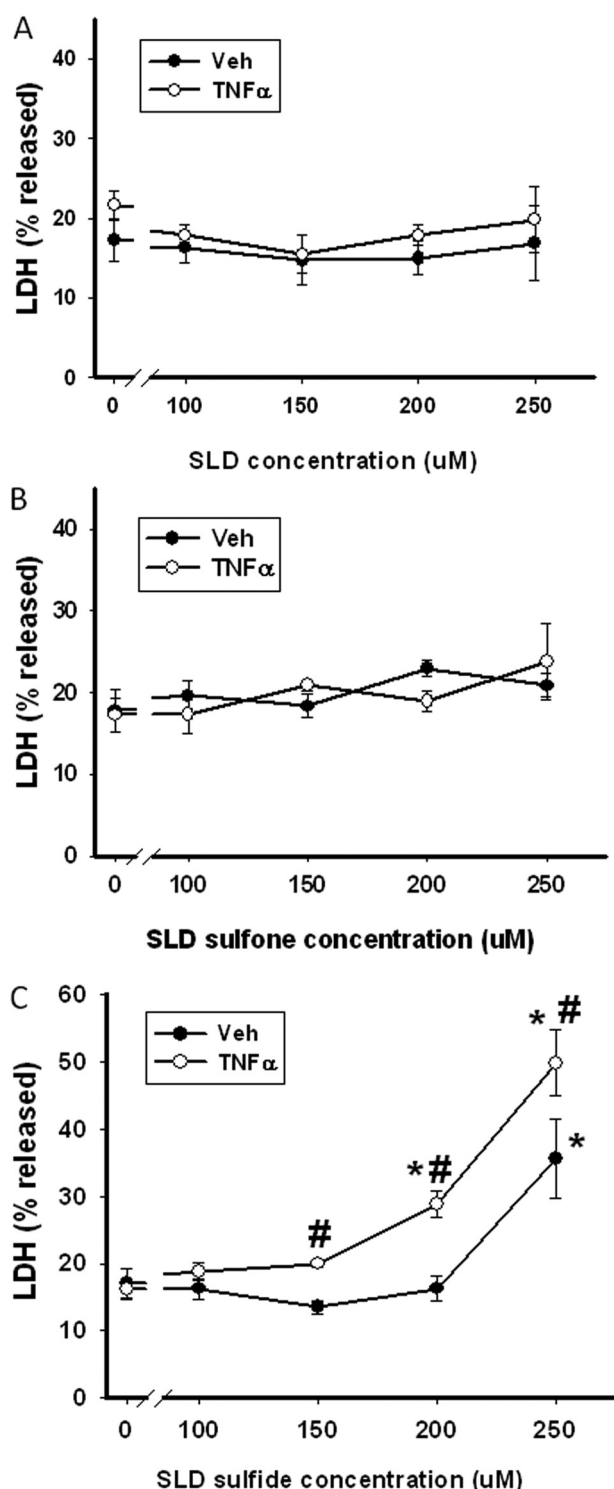


**Fig. 6.** Evaluation of cytotoxicity induced by SLD, SLD sulfone, or SLD sulfide. A, SLD, SLD sulfone, or SLD sulfide was administered at various concentrations to HepG2 cells. The percentage of LDH released into the medium after 24 h was determined as a marker of cytotoxicity. B, rat primary hepatocytes were treated with SLD, SLD sulfone, or SLD sulfide for 8 h, and the percentage of ALT activity released into medium was determined as described in *Materials and Methods*. \*, significantly different from vehicle (0 concentration). #, significantly different from SLD or SLD sulfone at the same concentration.  $P < 0.05$ ,  $n = 3$ .

caused cell death at concentrations as small as 30  $\mu\text{M}$ . The cytotoxicity of SLD sulfide was concentration-dependent, and 120  $\mu\text{M}$  SLD sulfide killed almost all of the hepatocytes.

**Effect of TNF- $\alpha$  on Cytotoxicity of SLD and Its Metabolites in HepG2 and Rat Primary Hepatocytes.** TNF- $\alpha$  alone did not affect the release of LDH from HepG2 cells (Fig. 7). Neither SLD nor SLD sulfone was cytotoxic in the presence or absence of TNF- $\alpha$ . Smaller concentrations (150, 200  $\mu\text{M}$ ) of SLD sulfide, which were not cytotoxic alone, induced cell death in the presence of TNF- $\alpha$ . TNF- $\alpha$  also enhanced the cytotoxicity of a larger concentration of SLD sulfide (250  $\mu\text{M}$ ).

The conversion of SLD to SLD sulfide is a reversible reaction (Duggan, 1981). To evaluate whether TNF- $\alpha$  affects metabolism of SLD sulfide in HepG2 cells, the amount of SLD sulfide in medium plus HepG2 cells was measured 24 h after SLD sulfide application to the cells. This amount in wells with TNF- $\alpha$  ( $5.4 \pm 0.2 \mu\text{g}$ ) was not significantly different from wells that received vehicle ( $5.3 \pm 0.1 \mu\text{g}$ ).



**Fig. 7.** Cytotoxicity induced by TNF- $\alpha$  and SLD or its metabolites. HepG2 cells were treated with SLD (A), SLD sulfone (B), or SLD sulfide (C) in the presence or absence of TNF- $\alpha$  (200 ng/ml). The percentage of LDH released was determined after 24 h as described in *Materials and Methods*. \*, significantly different from vehicle (0 concentration). #, significantly different from value in the absence of TNF- $\alpha$  at the same concentration of SLD or metabolite.  $P < 0.05$ ,  $n = 3$ . Veh, vehicle.

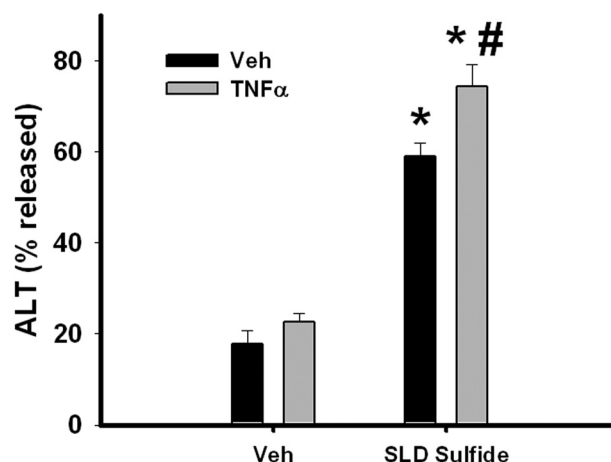
A potentiating effect of TNF- $\alpha$  was also observed on the cytotoxicity of SLD sulfide in primary hepatocytes. TNF- $\alpha$  alone did not affect ALT activity released into the culture medium compared with vehicle treatment. SLD sulfide alone

caused significant release of ALT activity into the medium (Fig. 8). When hepatocytes were treated with SLD sulfide and TNF- $\alpha$  together, TNF- $\alpha$  significantly enhanced the cell injury induced by SLD sulfide.

## Discussion

As reported previously (Zou et al., 2009), SLD/LPS cotreatment induced severe liver injury in rats. Proinflammatory cytokines, especially TNF- $\alpha$ , have proved to play a critical role in other drug/LPS-induced liver injury models (Shaw et al., 2007; Tukov et al., 2007). Moreover, studies suggest that reactive drug metabolites produced in liver are critical for idiosyncratic hepatotoxicity from some drugs (Kaplowitz, 2005). Therefore, we focused in this study on the roles of TNF- $\alpha$  and the toxic metabolite of SLD and their interaction in SLD/LPS-induced liver injury.

The concentration of TNF- $\alpha$  in serum was elevated in rats after exposure to LPS, and SLD significantly enhanced the LPS-mediated increase in TNF- $\alpha$  as early as 1 h. Besides SLD, other drugs associated with idiosyncratic hepatotoxicity in humans, such as ranitidine and trovafloxacin, also had a synergistic effect on the LPS-mediated increase in TNF- $\alpha$  in rodents (Shaw et al., 2007; Tukov et al., 2007). Sulindac and other NSAIDs enhanced TNF- $\alpha$  release from LPS-pretreated, macrophage-derived RAW264.7 cells at concentrations achieved clinically in humans (Cho, 2007). These findings suggest that enhancement of serum TNF- $\alpha$  concentration might be a common characteristic of drugs that induce idiosyncratic liver injury. The source of TNF- $\alpha$  and the mechanism by which SLD enhances TNF- $\alpha$  appearance are unknown. After LPS exposure, the increase in plasma TNF- $\alpha$  concentration is mirrored by elevated liver concentration (Fernández-Martínez et al., 2004). Therefore, the source of TNF- $\alpha$  after LPS exposure is probably liver. However, the source of enhanced TNF- $\alpha$  in serum after SLD cotreatment is not known. TNF- $\alpha$ -converting enzyme, which is required for release of biologically active TNF- $\alpha$ , is a possible contributor, because some NSAIDs can enhance the activity of this enzyme (Gómez-Gaviro et al., 2002). It is also possible that SLD



**Fig. 8.** Effect of TNF- $\alpha$  on SLD sulfide-induced injury to rat primary hepatocytes. SLD sulfide (60  $\mu$ M) and/or TNF- $\alpha$  (2  $\mu$ g/ml) was administered to rat primary hepatocytes. The percentage of ALT released was determined as described in *Materials and Methods*. \*, significantly different from corresponding vehicle group. #, significantly different from SLD sulfide alone group.  $P < 0.05$ ,  $n = 3$ . Veh, vehicle.

or its metabolites enhances TNF- $\alpha$  transcription or translation or interferes with TNF- $\alpha$  clearance.

The importance of TNF- $\alpha$  in SLD/LPS hepatotoxicity was explored by pretreating rats with etanercept, a soluble receptor that neutralizes TNF- $\alpha$ . TNF- $\alpha$  inhibition protected against SLD/LPS-induced liver injury, suggesting a critical role for TNF- $\alpha$  in this model. However, elevation in TNF- $\alpha$  concentration alone is not sufficient to cause liver damage, because much larger TNF- $\alpha$  concentrations have failed to induce liver injury (Deng et al., 2008). Thus, additional factors are probably involved in liver toxicity in SLD/LPS-cotreated rats.

The requirement for bioactivation of SLD raises the possibility that LPS treatment leads to liver injury in SLD-treated rats by increasing the conversion of SLD to a toxic metabolite. To study the effect of LPS on SLD metabolism, we examined the concentration of SLD and its metabolites in plasma, liver, and GI tract plus feces. According to previous studies, two enzymes are responsible for SLD metabolism; methionine sulfoxide reductase in both liver and gut flora reduces SLD to SLD sulfide, and a flavin-containing monooxygenase converts SLD to SLD sulfone and also catalyzes the conversion of SLD sulfide to SLD. SLD was maximally absorbed in 1 h, and SLD and its sulfone metabolite accumulated in liver, a result consistent with previous findings (Dugan et al., 1980). LPS can significantly down-regulate the expression of hepatic flavin-containing monooxygenase in mice (Zhang et al., 2008). Oxidative stress, a possible consequence of LPS exposure, can increase the expression of methionine sulfoxide reductase in bacteria (Vattanaviboon et al., 2005). Therefore, LPS might have an effect on shifting the metabolism of SLD toward SLD sulfide by regulating the expression of these two enzymes. However, LPS decreased the concentrations of SLD and SLD sulfide in plasma after the second administration of SLD. The liver concentrations of SLD and SLD sulfide were also decreased by LPS at 1 and 2 h and 2 and 4 h, respectively. These results suggested that LPS might decrease absorption of SLD from the GI tract. To address this possibility, we measured metabolite concentrations in the GI tract and feces at 2 h, a time at which we found a significant decrease in both SLD and SLD sulfide in plasma and liver after LPS exposure (Fig. 5). LPS increased the concentration of SLD in the GI tract and feces, suggesting that LPS decreased the bioavailability of SLD by reducing its absorption. This result does not rule out the possibility that LPS has an effect on the expression of enzymes that metabolize SLD. Moreover, the SLD metabolite concentrations in the plasma of cotreated rats were not changed at 8 h by etanercept pretreatment, suggesting that TNF- $\alpha$  does not play a role in the ability of LPS to reduce SLD absorption.

The cytotoxicity of SLD and its metabolites was compared in both HepG2 cells and primary rat hepatocytes. SLD and SLD sulfone were not toxic to HepG2 cells even up to 500  $\mu$ M, yet SLD sulfide showed significant toxicity. This result is consistent with previous findings, although a different medium was used and a different cytotoxicity assay was performed (Leite et al., 2006). It also has been widely reported that SLD sulfide can induce apoptosis of other cancer cell lines (Kim et al., 2005; Bock et al., 2007), which raised interest in treating cancer with SLD. However, in this study, we found that the active metabolite of SLD was also cytotoxic to primary hepatocytes and that primary rat hepatocytes

were more sensitive than HepG2 cells (Fig. 6). This might have implications for the use of SLD as an anticancer agent if normal host cells are more sensitive to the cytotoxic effects of SLD than are cancer cells.

Although the mechanisms of drug-induced idiosyncratic liver injury are still not clear, it is believed that accumulation of active metabolites in liver is an essential first step for many drugs (Watkins, 2005). Accordingly, excessive SLD sulfide in liver might be critical for SLD-induced idiosyncratic liver injury. This might be why two administrations of SLD were required in this model to effect hepatotoxicity. LPS decreased the concentration of SLD sulfide in the livers of rats, suggesting that SLD sulfide accumulation alone was not sufficient to induce liver injury, and that LPS might be activating pathways that enhance the toxicity of SLD sulfide, instead of increasing the concentration of SLD toxic metabolite.

Because TNF- $\alpha$  and SLD or its metabolites are both indispensable for the development of SLD/LPS-induced liver injury, we explored whether TNF- $\alpha$  acted synergistically with SLD or its metabolites using an *in vitro* system. Both HepG2 and primary rat hepatocytes were resistant to TNF- $\alpha$  toxicity. Even a much greater concentration of TNF- $\alpha$  than we used failed to kill HepG2 cells and primary rat hepatocytes (Adamson and Billings, 1992). SLD or SLD sulfone in combination with TNF- $\alpha$  was not cytotoxic; in contrast, this cytokine enhanced the toxicity of SLD sulfide to both cell types. There is evidence that SLD and TNF- $\alpha$  act synergistically to kill tumor cells in mice, which raised the possibility of using this combination of agents as a new anticancer therapy (Yasui et al., 2003). However, our results suggest that this therapy might also increase the chance of liver injury. The mechanism of SLD sulfide and TNF- $\alpha$  interaction is under investigation. TNF- $\alpha$  can lead either to hepatocyte proliferation through nuclear factor  $\kappa$ B (NF- $\kappa$ B) activation or to activation of cell death signaling (Wullaert et al., 2007). SLD, and particularly SLD sulfide, are potent inhibitors of the NF- $\kappa$ B pathway through inhibition of I $\kappa$  kinase activity (Yamamoto et al., 1999). It was reported that NF- $\kappa$ B plays an essential role in preventing TNF- $\alpha$ -induced cell death (Beg and Baltimore, 1996). As a result, it is possible that SLD sulfide sensitizes hepatocytes to TNF- $\alpha$ -induced cell death through inhibition of NF- $\kappa$ B prosurvival signaling. Moreover, SLD sulfide and TNF- $\alpha$  share a common toxic effect, which may add to enhance cell death. It has been reported that SLD sulfide can induce reactive oxygen species (ROS) *in vitro* (Sun et al., 2009) and lead to mitochondrial uncoupling (Leite et al., 2006). TNF- $\alpha$  can also cause the production of ROS (Schwabe and Brenner, 2006) and mitochondrial injury (Bradham et al., 1998). Therefore, ROS and mitochondria are two potential targets of interaction of SLD sulfide and TNF- $\alpha$ .

SLD sulfide and TNF- $\alpha$  are not the only mediators that contribute to hepatotoxicity in this model. Previously, we found that liver hypoxia is induced through the activation of the hemostatic system in SLD/LPS-cotreated rats and that inhibition of coagulation protects from liver damage (Zou et al., 2009). Hypoxia might contribute to liver injury through synergistic interplay with SLD sulfide. Furthermore, we cannot exclude the possible roles of other mediators. For example, proteases released from neutrophils are important in other drug/LPS models (Luyendyk et al., 2005; Deng et al., 2007).



The proinflammatory cytokine, interferon- $\gamma$ , has been shown to exacerbate TNF- $\alpha$ -induced cytotoxicity in hepatocytes (Adamson and Billings, 1993). These mediators might interact with SLD sulfide, TNF- $\alpha$ , and/or hypoxia to promote liver injury.

In summary, SLD and LPS interact to produce liver injury in rats. The LPS-stimulated increase in the concentration of TNF- $\alpha$  in rat serum was enhanced by SLD, and this cytokine plays a critical role in the pathogenesis. SLD sulfide was more toxic than SLD or SLD sulfone in vitro. Although LPS cotreatment reduced the bioavailability of SLD and the production of toxic SLD sulfide, the synergy of this toxic metabolite with TNF- $\alpha$  was sufficient to cause liver injury in rats. Such synergistic interactions might be a trigger for idiosyncratic liver injury from SLD in humans.

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