

Amiodarone Exposure During Modest Inflammation Induces Idiosyncrasy-like Liver Injury in Rats: Role of Tumor Necrosis Factor-alpha

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Amiodarone [2-butyl-3-(3',5'-diiodo-4' α -diethylaminoethoxybenzoyl)-benzofuran] (AMD), a class III antiarrhythmic drug, is known to cause idiosyncratic hepatotoxic reactions in human patients. One hypothesis for the etiology of idiosyncratic adverse drug reactions is that a concurrent inflammatory stress results in decreased threshold for drug toxicity. To explore this hypothesis in an animal model, male Sprague-Dawley rats were treated with nonhepatotoxic doses of AMD or its vehicle and with saline vehicle or lipopolysaccharide (LPS) to induce low-level inflammation. Elevated alanine aminotransferase (ALT), aspartate aminotransferase, alkaline phosphatase, and gamma-glutamyltransferase activities as well as increased total bile acid concentrations in serum and midzonal hepatocellular necrosis were observed only in AMD/LPS-cotreated rats. The time interval between AMD and LPS administration was critical: AMD injected 16 h before LPS led to liver injury, whereas AMD injected 2–12 h before LPS failed to cause this response. The increase in ALT activity in AMD/LPS cotreatment showed a clear dose-response relationship with AMD as well as LPS. The metabolism and hepatic accumulation of AMD were not affected by LPS coexposure. Serum concentration of tumor necrosis factor-alpha (TNF) was significantly increased by LPS and was slightly prolonged by AMD. In Hepac1c7 cells, addition of TNF potentiated the cytotoxicity of both AMD and its primary metabolite, mono-N-desethylamiodarone. *In vivo* inhibition of TNF signaling by etanercept attenuated the AMD/LPS-induced liver injury in rats. In summary, AMD treatment during modest inflammation induced severe hepatotoxicity in rats, and TNF contributed to the induction of liver injury in this animal model of idiosyncratic AMD-induced liver injury.

Key Words: amiodarone hepatotoxicity; inflammation; lipopolysaccharide; idiosyncratic adverse drug reactions; drug metabolism; tumor necrosis factor-alpha.

pharmacological target of the drug. They present a serious human health problem and are usually not predicted by current preclinical safety evaluation during drug development. During the period 1975–2000, 10% of newly approved drugs were withdrawn from the U.S. market or received black box warnings due to these adverse reactions (Roth *et al.*, 2003; Utrecht, 2007).

The mechanisms by which IADRs occur are not clear. Evidence from experimental animals indicates that mild inflammation can decrease the threshold for toxicity and thereby render an individual susceptible to an adverse drug reaction that would not otherwise occur (Roth *et al.*, 2003). Lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, is widely used as an inflammagen in these animal studies. A nonhepatotoxic dose of LPS can interact with nontoxic doses of several IADR-associated drugs from different pharmacologic classes to induce liver damage in rodents (Deng *et al.*, 2006; Luyendyk *et al.*, 2003; Waring *et al.*, 2006; Zou *et al.*, 2009b).

Amiodarone [2-butyl-3-(3',5'-diiodo-4' α -diethylaminoethoxybenzoyl)-benzofuran] (AMD), a class III antiarrhythmic drug, is effective in increasing the survival of patients after myocardial infarction or congestive heart failure (Singh, 1996). Since the approval of AMD by the U.S. Food and Drug Administration in 1985, the use of this drug has been associated with a variety of adverse effects, including liver dysfunction, pulmonary complications, thyroid dysfunctions, and ocular disturbance (Rotmensch *et al.*, 1984). The reported frequency of liver abnormalities in patients receiving AMD varies from 14 to 82% (Lewis *et al.*, 1989). Most of these reactions are mild, with serum transaminase elevation within threefold of the upper limit of normal (ULN), but some are more severe (Babatin *et al.*, 2008). Cases of liver reactions after intravenous administration of AMD are rare, but damage can be acute and marked (Rätz Bravo *et al.*, 2005). Fulminant hepatic failure or death associated with AMD hepatotoxicity has also been reported (Babatin *et al.*, 2008).

Idiosyncratic adverse drug reactions (IADRs) typically occur only in a small fraction of patients who are treated with certain drugs at therapeutic doses. IADRs are usually unrelated to the

There is evidence that the interaction between LPS-induced cytokines and drugs or their metabolites plays an important role in the LPS-drug interaction (Zou *et al.*, 2009a). Tumor necrosis factor- α (TNF) is a proximal mediator of the inflammatory cascade induced by LPS (Beutler and Kruys, 1995) and is critically involved in many models of liver injury, such as ischemia/reperfusion (Teoh *et al.*, 2004), alcoholic liver disease (Yin *et al.*, 1999) and some drug/LPS-induced liver injury models (Shaw *et al.*, 2009b; Tukov *et al.*, 2007; Zou *et al.*, 2009a). As an example, TNF selectively augmented the cytotoxicity of sulindac sulfide, which is the major toxic metabolite of sulindac (Zou *et al.*, 2009a). In the case of amiodarone, the major metabolite of AMD is mono-N-desethylamiodarone (DEA), which shares similar pharmacological (Talajic *et al.*, 1987) and pharmacokinetic (Shayeganpour *et al.*, 2008) characteristics with AMD. DEA has antiarrhythmic properties, a very long half-life, and accumulates in the liver and many other tissues. In primary hepatocytes, HepG2 cells and other cell types, DEA is much more cytotoxic than AMD (Waldhauser *et al.*, 2006). The plasma concentration of DEA is greater in cases of AMD-associated IADRs (O'Sullivan *et al.*, 1995), suggesting a possible role for this metabolite in AMD toxicity.

The purpose of this study was to test the hypothesis that inflammatory stress induced by LPS potentiates amiodarone-induced hepatotoxicity in rats. When the results demonstrated a hepatotoxic interaction between inflammatory stress and amiodarone, the roles of metabolism and TNF were explored.

MATERIALS AND METHODS

Materials. Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (St Louis, MO). The activity of LPS (Lot 075K4038, derived from *Escherichia coli* serotype O55:B5) was 3.3×10^6 endotoxin units (EU)/mg, which was determined by a Limulus Amebocyte Lysate Kinetic-QCL kit from Cambrex Corp. (Kit 50-650U; East Rutherford, NJ). The reagents for the measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and gamma-glutamyltransferase (GGT) activities were purchased from Thermo Electron Corp. (Waltham, MA). The kit for total bile acids measurement was purchased from Diazyme Laboratories (Poway, CA).

Animals. Male Sprague-Dawley rats (CrI:CD(SD)IGS BR; Charles River, Portage, MI) weighing 250–370 g were used for *in vivo* studies. They were fed standard chow (Rodent Chow/Tek 8640; Harlan Teklad, Madison, WI) and allowed access to water ad libitum. Animals were allowed to acclimate for 1 week in a 12-h light/dark cycle prior to experiments. They received humane care according to the criteria in the Guide for the Care and Use of Laboratory Animals.

Experimental protocol. In all the experiments, rats were fasted for 12 h before administration of LPS and food was returned thereafter. A 20 mg/ml solution of AMD was made in its vehicle (0.18% Tween 80), and 4.1×10^5 EU/ml solution of LPS was made in sterile saline. To determine the optimal time interval between AMD and LPS treatments, rats were treated with AMD (300 mg/kg, ip) 2 h, 8 h, 12 h, 16 h, or 20 h before LPS (1.6×10^6 EU/kg, iv). For the evaluation of the dose-response relationship for AMD, rats were treated with AMD (0–400 mg/kg, ip) and 16 h later with LPS (1.6×10^6 EU/kg, iv) or saline. For the evaluation of the dose-response relationship for LPS, rats were

treated with AMD (400 mg/kg, ip) or vehicle and 16 h later with LPS (0– 1.6×10^6 EU/kg, iv). In subsequent studies, rats were treated with AMD (400 mg/kg, ip) or vehicle and 16 h later with LPS (1.6×10^6 EU/kg, iv) or saline. In the etanercept treatment study, rats were treated with etanercept (8 mg/kg) or sterile water by sc injection 1 h before LPS.

Rats were anesthetized with isoflurane, and blood and liver samples were taken. Serum was prepared from blood, and plasma was prepared from blood collected into a syringe containing 3.2% sodium citrate (BD Biosciences, San Diego, CA). The right medial lobe of the liver was rapidly frozen for immunohistochemistry, and the left lateral lobe of liver was fixed in 10% neutral-buffered formalin and stored in 70% ethanol for histopathology.

Evaluation of liver injury. Liver injury was estimated from the serum activities of ALT, AST, ALP, and GGT and from the serum concentration of total bile acids.

Formalin-fixed liver samples were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) staining. The stained liver sections were examined using light microscopy.

Drug and metabolite analysis. Serum samples and liver homogenates were mixed with acetonitrile containing ethopropazine as internal standard (IS). After vortexing and centrifugation, protein was removed, and the supernatant was diluted and transferred to autosampler vials for liquid chromatography-tandem mass spectrometry (LC/MS/MS) analysis. LC/MS/MS analysis was performed by use of a Shimadzu LC-20 high performance liquid chromatography (HPLC) system coupled to a QTRAP 3200 tandem quadrupole mass spectrometer (AB SCIEX, Foster City, CA) operated under control of Analyst v. 1.4.2 software. The Ascentis Express C18 HPLC Column (5 cm \times 2.1 mm, 2.7 μ m) was maintained at 50°C. A volume of 2 μ l was injected into the HPLC system and eluted with a gradient based on 10mM ammonium acetate in H₂O (solvent A) and methanol (solvent B): 0–0.5 min, 10% solvent B; 0.5–1 min, 10–98% solvent B; 1–4 min, 98% solvent B; 4–6 min, 10% solvent B; flow rate, 0.3 ml/min. Positive mode electrospray ionization was used for all analyses. Mass spectrometry parameters, including declustering potential and collision energy, were optimized independently for each analyte and IS. Multiple reactions monitoring the *m/z* transitions were used for the quantitative analysis of AMD (*m/z* 646.1 \rightarrow 201.1), DEA (*m/z* 618.1 \rightarrow 547.0), and ethopropazine (*m/z* 313.1 \rightarrow 114.1). The LC/MS/MS method achieved lower limits of quantification: ≤ 50 ng/ml for AMD and ≤ 5 ng/ml for DEA. Analytical reproducibility was judged to be $\pm 10.2\%$ in the middle of the calibrated range of concentrations. The Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL) was used to determine protein concentration in the liver homogenates.

Serum TNF concentration. TNF concentration in serum was measured with an ELISA kit (BD Biosciences).

Assessment of cytotoxicity in vitro. The murine hepatoma cell line Hepa1c1c7 purchased from American Type Culture Collection (Manassas, VA) was used to assess cytotoxicity *in vitro*. Hepa1c1c7 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) with 1% antibiotic-antimycotic (Invitrogen) and 10% heat-inactivated fetal bovine serum (SAFC Biosciences, Lenexa, KS) in 75-cm² tissue culture flasks at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Cells were plated in 96-well plates at 15,000 cells per well and allowed to attach for 8 h before medium was replaced. Various concentrations of AMD, DEA, and/or TNF were added to designated wells, and cells were incubated under maintenance conditions. Twenty-four hours later, lactate dehydrogenase (LDH) activity released into the culture medium was measured using the Cytotox-One Homogeneous Membrane Integrity Assay (Promega, Madison, WI). The percent LDH release was calculated as LDH in supernatant/(LDH in supernatant + LDH in cell lysate). Lysate LDH was determined after addition of Triton to lyse the cells.

Statistical analysis. The results are expressed as means \pm SEM. One-way or two-way ANOVA was applied as appropriate; Tukey's method was employed as a *post hoc* test. Grubb's test was used to detect outliers. At least three biological repetitions were performed for each experiment. The *p* value < 0.05 was set as the criterion for statistical significance.

RESULTS

The Time Interval Between AMD and LPS Administration is Important for the Production of Liver Injury

Serum ALT activity did not increase from treatment with either LPS or AMD alone (Fig. 1). In the AMD/LPS group, administration of AMD at 16 or 20 h before LPS resulted in significant serum ALT activity increase, whereas AMD injected 2–12 h before LPS failed to cause this response. The 16-h interval between AMD and LPS treatments was selected for future studies.

Dose-Response Relationships and Time Course of Liver Injury

Neither AMD alone nor LPS alone affected ALT activity at any of the doses tested. In rats cotreated with AMD and LPS, serum ALT activity was dependent on both AMD (Fig. 2A) and LPS (Fig. 2B) doses. Significant increases in ALT activity were observed with AMD doses ≥ 300 mg/kg ($p < 0.05$) and with LPS doses $\geq 1.2 \times 10^6$ EU/kg ($p < 0.05$). 400 mg/kg and 1.6×10^6 EU/kg were selected as AMD and LPS doses for subsequent studies, respectively.

In the time course study, the serum activities of both ALT and AST were measured as markers for hepatocellular injury (Figs. 3A and B). Neither AMD nor LPS alone affected serum ALT activities at any time examined. For the AST activity, LPS alone had no effect at any time examined, and AMD alone caused a slight increase at 2, 4, and 10 h. Significant elevation of serum ALT and AST activities were only observed in AMD/LPS cotreatment, and the increases started between 4 and 6 h after LPS administration and continued to increase through 10 h.

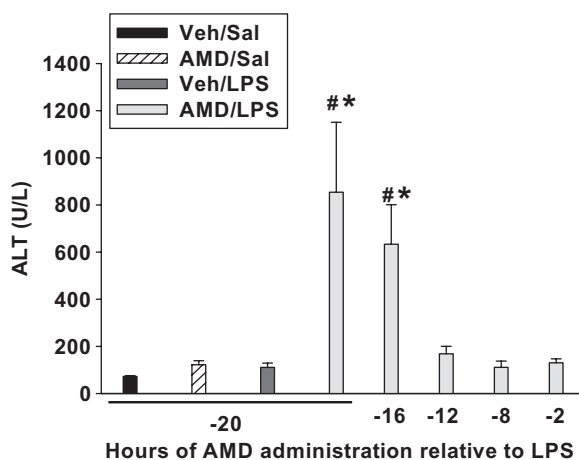


FIG. 1. Effect of time interval between AMD and LPS administrations on the induction of liver injury. Rats were treated with AMD (300 mg/kg, ip) 2, 8, 12, 16, or 20 h before LPS (1.6×10^6 EU/kg, iv). Serum ALT activity was measured 10 h after LPS injection. # and * indicate significantly different from AMD/Sal or Vehicle/LPS, respectively. $p < 0.05$, $n = 3-8$.

At 10 h after LPS administration, serum activities of ALP and GGT and concentration of bile acids were measured as indicators of cholestatic injury (Figs. 4A–C). Only AMD/LPS cotreatment caused significant increases in these serum markers, whereas AMD or LPS treatment alone had no effect.

Hepatic Histopathology

All the saline-treated control rats were free of liver lesions (Fig. 5A). No microscopic evidence of hepatic pathology was found in four of the six LPS-treated rats (Fig. 5B). Liver sections from two of the LPS-treated rats had a few small foci of midzonal hepatocellular necrosis with an associated neutrophilic influx. In contrast, a widespread, mild-to-marked fibrinopurulent capsulitis was present in the liver sections from all the AMD-treated rats (Fig. 5C). This was characterized by a thickening of the hepatic capsule due to edema and a conspicuous inflammatory exudate comprising mainly neutrophils, lesser numbers of mononuclear cells, and various amounts of amorphous proteinaceous material. This fibrinopurulent exudate was also often scattered along the outer peritoneal surface of the capsule. Focal areas of subcapsular hepatocellular necrosis were occasionally associated with the capsulitis.

The most profound hepatic histopathology was found in animals treated with both AMD and LPS (Fig. 5D). All these rats had a mild-to-marked fibrinopurulent capsulitis with occasional subcapsular necrosis similar to that found in the AMD-treated rats, but in addition, all these animals had conspicuous areas of midzonal hepatocellular necrosis. The latter lesion ranged from widely scattered focal areas of necrosis in midzonal regions to widespread hepatocellular necrosis with coalescence of affected midzonal and occasionally centriacinar regions (bridging necrosis). Accumulations of neutrophils (inset, Fig. 5D) were present in all these necrotic regions. Periportal regions were spared of AMD/LPS treatment-related injury.

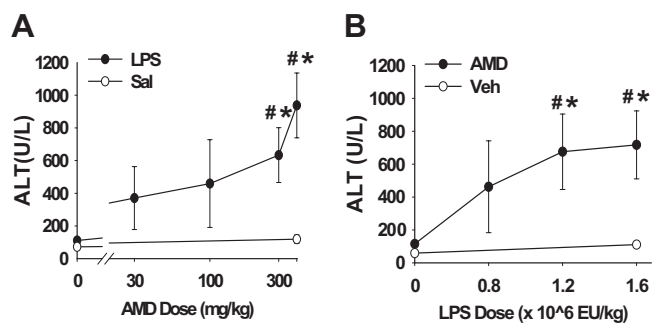


FIG. 2. Dose-response relationships for AMD and LPS. (A) Rats were treated with AMD (0–400 mg/kg, ip) and 16 h later with saline or LPS (1.6×10^6 EU/kg, iv). (B) Rats were treated with AMD (400 mg/kg, ip) or its vehicle and 16 h later with LPS (0– 1.6×10^6 EU/kg, iv). Serum ALT activity was measured at 10 h after LPS administration for both data sets. # indicates significantly different from respective groups not given LPS; * indicates significantly different from respective group not given AMD. $p < 0.05$, $n = 3-14$.

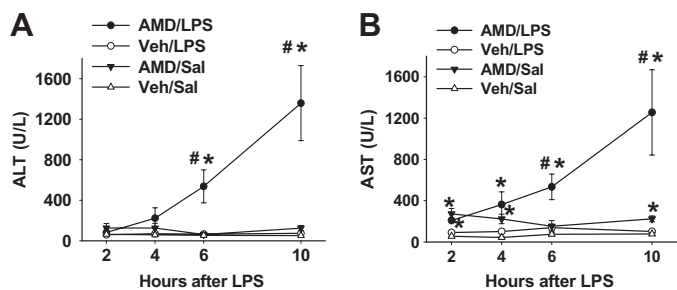


FIG. 3. Development of liver injury. Rats were treated with AMD (400 mg/kg, ip) or vehicle and 16 h later with LPS (1.6×10^6 EU/kg, iv) or saline. They were examined 2, 4, 6, or 10 h after LPS injection. Activities of (A) ALT and (B) AST in serum were measured. # indicates significantly different from respective groups not given LPS; * indicates significantly different from respective group not given AMD. $p < 0.05$, $n = 4-9$.

LPS Did Not Affect the Metabolism or Hepatic Accumulation of AMD

AMD and DEA concentrations in rat serum and liver homogenates were determined at various times after LPS administration (Figs. 6A–D). From 2 to 10 h after LPS or saline administration (i.e., 18–28 h after AMD administration), the serum and tissue concentrations of AMD and DEA were unaffected by LPS cotreatment. The average serum concentrations of AMD and DEA were 1100 and 128 ng/ml, respectively; and the average liver concentrations of AMD and DEA were 157 and 53 ng/mg protein, respectively.

AMD Affected the Concentration of TNF in Serum

Serum TNF concentration was measured at 2 and 4 h after LPS administration (Fig. 7). AMD by itself had no effect on serum TNF concentration. At 2 h after LPS, the serum TNF

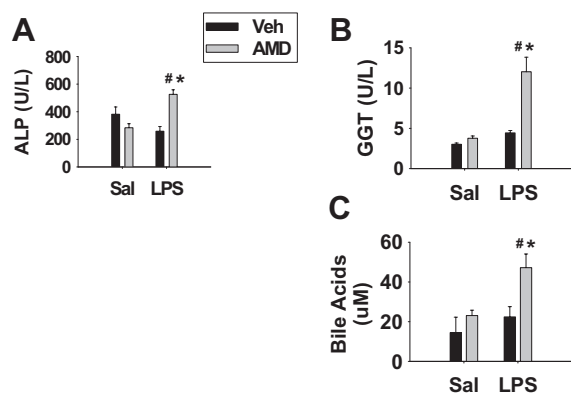


FIG. 4. Markers of cholestasis after AMD/LPS cotreatment. Rats were treated with AMD (400 mg/kg, ip) or vehicle and 16 h later with LPS (1.6×10^6 EU/kg, iv) or saline. Blood samples were collected at 10 h after LPS administration. Serum markers for cholestatic liver injury were measured: (A) ALP; (B) GGT; (C) concentration of total bile acids. # indicates significantly different from respective groups not given LPS; * indicates significantly different from respective group not given AMD. $p < 0.05$, $n = 4-9$.

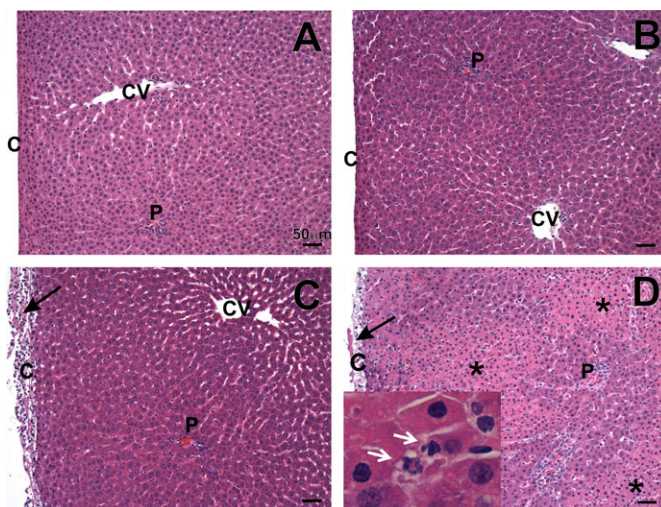


FIG. 5. Hepatic histopathology. Rats were treated with AMD (400 mg/kg, ip) or vehicle and 16 h later with LPS (1.6×10^6 EU/kg, iv) or saline. Liver tissue samples were collected at 10 h after LPS administration. Formalin-fixed liver samples were embedded in paraffin, sectioned, and stained with H&E staining. Treatment groups: (A) vehicle/sal; (B) vehicle/LPS; (C) AMD/sal; (D) AMD/LPS. CV, central vein; P, portal vein; C, capsule. Black arrows indicate fibrinopurulent capsulitis; * depicts necrotic foci. Inset in panel D is a higher magnification photomicrograph from a rat treated with AMD/LPS demonstrating neutrophils (white arrows).

concentrations in rats treated with AMD/LPS or with vehicle/LPS were similar. However, by 4 h after LPS, the concentration of TNF in serum of AMD/LPS-treated rats was significantly greater than that in vehicle/LPS-treated rats.

TNF Potentiated the Cytotoxicity of AMD and DEA in Hepa1c7 Cells

Hepa1c7 cells were exposed to AMD or DEA, and 24 h later, cytotoxicity was assessed by measuring LDH activity released into the culture medium (Figs. 8A and B). Both AMD and DEA caused concentration-dependent LDH release. Significant cytotoxicity was observed with AMD concentrations greater than 20 μ g/ml and DEA concentrations greater than 7 μ g/ml. Addition of TNF (3 ng/ml) did not cause cytotoxicity alone but significantly potentiated the cytotoxicity of AMD and DEA.

Neutralization of TNF *In Vivo* Attenuated AMD/LPS-Induced Liver Injury

Etanercept is a soluble TNF receptor construct that inactivates TNF. Etanercept (8 mg/kg, sc) injected 1 h before LPS inhibited the biological activity of TNF in rats and was not hepatotoxic by itself (Tukov *et al.*, 2007; Zou *et al.*, 2009a). The same treatment was used in this study. AMD/LPS cotreatment increased serum ALT activity, and etanercept significantly attenuated this increase (Fig. 9A). Changes in serum ALT activity were supported by histological

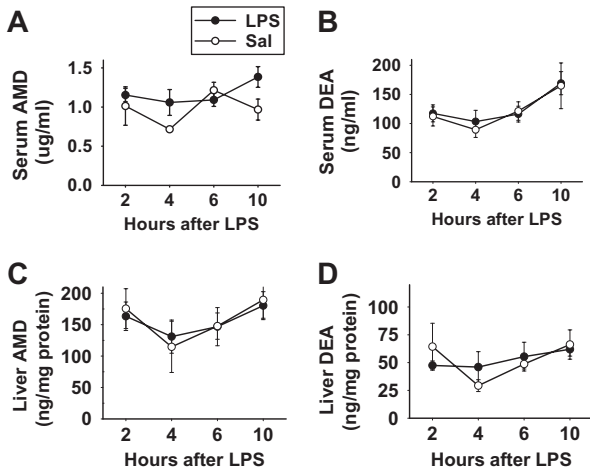


FIG. 6. Serum and liver concentrations of AMD and DEA. Rats were treated with AMD (400 mg/kg, ip) and 16 h later with LPS (1.6×10^6 EU/kg, iv) or saline. Blood and tissue samples were collected at 2, 4, 6, or 10 h after LPS injection. Serum concentrations of (A) AMD and (B) DEA and liver concentrations of (C) AMD and (D) DEA were measured with LC/MS/MS as described in "Materials and Methods" section. $n = 4-9$.

examination of H&E-stained liver sections: the severity and frequency of necrotic foci were markedly reduced in rats cotreated with etanercept (Fig. 9B).

DISCUSSION

Since its introduction in Europe in 1962, amiodarone has been associated with idiosyncratic hepatotoxicity (Lewis *et al.*, 1989). A linear correlation between serum AMD concentration and serum ALT activities has been established (Pollak and You, 2003); however, in that study, the ALT values did not exceed $3 \times$ ULN and were not considered to be clinically significant. For the cases of severe liver injury caused by intravenous

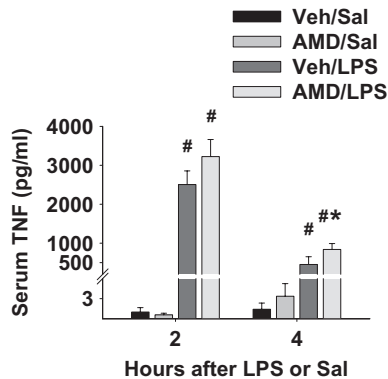


FIG. 7. Serum concentration of TNF. Rats were treated with AMD (400 mg/kg, ip) or vehicle and 16 h later with LPS (1.6×10^6 EU/kg, iv) or saline. Blood samples were collected at 2 or 4 h after LPS administration. Serum concentrations of TNF were measured with ELISA. # indicates significantly different from respective groups not given LPS; * indicates significantly different from respective group not given AMD. $p < 0.05$, $n = 5-8$.

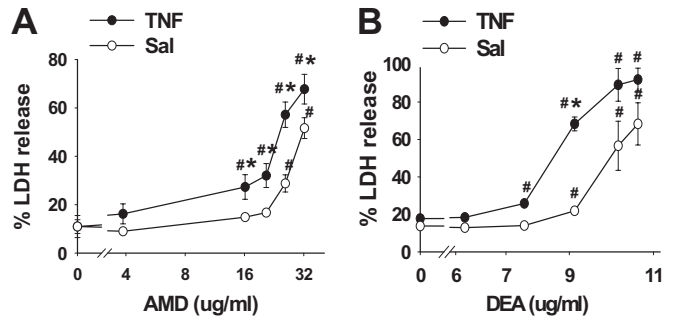


FIG. 8. Effect of TNF on cytotoxicity of AMD and DEA *in vitro*. Various concentrations of (A) AMD or (B) DEA were added to cultures of Hepa1c1c7 cells together with TNF (3 ng/ml) or saline. Twenty-four hours after treatment, LDH activity released into the culture medium was measured. The percent LDH release was calculated as described in "Materials and Methods" section. * indicates significantly different from respective groups not given TNF; # indicates significantly different from respective groups not given AMD or DEA. $p < 0.05$, $n = 3$.

amiodarone loading, patients' serum ALT activities were up to $10-206 \times$ ULN. There was usually a 24-72 h delay between the initial loading of AMD and the onset of elevation in ALT activities; and in many cases, the ALT activity returned to normal after a few days of continuation of maintenance dosing (Rätz Bravo *et al.*, 2005). Accordingly, it is hard to draw a simple linear relationship between the magnitude or frequency of severe hepatotoxicity and serum AMD concentration. An effort to establish a model for AMD-induced liver injury in healthy rodents was unsuccessful. Neither short-term, large dose nor long-term, small dose administration of AMD led to observable liver damage (Young and Mehendale, 1989). The cause of severe AMD hepatotoxicity is more likely to be a combination of AMD and other factors, e.g., inflammatory

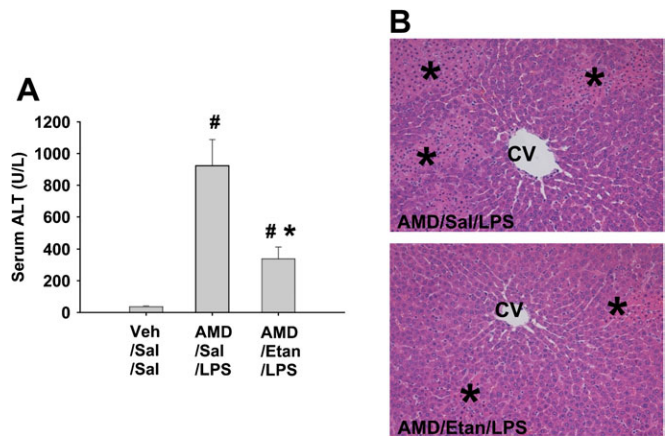


FIG. 9. Effect of TNF inhibition on AMD/LPS-induced liver injury. Rats were treated with AMD (400 mg/kg, ip) or vehicle and 16 h later with LPS (1.6×10^6 EU/kg, iv) or saline. Etanercept (8 mg/kg, sc) or its saline vehicle was given 1 h before LPS. Rats were killed 10 h after LPS. (A) Serum ALT activity. # indicates significantly different from vehicle/Sal/Sal; * indicates significantly different from AMD/Sal/LPS. $p < 0.05$, $n = 5-8$. (B) H&E-stained liver slides. CV, central vein; * depicts necrotic foci.

episodes. In the present study, regardless of cotreatment with LPS, the serum concentration of AMD was about 1100 ng/ml, which is very close to the steady-state serum concentration of AMD in human patients (1500 ng/ml) under long-term oral amiodarone therapy (Pollak *et al.*, 2000). Our findings support that at this clinically relevant concentration of AMD in serum, hepatotoxicity can be induced by a concurrent inflammatory episode related to LPS exposure.

Previous studies in rodents have suggested a possible association between inflammation and liver injury for several drugs associated with human IADRs, including chlorpromazine (Buchweitz *et al.*, 2002), ranitidine (Luyendyk *et al.*, 2003), diclofenac (Deng *et al.*, 2006), trovafloxacin (Shaw *et al.*, 2007), sulindac (Zou *et al.*, 2009b), and halothane (Dugan *et al.*, 2010). The results of the present study expand these findings and demonstrate that a nonhepatotoxic dose of AMD is rendered hepatotoxic when acute inflammation is triggered by LPS administration. Acute increases in serum markers for hepatocellular and cholestatic injury were found in rats cotreated with AMD/LPS. These resemble the clinical hepatic chemistry changes in human idiosyncrasy during AMD therapy (Rätz Bravo *et al.*, 2005). The midzonal and bridging necrosis and infiltration of inflammatory cells in AMD/LPS are also consistent with some of the histological changes found in human patients (Babatin *et al.*, 2008; Rätz Bravo *et al.*, 2005). However, the histological characteristics in people with AMD-induced liver injury were variable. Different patterns of hepatocellular necrosis, such as midzonal, centrilobular, bridging, and panlobular, have been reported (Lewis *et al.*, 1989). Genetic differences, concurrent medications, and even different origins of inflammation might account for these varied responses; nevertheless, the AMD/LPS interaction model in rats mimics important aspects of AMD-induced IADRs in human patients.

The timing of AMD and LPS dosing in this model was important for the development of severe liver damage. A minimal interval of 16 h was required for AMD and LPS to interact to induce liver injury. When LPS was injected within 16 h after AMD, no liver injury was observed. Absorption, distribution, metabolism, and clearance as well as toxicological actions could contribute to this timing requirement. The elimination of AMD is primarily through hepatic metabolism and biliary excretion, and its half-life in plasma is very long (55 days in humans) (Pollak *et al.*, 2000). Accordingly, the loss of AMD due to elimination within 16 h is minimal. AMD has a dose-dependent effect on the respiratory chain and β -oxidation in the mitochondria (Fromenty *et al.*, 1990a, b), and it can also affect the function of lysosomes and other acidic organelles (Stadler *et al.*, 2008). The 16-h interval may be required for AMD to distribute into the liver, accumulate in organelles, and sensitize hepatocytes to interact with LPS or its downstream cytokines.

In other drug/LPS models, there is also a dependence on the temporal relationship between administrations of drug and LPS, and the time interval needed for a maximal hepatotoxic

response is different for different drugs (Shaw *et al.*, 2007; Zou *et al.*, 2009b). This time interval requirement might help to explain the low frequency of IADRs in human patients: i.e., only when the inflammatory episode happens at a certain time during drug therapy would idiosyncratic hepatotoxicity occur.

In rats, AMD is deethylated by cytochromes P450 (CYPs) 3A4, 1A1, 2D1, and 2C11 in the liver (Elsherbiny *et al.*, 2008). DEA, the major metabolite, is three- to fivefold more toxic than AMD to cultured HepG2 cells (Waldhauser *et al.*, 2006) and to primary rat hepatocytes (Gross *et al.*, 1989). In our treatment of Hep1c1c7 cells, a similar trend was observed (Fig. 8). The administration of LPS affects the expression and activities of CYPs in rats (Sewer *et al.*, 1997). This raised the possibility that LPS might potentiate the toxicity of AMD by increasing its metabolism to DEA. To evaluate this, serum and liver concentrations of AMD and DEA were measured with LC/MS/MS. The average serum concentration of DEA in AMD-treated rats was 128 ng/ml, which is about 1/10 of the serum AMD concentration. This ratio is commonly seen in the serum after an intravenous loading dose of AMD, both in people (Ha *et al.*, 2005) and rats (Shayeganpour *et al.*, 2008). Neither the serum nor the liver concentration of AMD or DEA was affected by LPS cotreatment, suggesting that neither AMD accumulation nor DEA generation was affected by LPS.

Intratracheal instillation of AMD *in vivo* or exposure of alveolar macrophages to AMD *in vitro* led to TNF production (Futamura, 1996; Reinhart and Gairola, 1997). AMD treatment also increased TNF production by alveolar macrophages on LPS stimulation (Punithavathi *et al.*, 2003). In the present study, treatment of rats with AMD alone did not cause serum TNF elevation, but it did increase the concentration of TNF in serum of LPS-treated rats. These results suggest that the increased appearance of TNF in AMD/LPS-cotreated rats was probably not an additive effect; rather, AMD appeared to potentiate the production or diminish the clearance of TNF caused by LPS. The concentration of TNF in serum increases rapidly in LPS-treated rats, peaks at around 1.5–2 h, and then returns to basal levels at around 6 h (Tukov *et al.*, 2007). In the present study, the concentration of TNF around the peak time (i.e., 2 h) in LPS-cotreated rats was not affected by AMD, but the TNF concentration was greater in AMD-cotreated rats at a later time (4 h). These data suggest that AMD prolonged the elevation in TNF caused by LPS administration. This seemingly small difference in TNF concentration was shown to be critical to liver pathogenesis in another drug/LPS model of liver injury involving trovafloxacin (Shaw *et al.*, 2009b). Accordingly, it is possible that prolongation of the LPS-induced TNF response is a critical event across models of LPS–drug interaction.

The importance of TNF in the AMD model was explored by preventing its binding to cellular receptors with etanercept. Etanercept pretreatment reduced hepatotoxicity, indicating that TNF has an important role in AMD/LPS-induced liver injury. Because no liver injury was observed after treatment with LPS

alone, the large TNF peak caused by LPS was not hepatotoxic by itself; however, this amount of TNF could be critical for the induction of hepatocellular injury by potentiating the toxic effect of AMD and/or DEA. Signaling from an activated TNF receptor can lead to lysosomal leakage, mitochondrial damage, and caspase activation (Wullaert *et al.*, 2007). All three of these events were also found in AMD and DEA cytotoxicity *in vitro* (Agoston *et al.*, 2003; Spaniol *et al.*, 2001). Further support for a critical role for TNF came from our *in vitro* study in Hepa1c1c7 cells in which TNF increased the cytotoxicity of both AMD and DEA. As a proximal proinflammatory cytokine, TNF can also contribute to liver damage by inducing downstream inflammatory events, such as coagulation activation and neutrophil activation (Shaw *et al.*, 2009b; Tukov *et al.*, 2007). The etanercept treatment herein reduced the ALT activity to half of the level seen in the absence of this inhibitor, whereas the same dose of etanercept reduced the ALT activity almost to control level in trovafloxacin/LPS and sulindac/LPS models (Shaw *et al.*, 2007; Zou *et al.*, 2009a). This suggests that other factors induced by LPS might act in parallel with TNF in the AMD/LPS model. In other models of potentiation of xenobiotic toxicity by LPS, factors such as neutrophils (Luyendyk *et al.*, 2005), the coagulation system (Shaw *et al.*, 2009a), and prostanoids (Ganey *et al.*, 2001) play important roles, and these factors might be relevant in the model presented here.

In summary, AMD was rendered hepatotoxic in rats in the presence of a coexisting inflammatory stress induced by LPS. AMD/LPS-cotreated rats developed liver pathology and blood chemistry changes that resemble AMD-induced idiosyncratic hepatotoxicity in human patients. LPS did not interact with AMD by changing the metabolism or distribution of AMD. AMD enhanced the increase in plasma TNF concentration caused by LPS, and neutralizing TNF reduced liver injury from AMD/LPS coexposure. Moreover, TNF potentiated the cytotoxicity of both AMD and DEA *in vitro*. These findings add support to the idea that inflammatory stress can interact with IADR-associated drugs to cause liver injury by a mechanism involving TNF and suggest that a similar mode of action might apply to several drugs that cause idiosyncratic hepatotoxicity in humans.

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