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Chlorpromazine-induced hepatotoxicity during inflammation is mediated by TIRAP-dependent signaling pathway in mice

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Abstract

Inflammation is a major component of idiosyncratic adverse drug reactions (IADRs). To understand the molecular mechanism of inflammation-mediated IADRs, we determined the role of the Toll-like receptor (TLR) signaling pathway in idiosyncratic hepatotoxicity of the antipsychotic drug, chlorpromazine (CPZ). Activation of TLRs recruits the first adaptor protein, Toll-interleukin 1 receptor domain containing adaptor protein (TIRAP) to the TIR domain of TLRs leading to the activation of the downstream kinase, c-Jun-N-terminal kinase (JNK). Prolonged activation of JNK leads to cell-death. We hypothesized that activation of TLR2 by lipoteichoic acid (LTA) or TLR4 by lipopolysaccharide (LPS) will augment the hepatotoxicity of CPZ by TIRAP-dependent mechanism involving prolonged activation of JNK. Adult male C57BL/6, TIRAP^{+/+} and TIRAP^{-/-} mice were pretreated with saline, LPS (2 mg/kg) or LTA (6 mg/kg) for 30 min or 16 h followed by CPZ (5 mg/kg) or saline (vehicle) up to 24 h. We found that treatment of mice with CPZ in presence of LPS or LTA leads to ~3–4 fold increase in serum ALT levels, a marked reduction in hepatic glycogen content, significant induction of serum tumor necrosis factor (TNF) α and prolonged JNK activation, compared to LPS or LTA alone. Similar results were observed in TIRAP^{+/+} mice, whereas the effects of LPS or LTA on CPZ-induced hepatotoxicity were attenuated in TIRAP^{-/-} mice. For the first time, we show that inflammation-mediated hepatotoxicity of CPZ is dependent on TIRAP, and involves prolonged JNK activation *in vivo*. Thus, TIRAP-dependent pathways may be targeted to predict and prevent inflammation-mediated IADRs.

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Conflict of interest statement

There are no conflicts of interest to declare

Keywords

Inflammation; chlorpromazine; lipopolysaccharide; lipoteichoic acid; c-Jun-N-terminal kinase; Toll-like receptor signaling; idiosyncratic adverse drug reactions

Introduction

Drug-induced liver injury (DILI) accounts for more than 50% of acute liver failure cases in the United States (Bissell *et al.*, 2001) and remains a major concern in significant attrition of new drug molecules reaching phase III clinical trials and is the single major cause of “black-box” warning for several clinically relevant drugs. Inflammation, induced by bacterial or viral pattern recognition molecules, or by underlying disease conditions, has been shown to increase the toxic responses to known hepatotoxic drugs such as trovafloxacin (Shaw *et al.*, 2009b), diclofenac (Deng *et al.*, 2006), sulindac (Zou *et al.*, 2009) or chlorpromazine (Buchweitz *et al.*, 2001). The gram-negative bacterial endotoxin, lipopolysaccharide (LPS), is commonly used to induce inflammation in animal models and in cell-culture, and toxicity of drugs was shown to be increased in LPS-treated rodent models (Shaw *et al.*, 2007; Tukov *et al.*, 2007). Similarly, the gram-positive bacterial outer membrane component, lipoteichoic acid (LTA) has been shown to augment drug-induced hepatotoxicity in animals (Shaw *et al.*, 2009b). However the molecular mechanism by which LPS or LTA increases the toxicity of drugs is not completely known. In this study, we sought to determine the mechanism by which LPS or LTA regulate the hepatotoxicity of the anti-psychotic agent, chlorpromazine.

Chlorpromazine (CPZ) is widely used as a sedative or antiemetic. Although replaced by the newer 2nd generation atypical antipsychotics, CPZ remains to be the primary drug for treating schizophrenia (Liu and De Haan, 2009). There is a long known history of CPZ-induced hepatotoxicity in humans characterized by elevated serum alkaline phosphatases or biliary cirrhosis (Breuer, 1965; Read *et al.*, 1961). Animal models and *in vitro* studies indicate that CPZ is an intrinsic hepatotoxin (Abernathy *et al.*, 1977; Mullock *et al.*, 1983). CPZ was shown to be toxic to the liver in rats treated with LPS (Buchweitz *et al.*, 2002; Gandhi *et al.*, 2010). Furthermore, in our recent *in vitro* study using primary mouse hepatocytes, we showed that hepatotoxicity of CPZ was markedly augmented when pre-treated with the pro-inflammatory cytokine, tumor necrosis factor (TNF)- α , or bacterial endotoxins, LPS or LTA (Gandhi *et al.*, 2010). Thus, we selected CPZ as the model drug for our *in vivo* studies.

The bacterial components, LPS or LTA activate Toll-like receptors (TLRs) 4 or 2, respectively. This leads to the recruitment of the first adaptor protein, TIRAP (Toll-interleukin 1 receptor domain containing adaptor protein) to the intracellular domain of TLRs (Akira *et al.*, 2001). Using TIRAP^{-/-} mice, we and others have shown that, TIRAP played a significant role in attenuating the production of inflammatory cytokines in response to activated TLR4 or TLR2 (Fitzgerald *et al.*, 2001; Ghose *et al.*, 2008). However, TIRAP was shown to have a differential role in mediating the effects of LPS or LTA on hepatic detoxification genes (Ghose *et al.*, 2011; Ghose *et al.*, 2008). In particular, TIRAP was involved in mediating the alteration of hepatic detoxification genes by LTA, but was not involved in mediating the effects of LPS on these genes. Thus, in this study, our goal is to determine the role of TIRAP in mediating the effects of LPS or LTA on the hepatotoxicity of CPZ.

A key component of the TIRAP-dependent TLR-signaling pathway is the MAP-kinase, c-Jun N-terminal kinase (JNK). JNK exists in 3 distinct isoforms (JNK1–3) and is expressed in various tissues such as liver, heart, brain, etc. (Ip and Davis, 1998). JNK activation by

TNF α or UV radiation (Whitmarsh and Davis, 1996) displays two distinct activation profiles; i.e. early/transient and late/sustained (Guo *et al.*, 1998; Roulston *et al.*, 1998). Transient activation of JNK was shown to be involved in cell survival, whereas sustained activation led to apoptosis. Recently, sustained activation of JNK was shown to play a significant role in acetaminophen (APAP)-induced liver injury *in vivo* (Gunawan *et al.*, 2006; Hanawa *et al.*, 2008; Henderson *et al.*, 2007). We had observed that augmentation of CPZ toxicity by TNF α in primary mouse hepatocytes was associated with sustained activation of JNK (Gandhi *et al.*, 2010). Furthermore, TNF α mediated increase in CPZ hepatotoxicity in primary mouse hepatocytes was attenuated by treatment with a JNK inhibitor (Gandhi *et al.*, 2010).

In order to predict and prevent idiosyncratic drug-induced hepatotoxicity and to improve the quality of treatment options to patients, we need to understand the mechanism by which certain drugs have the propensity of causing serious hepatotoxicity in a subset of population. In this study, we tested the hypothesis that activation of TLR2 by LTA or TLR4 by LPS will augment the hepatotoxicity of CPZ by TIRAP-dependent mechanism involving prolonged activation of JNK. We observed that LPS or LTA treatment led to increased CPZ-induced hepatotoxicity involving sustained activation of JNK and release of serum TNF α . Furthermore, TIRAP played a central role in regulating the effects of LPS or LTA on CPZ-induced hepatotoxicity.

Materials and methods

Materials

Highly purified LPS (*Escherichia coli* serotype 0111:B4, # tlr1-pelps) and lipoteichoic acid (*Staphylococcus aureus*, # tlr1-pslta) were purchased from InvivoGen (San Diego, CA, USA) and freshly diluted to the desired concentration in pyrogen-free 0.9% saline before injection. Anti-JNK (# 9252) and anti-phospho-JNK (# 9251) (Cell-Signaling, Beverly, MA, USA) were used according to the manufacturer's instructions. CPZ and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals

C57BL/6 mice (~8 weeks) weighing 20–25 g were obtained from Harlan Laboratories (Houston, Texas, USA). TIRAP^{+/+} and TIRAP^{-/-} mice (C57BL/6 $\times$ SV129; F3) were obtained from Dr. Ruslan Medzhitov (Yale University School of Medicine, New Haven, CT, USA) as described in our earlier publication (Ghose *et al.*, 2008). The animals were maintained in a temperature and humidity-controlled environment and were provided with water and rodent chow *ad libitum*. All animal experimental and surgical procedures were approved by the Institutional Animal Care and Use Committee (IACUC).

Treatment of animals and sample collection

For toxicity studies, C57BL/6, TIRAP^{+/+} or TIRAP^{-/-} mice were intraperitoneally (i.p.)-injected with saline, LPS (2 mg/kg body wt.) or LTA (6 mg/kg body wt.) for 16 h. The doses for LPS or LTA were based on our previous studies (Ghose *et al.*, 2009; Ghose *et al.*, 2008). After 16 h, the mice were i.p.-injected with CPZ (5 mg/kg) or saline (vehicle control) for 24 h. In order to evaluate the role of CPZ in eliciting a hepatotoxic response in the presence of inflammation, CPZ was administered by the i.p. route to have direct effects on the liver, if any, in presence or absence of inflammation induced by LPS or LTA. Blood was collected by cardiac puncture and serum was separated and stored at -80°C for further analysis. Livers were removed and snap frozen in liquid nitrogen and stored at -80°C for further studies. For immunoblotting studies, C57BL/6, TIRAP^{+/+} or TIRAP^{-/-} mice were pre-treated with

saline, LPS or LTA for 30 min followed by CPZ and the animals were sacrificed and livers harvested at 1, 2 4 or 8 h after CPZ treatment and stored as described above.

Alanine aminotransferase (ALT) assay

ALT assay was performed according to the manufacturer's instructions (ALT-GPT, Thermo Scientific, Middletown, VA, USA, # TR18503) with slight modifications. Briefly, 5 μ L of serum was added to 50 μ L of ALT reagent and incubated at 37°C for 3 min. The change in absorbance per min was recorded at 340 nm. Change in ALT activity was reported as ALT release in U/L.

Serum TNF α analysis

Serum TNF α was quantified by ELISA kit according to the manufacturer's instructions (BioLegend, San Diego, CA, USA, # 430904). A standard curve was performed in duplicate and concentration of TNF α was calculated using the standard curve as reference. The optical density at 450 nm and background at 570 nm were measured on a Synergy II microplate reader (BioTek, Winooski, VT, USA). Calculation of validation parameters of the ELISA shows that the sensitivity of limit of detection (LOD) for the assay was 0.37 ng/ml. The intra-assay variability was less than 12% and the inter-assay variability was less than 7% between 3 separate days.

Immunoblotting

Homogenates were prepared from liver tissues pre-treated with LPS/LTA as described previously (Gandhi *et al.*, 2010; Ghose *et al.*, 2009; Ghose *et al.*, 2008). The protein concentration was determined by BCA assay according to the manufacturer's protocol (Pierce Chemical, Rockford, IL, USA). The samples (~ 30 μ g protein per well) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane and probed with JNK (1:500) or phospho (P)-JNK (1:1000) antibodies. Total JNK expression was control for equal loading of the protein among different samples as described earlier (Ghose *et al.*, 2008; Ghose *et al.*, 2009). Membranes were subsequently washed and probed with a goat anti-rabbit IgG-AP secondary antibody (1:2000) (Santa Cruz Biotechnology, Santa Cruz, CA, # sc-2007) for 1 h at room temperature. The blots were then washed with TBS-Tween-20 and incubated with Tropix® CDP Star® Nitro block II™ ECL reagent as per the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Membranes were analyzed on FluorChem FC2 Imaging System (Cell Biosciences, Inc., Santa Clara, CA, USA) and quantified by densitometry using the AlphaEase software.

Histopathological analysis

The liver tissues were fixed in 10% neutral buffered formalin. They were processed with different alcohol gradients, embedded in paraffin, sectioned to a thickness of 4–5 μ m and stained with Periodic acid Schiff's reagent (PAS) for qualitative analysis of glycogen content. Quantitative assay of glycogen content in the liver was performed by using the Glycogen assay Kit (Abcam, Cambridge, MA) according to the manufacturer's instructions. Formalin-fixed/paraffin-embedded liver sections were also stained with hematoxylin/eosin (H&E). Liver pathology was evaluated in a blind manner by a certified pathologist. The processing and staining of the liver tissue sections was performed at the core facility of Baylor College of Medicine, Houston, TX.

Data analysis

All the data are presented as mean $\pm$ S.D. with 4–5 animals per group. Differences between treatment groups were assessed for statistical significance by one-way, or two-way ANOVA

where appropriate, using GraphPad Prism 4.0 (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance was demonstrated at $p < 0.05$.

Results

Hepatotoxicity of CPZ is augmented upon LPS or LTA treatment

Hepatotoxicity of CPZ is very well established in humans (Breuer, 1965; Derby *et al.*, 1993). Treatment with the gram-negative bacterial endotoxin, LPS or gram-positive bacterial component, LTA, is known to augment the hepatotoxicity of known hepatotoxins (Luyendyk *et al.*, 2003; Shaw *et al.*, 2009b). In the present study, mice were pretreated with the TLR4 ligand, LPS (2 mg/kg), or the TLR2 ligand, LTA (6 mg/kg) 16 h prior to saline (vehicle control) and CPZ treatment (5 mg/kg). Serum samples collected after 24 h were analyzed for ALT release. The 5 mg/kg dose of CPZ was chosen as a non-hepatotoxic dose based on the data from our dose escalation studies (data not shown). We found that hepatotoxicity of CPZ, was significantly increased by 3–4 fold in mice pretreated with LPS (Fig. 1A) or LTA (Fig. 1B), as demonstrated by ALT release. The ALT levels did not differ significantly in mice treated with CPZ, LPS, or LTA alone from saline controls up to 24 h (Figs. 1A and 1B). These results were confirmed by aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT) analysis (data not shown).

Histopathological examination of liver tissues upon LPS or LTA treatment

Changes in liver histopathology such as hepatocellular necrosis, midzonal necrotic foci or accumulation of neutrophils have been observed in LPS-drug co-stimulation hepatotoxicity animal models. However, we did not observe any marked hepatocellular damage in our studies by H & E staining (data not shown). Hepatic glycogen is known to prevent the liver from injury induced by drugs or ischemia-reperfusion (Tang *et al.*, 2002). Depletion of glycogen stores can lead to a deficiency in energy reserves, further affecting the cellular metabolic processes and can lead to cell death. As glycogen is involved in maintaining the plasma membrane stability, we wanted to study whether the increase in ALT levels was due to any changes in hepatic glycogen content. Thus, using PAS staining, liver glycogen content (as depicted by intense red staining) in mice pretreated with LPS or LTA prior to saline or CPZ was determined qualitatively. We observed marked depletion of glycogen content (diminished red areas) in the liver sections of LPS- (Fig. 2A) or LTA-pretreated mice (Fig. 2B) only in the presence of CPZ. The glycogen content in liver sections of CPZ-, LPS- or LTA-treated mice alone did not differ significantly compared to saline treatment (Figs. 2A and 2B).

The data from these qualitative assays were further quantified as described in the *Materials and methods* section. As expected, we observed significant depletion (~2 fold) of liver glycogen content in LPS- (Fig. 2C) or LTA-pretreated mice (Fig. 2D) only in the presence of CPZ compared to their respective saline controls. There were no significant changes upon treatment with LPS, LTA or CPZ only (Figs 2C and 2D).

Increase in serum TNF α concentration is associated with CPZ-induced hepatotoxicity in the presence of LPS or LTA

Previous studies with LPS have shown that TNF α plays an important role in exacerbating the hepatotoxic responses to drugs in animal models of DILI (Lu *et al.*, 2012; Tukov *et al.*, 2007). We have also previously shown that TNF α played a major role in increasing the hepatotoxicity of CPZ in primary mouse hepatocytes (Gandhi *et al.*, 2010). On the contrary, IL-1 β or IL-6 were not involved. In the present study, mice were pretreated with LPS or LTA 30 min prior to saline or CPZ up to 4 h. Our results demonstrated that serum TNF α levels at 1 h were comparable in LPS or LTA treated mice in presence or absence of CPZ

(Figs. 3A and 3B). However, at 2 h, compared to LPS/Sal or LTA/Sal group, serum TNF α levels were 5–6 fold higher in LPS/CPZ or LTA/CPZ group (Figs. 3A and 3B). Similarly, at 4 h, serum TNF α levels were 3–4 fold higher in LPS- or LTA-treated mice only in the presence of CPZ. Serum TNF α levels in only CPZ-treated mice were similar to the saline-treated vehicle controls.

Sustained activation of JNK by CPZ in the presence of LPS or LTA

Although JNK plays a crucial role in regulation of cell growth, differentiation and proliferation, prolonged activation by inflammatory stress can lead to apoptosis or even hepatic necrosis. We have previously shown that TNF α -induced hepatotoxicity of CPZ was associated with prolonged activation of JNK, whereas the hepatotoxicity of CPZ was attenuated in presence of JNK inhibitor in primary mouse hepatocytes (Gandhi *et al.*, 2010). In the present study, liver tissues were harvested from mice pretreated for 30 min with saline, LPS or LTA followed by saline or CPZ up to 4 h. Immunoblotting studies indicated that LPS moderately activated JNK (as evidenced by phosphorylation of JNK) up to 1 h only (Fig. 4A). However in combination with CPZ, the activation of JNK was sustained up to 4 h (Fig. 4A). LTA by itself had minimal effect on JNK activation at 1h. However, JNK was activated up to 4 h in the livers of LTA-pretreated mice only in the presence of CPZ (Fig. 4B). JNK activation was not detected at the 8 h time point in LPS- or LTA-treated mice in presence or absence of CPZ (data not shown). Saline or CPZ by itself did not activate JNK at any of the time points tested.

TIRAP mediates CPZ-induced hepatotoxicity upon LPS or LTA treatment by regulating serum TNF α release and JNK activation

To study the role of TIRAP in regulating CPZ-induced hepatotoxicity and hepatic glycogen content in LPS- or LTA-induced inflammation, TIRAP^{+/+} or TIRAP^{-/-} mice were pretreated with saline, LPS (2 mg/kg) or LTA (6mg/kg) 16 h prior to saline or CPZ (5 mg/kg). Serum samples collected after 24 h were analyzed for ALT release. Only in the presence of CPZ, ALT levels were significantly increased (3–4 fold) in LPS- or LTA-treated TIRAP^{+/+} mice. This rise in ALT level was attenuated in TIRAP^{-/-} mice (Figs. 5A and 5B). ALT levels did not differ significantly in saline, LPS, LTA or CPZ treatment alone in both TIRAP^{+/+} and TIRAP^{-/-} mice (Figs. 5A and 5B).

TIRAP also plays an important role in regulating CPZ-mediated increase in serum TNF α levels (Ghose *et al.*, 2011; Ghose *et al.*, 2008). Therefore, to study the role of TIRAP in regulating serum TNF α levels in CPZ-induced hepatotoxicity, TIRAP^{+/+} or TIRAP^{-/-} mice were pretreated with LPS or LTA followed by saline or CPZ for up to 2 h. We observed significant increase in serum TNF α levels in LPS or LTA treated TIRAP^{+/+} mice only in the presence of CPZ (Fig. 6A and 6B). Serum TNF α levels were attenuated in TIRAP^{-/-} mice in both, LPS/CPZ or LTA/CPZ groups.

In order to study the role of TIRAP in activation of JNK by CPZ, mouse liver tissues pretreated with LPS or LTA 30 min prior to CPZ were harvested after 4 or 2 h, respectively. We observed activation of JNK in LPS/CPZ group at 4 h or LTA/CPZ group at 2 h in TIRAP^{+/+} mice. However, JNK activation in LPS/CPZ or LTA/CPZ groups was blocked in TIRAP^{-/-} mice (Figs. 7A and 7B). Also, CPZ, LPS or LTA did not activate JNK by itself up to 4 h in either of the groups (Figs 7A and 7B). Expression of total JNK was same in all the saline-treated controls, and there was no activation of JNK in these samples, as evidenced by the absence of phospho-JNK bands (data not shown). Thus, CPZ-induced hepatotoxicity may likely be mediated by TIRAP involving sustained activation of JNK.

Discussion

The underlying mechanisms involved in idiosyncratic DILI in humans are widely debated. Immune-mediated hypersensitivity reactions (Uetrecht, 2003), role of the hemostatic system (Shaw *et al.*, 2009a), accumulation of polymorphonuclear neutrophils (Zou *et al.*, 2011), genetic polymorphisms in drug metabolizing enzymes (Poolsup *et al.*, 2000) are some of the contributing factors to idiosyncratic DILI.

Inflammation is considered to be an underlying factor determining the susceptibility for the toxic effects of xenobiotic agents including CPZ (Buchweitz *et al.*, 2002; Roth *et al.*, 1997). Buchweitz *et al.* reported ~1.5-fold increase in ALT levels in CPZ-treated rats in the presence of LPS (Buchweitz *et al.*, 2002). In the present study, we observed that CPZ treatment in mice led to ~3–4 fold rise in serum ALT levels in LPS/CPZ or LTA/CPZ groups (Figs. 1A and 1B). Thus, similar hepatotoxic responses were observed in both rats and mice. Therefore, development of CPZ-inflammation interaction observed in our study could be extrapolated in humans to study CPZ-mediated adverse drug reactions in humans.

The molecular mechanism by which LPS or LTA increases hepatotoxic responses to CPZ is unknown. LPS- or LTA-treatment of rodents or cells are known to decrease the expression of drug metabolizing enzymes, leading to altered drug metabolism (Gandhi *et al.*, 2012; Ghose *et al.*, 2009; Ghose *et al.*, 2008; Richardson *et al.*, 2006; Sewer *et al.*, 1996). Metabolism of CPZ is very complex. The major metabolic pathways include 7-hydroxylation, N-dealkylation, N-oxidation and S-oxidation (Hartmann *et al.*, 1983). Of these, the 7-hydroxylation pathway, catalyzed mainly by CYP2D6 and partially by CYP1A2, is considered to be the major metabolic pathway for CPZ metabolism in humans (Yeung *et al.*, 1993; Yoshii *et al.*, 2000). It was shown that the ring-hydroxylated metabolites of CPZ are highly potent than the sulfoxidation product in causing jaundice in humans (Watson *et al.*, 1988). Another study showed that the demethylated metabolites, mono- and didesmethyl-CPZ, were three and six times, respectively, more potent than CPZ in causing the release of aspartate aminotransferase from isolated rat hepatocytes (Abernathy *et al.*, 1977). 7-hydroxyhchlorpromazine is equally potent as CPZ (Manian *et al.*, 1965). Although LPS down-regulates the gene expression of several CYP isoforms in mice, the effects of LPS or LTA on regulation of CYP2D6 and the enzymes responsible for catalyzing the demethylation of CPZ are not known. Therefore, the possibility that the hepatotoxic effects of CPZ is due to a reactive metabolite warrants further studies.

In the current study, histopathological examinations with hematoxylin and eosin staining revealed no significant liver damage in CPZ-, LPS- or LTA-treated mice compared to the saline controls (data not shown). The increase in serum ALT levels in these mice can be attributed to glycogen depletion in the liver. Although, depletion of hepatic glycogen does not always correlate with the rise in ALT levels, as observed in the case of APAP, it can be used as a marker for the early detection of drug-induced hepatotoxicities (Beitia *et al.*, 2000; Hinson *et al.*, 1983). Studies have shown that, reduction in hepatic glycogen correlated with increased hepatocellular damage in endosulfan-treated catfish (Rawat *et al.*, 2002). Similarly, hepatotoxicity of the anxiolytic benzodiazepine, pindolol, correlated with depletion of hepatic glycogen content in Dutch-belted rabbits as well in primary cultures of rabbit hepatocytes (Bacon *et al.*, 1996; Ulrich *et al.*, 1995). This was also accompanied by increase in serum transaminases and hepatic and serum triglyceride levels. In addition, CPZ has been shown to cause glycogen depletion in mice and rats (Mullock *et al.*, 1983; Samorajski *et al.*, 1965; Siddiqui *et al.*, 1979). Significant loss of liver glycogen can be detrimental. If hepatocytes do not have sufficient glycogen levels to provide glucose to drive glycolysis, cellular ATP levels may drop below critical levels. This may have deleterious consequences as ATP plays an important role in stabilizing cell membrane and metabolic

functionality of drug metabolizing enzymes (Fouts, 1963; Tang *et al.*, 2002). As less than 1% of CPZ is excreted unchanged, a decrease in drug metabolism can lead to drug accumulation, which can contribute to hepatotoxic effects of CPZ during inflammation. However, the exact mechanism by which CPZ may lead to a decrease in liver glycogen needs to be further investigated. CPZ in combination with LPS or LTA increased the levels of serum transaminases and depleted glycogen without causing hepatocellular damage. Increase in the levels of serum transaminase such as AST and ALT have been shown previously without the detection of liver injury by immunohistochemistry analysis (Radi *et al.*, 2011). Serum enzymes such as ALT and AST were shown to undergo endocytosis by non-parenchymal cells such as Kupffer cells and endothelial cells. Modulation of Kupffer cells was shown to affect the steady-state levels of these enzymes without any histopathological changes in the liver. It is possible that CPZ in combination with LPS or LTA affects non-parenchymal cells and induces these enzymes without causing any marked hepatocellular damage.

The pro-inflammatory cytokine, TNF α , plays an important role in mediating hepatotoxic responses of several xenobiotics (Shaw *et al.*, 2007; Tukov *et al.*, 2007; Zou *et al.*, 2009). We find that LPS- or LTA-mediated increase in serum TNF α levels were sustained up to 4 h only in the presence of CPZ (Figs. 3A and 3B). However, the data from the present study are in contrast to the previous reports in which it was shown that, CPZ injected prior to LPS plays a hepatoprotective role in attenuating the toxic effects of LPS and increased TNF α levels in mice (Gadina *et al.*, 1991; Ghezzi *et al.*, 1996). This discrepancy can arise due to several factors: (1) Although the doses of CPZ were not significantly different (4 mg/kg in earlier studies vs. 5 mg/kg in the current study), Gadina *et al* used 75 μ g/kg and 30 mg/kg of LPS and Ghezzi *et al* used 2.5 μ g/mouse of LPS, respectively. (2) Also, the earlier studies used CD-1 mice (Gadina *et al.*, 1991; Ghezzi *et al.*, 1996), where-as C57/BL6 mice were used in the present study. (3) Also, CPZ was administered before LPS in the previous studies as opposed to 30 min post-treatment of CPZ in the present study. Thus, strain differences and dosing regimen of LPS may account for the disparate observations. In addition, *in vitro* studies also showed that CPZ blocked the cytotoxic effects of TNF α by inhibiting its synthesis in human monocytic leukemia cells or inhibition of mRNA for TNF α in human thymocytes (Schleuning *et al.*, 1989; Zinetti *et al.*, 1995). Thus, the dosing regimen of CPZ may determine its hepatoprotective or hepatotoxic nature. However, the therapeutic effectiveness of CPZ in lowering TNF α in humans requires further investigation.

Recently, we showed that TNF α -induced hepatotoxicities of APAP or CPZ in primary mouse hepatocytes were blocked when pretreated with JNK inhibitor (Gandhi *et al.*, 2010). These findings are in accordance with our data from the present study in which we observed sustained activation of JNK (up to 4 h) in LPS- or LTA-treated mice only in the presence of CPZ (Figs. 4A and 4B). Although JNK plays an important role in stress response in cells, sustained activation is believed to promote cell injury and death by activation of downstream pro-apoptotic genes (Singh and Czaja, 2007). Furthermore, recent studies have shown that, JNK is a major contributor to APAP-induced liver injury (Gunawan *et al.*, 2006; Hanawa *et al.*, 2008; Henderson *et al.*, 2007). The link between sustained activation of JNK and cell death may possibly be related to increased TNF α production (Henderson *et al.*, 2007).

TIRAP plays an important role in regulating gene expression of key hepatic phase I and phase II drug metabolizing enzymes only in TLR2 and not in TLR4-mediated signaling pathway (Ghose *et al.*, 2011; Ghose *et al.*, 2008). The increase in serum ALT upon CPZ treatment in LPS- or LTA-pretreated TIRAP^{+/+} mice was blocked in TIRAP^{-/-} mice (Figs. 5A and 5B). TIRAP is the proximal adaptor molecule involved in LPS-induced TLR4 or LTA-induced TLR2 activation. It is also known to regulate the LPS-induced apoptosis in macrophages by regulating the activation of NF- κ B which plays an important role in

transcription of genes involved in apoptosis (Fitzgerald *et al.*, 2001; Horng *et al.*, 2001). Recently, TIRAP was shown to regulate LPS-mediated caspase activation in endothelial cells (Bannerman *et al.*, 2002). However, further studies will be needed to determine if TIRAP-dependent hepatotoxicity of CPZ is due to altered drug metabolism due to the fact that TIRAP is differentially involved in regulation of drug metabolizing enzyme genes in LPS- or LTA-induced inflammation.

We previously showed that serum cytokine levels were significantly attenuated in LPS- or LTA-treated TIRAP^{-/-} mice (Ghose *et al.*, 2011; Ghose *et al.*, 2008). Similarly, in the present study, significant induction of serum TNF α levels by CPZ in LPS- or LTA-treated TIRAP^{+/+} mice was attenuated in TIRAP^{-/-} mice (Figs. 6A and 6B). Activation of JNK in LPS or LTA-treated TIRAP^{+/+} mice in presence of CPZ was attenuated in TIRAP^{-/-} mice (Figs. 7A and 7B). We and others have shown that TIRAP is essential for regulation of JNK in LPS or LTA-induced inflammation (Ghose *et al.*, 2011; Horng *et al.*, 2001).

In conclusion, the novel finding in our study was that a non-hepatotoxic dose of CPZ was rendered hepatotoxic by LPS or LTA, by a TIRAP-dependent pathway. Thus, the underlying mechanisms responsible for precipitating idiosyncratic hepatotoxicity of CPZ could be attributed to TIRAP which was shown to regulate serum secretion of the pro-inflammatory cytokine, TNF α , and activation of JNK in presence of LPS or LTA. Thus, TIRAP-dependent signaling pathway may be effectively targeted to prevent DILI to restrict the progression and severity of liver injury during acute inflammatory episodes.

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Abbreviations

CPZ	chlorpromazine
IADRs	idiosyncratic adverse drug reactions
JNK	c-Jun-N-terminal kinase
LPS	lipopolysaccharide
LTA	lipoteichoic acid
TIRAP	Toll-interleukin 1 receptor domain containing adaptor protein
TLR	Toll-like receptor
TNF	tumor necrosis factor

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Highlights

- Inflammation augments the toxicity of an idiosyncratic hepatotoxin chlorpromazine
- Activation of Toll-like receptor signaling by lipopolysaccharide (LPS) or lipoteichoic acid (LTA) induces chlorpromazine toxicity
- Sustained stress kinase (JNK) activation is associated with chlorpromazine toxicity
- These studies provide novel mechanistic insights into idiosyncratic hepatotoxicity

Fig. 1. Regulation of hepatotoxicity of CPZ in presence of LPS or LTA

C57BL/6 mice were pre-treated with saline, LPS (2 mg/kg) (1A) or LTA (6 mg/kg) (1B) for 16 h followed by CPZ (5 mg/kg, i.p) or saline (vehicle control). Blood samples were collected 24 h after CPZ treatment and ALT assays performed. All the data represented are mean $\pm$ S.D. from 4–5 mice per group. * $p < 0.05$ indicates statistical significance when compared to LPS/Sal or LTA/Sal.

Fig. 2. Qualitative evaluation of hepatic glycogen content

C57BL/6 mice were pre-treated with saline, LPS (2 mg/kg) (2A) or LTA (6 mg/kg) (2B) for 16 h followed by CPZ (5 mg/kg, i.p) or saline (vehicle control). Liver tissues were harvested 24 h after CPZ treatment and hepatic glycogen content was determined using PAS staining. All the data represented are mean $\pm$ S.D. from 4–5 mice per group. The dark red portions represent areas with abundant glycogen content as depicted by the black arrows. Liver glycogen levels were determined in saline, LPS (2C) or LTA (2D) CPZ-treated mice as described in the *Materials and Methods* section. * $p < 0.05$, compared to saline/saline-treated control.

Fig. 3. Induction of serum TNF α level upon LPS or LTA treatment

C57BL/6 mice were pretreated with saline, LPS (2 mg/kg) (3A) or LTA (6 mg/kg) (3B) for 30 min followed by CPZ (5 mg/kg, i.p) or saline (vehicle control). Blood samples were collected up to 4 h after CPZ treatment. Determination of serum TNF α was performed by ELISA as described in the *Materials and Methods* section. All the data represented are mean $\pm$ S.D. from 4–5 mice per group. * $p < 0.05$ indicates statistical significance when compared to LPS/Sal or LTA/Sal at the corresponding time point.

Fig. 4. Activation of JNK by LPS or LTA is prolonged in presence of CPZ

C57BL/6 mice were pre-treated with saline, LPS (2 mg/kg) (4A) or LTA (6 mg/kg) (4B) for 30 min followed by CPZ (5 mg/kg, i.p) or saline (vehicle control). Liver tissues were collected up to 4 h after CPZ treatment. Activation of JNK was determined by western blotting as described under the *Materials and Methods* section. Protein bands were quantified using densitometry software. # $p < 0.05$ indicates statistical significance when

compared to Sal/Sal. * $p < 0.05$ indicates statistical significance when compared to LPS/Sal or LTA/Sal at the corresponding time point.

Fig. 5. Role of TIRAP in LPS- or LTA-induced hepatotoxicity of CPZ

TIRAP^{+/+} and TIRAP^{-/-} mice were pre-treated with saline, LPS (2 mg/kg) (5A) or LTA (6 mg/kg) (5B) for 16 h followed by CPZ (5 mg/kg, i.p) or saline (vehicle control). Blood samples were collected 24 h after CPZ treatment and ALT assays performed. All the data represented are mean $\pm$ S.D. from 4–5 mice per group. * $p < 0.05$ indicates statistical significance when compared to LPS/Sal or LTA/Sal in TIRAP^{+/+} mice.

Fig. 6. Regulation of serum TNF α upon LPS or LTA treatment

TIRAP^{+/+} and TIRAP^{-/-} mice were pre-treated with saline, LPS (2 mg/kg) (6A) or LTA (6 mg/kg) (6B) for 30 min followed by CPZ (5 mg/kg, i.p) or saline (vehicle control). Blood samples were collected 2 h after CPZ treatment. Determination of serum TNF α was performed by ELISA as described in the *Materials and Methods* section. All the data represented are mean $\pm$ S.D. from 4–5 mice per group. * p <0.05 indicates statistical significance when compared to LPS/Sal or LTA/Sal in TIRAP^{+/+} mice.

Fig. 7. Activation of JNK by LPS or LTA in presence of CPZ is TIRAP-dependent

TIRAP^{+/+} and TIRAP^{-/-} mice were pre-treated with saline, LPS (2 mg/kg) (7A) or LTA (6 mg/kg) (7B) for 30 min followed by CPZ (5 mg/kg, i.p) or saline (vehicle control). Liver tissues were collected 2 h after CPZ treatment. Activation of JNK was determined by western blotting as described under the *Materials and Methods* section. Protein bands were quantified using densitometry software. * $p < 0.05$ indicates statistical significance when compared to LPS/Sal or LTA/Sal in TIRAP^{+/+} mice.