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Lactoferrin Protects Against Acetaminophen-Induced Liver Injury in Mice

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Abstract

Acetaminophen-induced liver injury (AILI) is a significant health problem and represents the most frequent cause of drug-induced liver failure in the United States. The development and implementation of successful therapeutic intervention strategies have been demanding, due to significant limitations associated with the current treatment for AILI. Lactoferrin (Lac), a glycoprotein present in milk, has been demonstrated to possess a multitude of biological functions. Our study demonstrated a profound protective effect of Lac in a murine model of AILI, which was not dependent on its iron binding ability, inhibition of acetaminophen (APAP) metabolism, or a direct cytoprotective effect on hepatocytes. Instead, Lac treatment significantly attenuated APAP-induced liver sinusoidal endothelial cell dysfunction and ameliorated hepatic microcirculation disorder. This protective effect of Lac appeared to be dependent on hepatic resident macrophages (Kupffer cells, KC). Collectively, our data indicated that Lac, through activation of KC, inhibited APAP-induced liver sinusoidal endothelial cell damage and improved hepatic congestion, thereby protecting against AILI. These findings reveal the significant therapeutic potential of Lac during AILI and other types of liver diseases.

Keywords

Lactoferrin; Acetaminophen; Hepatotoxicity; Microcirculation

Introduction

Acetaminophen (APAP) is a widely used analgesic and antipyretic that is safe and effective, with few adverse effects, when taken at therapeutic doses. [1] However, an acute or cumulative overdose of APAP can cause severe liver damage with the potential to progress to liver failure. [2] APAP overdose accounts for more than 56,000 emergency room visits, 2,600 hospitalizations, and an estimated 458 deaths due to acute liver failure each year in the United States. [2] The current use of N-acetylcysteine (NAC) in the treatment of APAP-induced liver failure has considerable limitations. A variety of adverse reactions to NAC have been observed in the clinic, ranging from nausea to death. [3] In addition, the therapeutic window of NAC administration is quite narrow, as NAC only demonstrates effectiveness for

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patients who present within hours of APAP ingestion. Later treatment of Lac is much less effective and even impairs liver regeneration. [4]

The murine model of APAP-induced liver injury (AILI), which has similar characteristics to human patient based on clinical observation and biochemical and histopathological determinations, has been widely studied to gain insight into the underlying mechanisms of AILI and discover potential therapeutic interventions. AILI is initiated by the formation of a reactive metabolite, N-acetyl-*p*-benzoquinone imine (NAPQI), which depletes hepatic glutathione (GSH), covalently binds to cellular proteins, [5,6] and directly damages hepatocytes following mitochondrial dysfunction, lipid peroxidation, oxidative stress and DNA fragmentation. [7] Evidence also suggests that APAP-induced cytotoxicity of liver sinusoidal endothelial cells (LSEC) contributes to the overall initiation and progression of overt tissue injury. [8,9] It has been demonstrated that NAPQI-induced direct hepatocellular damage is preceded by the impairment of hepatic microcirculation. This impairment occurs as early as 30 min after APAP challenge, evident by the swelling of LSEC and their loss of ability to endocytose formaldehyde-treated serum albumin, a ligand for the scavenger receptor. [8,9] In addition, LSEC become swelling or rounding-up, and begin to lose the Gap formation in the cytoplasm of LSEC results in red blood cell (RBC) penetration into the Space of Disse as early as 2 h and is markedly increased by 6 h post-APAP treatment. [8] Sinusoidal perfusion has been shown to be reduced between 1 and 12 h, with a nadir (approximately 30% decrease) 4 to 6 h after APAP challenge. [8] It has also been reported that APAP-induced damage to LSEC, assessed by serum hyaluronic acid (HA), was greater in non-survivors than survivors of AILI. [10] Furthermore, inhibition of LSEC damage using matrix metalloproteinase (MMP) inhibitors, [11] improvement of haemodynamics using nitric oxide (NO) donors, [12,13] and amelioration of microvascular dysfunction using α1-adrenoceptor antagonists [14] or heparin [15] have been demonstrated to significantly attenuate AILI. These studies provide strong evidence that APAP-induced LSEC injury and microcirculatory disorder, which precede hepatocyte toxicity, play a key role in the pathogenesis of AILI, and suggest that their prevention may be critical for inhibiting AILI.

Lactoferrin (Lac) belongs to the family of transferrins and is a major protein component of human colostrums and milk. [16] Lac has been demonstrated to possess anti-inflammatory activity, as it can modulate cytokine production by immune cells through receptor-mediated signaling pathways [17] and down-regulate inflammatory responses via the prevention of iron-catalyzed free-radical damage at inflammation sites. [18] Although a number of clinical trials have evaluated the efficacy of Lac in the treatment of inflammation, hepatitis C infection, and cancer [19–21], the potential for Lac in the treatment of drug-induced liver injury (DILI) remains unknown. The present report reveals a profound protective effect of Lac against AILI in mice. Our data demonstrates that Lac, through activation of hepatic macrophages, inhibits APAP-induced LSEC damage and mitigates hepatic microcirculatory dysfunction, thereby attenuating AILI.

Materials and Methods

Animal treatment and assessment of hepatotoxicity

Male Balb/cJ and C57Bl/6J mice (8–10 weeks of age) were purchased from The Jackson Laboratory (Bar Harbor, ME) and kept in the Center for Laboratory Animal Care at the University of Colorado Denver (UC Denver) for one week before treatment. All animal experiments were performed according to guidelines from the UC Denver Institutional Animal Care and Use Committee. Mice were intraperitoneally (i.p.) injected with 300 mg/kg of APAP (dissolved in PBS). Bovine Lac (50 mg/kg, dissolved in PBS; Sigma-Aldrich, St. Louis, MO) was administered intravenously (i.v.) to mice concurrent with or after APAP challenge. Five, 8 and 24 h after APAP treatment, blood was collected by retro-orbital

puncture and serum alanine trasaminase (ALT) levels were determined using a diagnostic assay kit (Teco Diagnostics, Anaheim, CA). Twenty-four h after APAP challenge, livers were excised and histopathology was examined from formalin-fixed, paraffin-embedded sections after staining with hematoxylin and eosin (H&E; Department of Pathology, UC Denver). To deplete hepatic Kupffer cells (KC), mice were i.v. injected with liposome-entrapped clodronate (liposome/clodronate; Sigma) 2 days prior to APAP challenge. [22] Control mice were i.v. injected with empty liposomes.

Intravital microscopy

Male Balb/cJ mice were treated with APAP/PBS or APAP/Lac as described above. Naïve mice treated with PBS were used as controls. Five h after APAP challenge, mice were i.v. injected with 100 μL Quantum dots (QD705, 1:5 dilution in PBS; Invitrogen) immediately prior to liver sinusoidal visualization using an inverted LSM 510 NLO multiphoton microscope.

Hypoxia measurement

Male Balb/cJ mice were treated with APAP/PBS or APAP/Lac as described above. Naïve mice treated with PBS were used as controls. Five h after APAP challenge, mice were i.p. injected with 60 mg/kg of pimonidazole hydrochloride (provided in Hypoxyprobe™-1 kit, NPI Inc., Burlington, MA). Fifteen min after injection, livers were collected for preparation of tissue homogenate and formalin-fixed and paraffin-embedded sections. Immunohistochemical (IHC) analysis was performed using a mouse monoclonal antibody (1:50 dilution; provided in the Hypoxyprobe™-1 kit) that recognizes pimonidazole-protein adducts formed in hypoxic cells. A rabbit anti-mouse secondary antibody (1:500 dilution; Chemicon) was then applied to detect the protein adducts. Hepatic hypoxia was further examined by immunoblot analysis using the same anti-pimonidazole antibody (1:50 dilution).

LSEC isolation

Livers were perfused as described above and the cell suspensions were centrifuged at 30 × g for 3 min to pellet hepatocytes. The supernatants were then centrifuged at 330 × g for 5 min and the resulting cell pellet fractionated using 30% (w/v) Nycodenz (Axis-Shield, Scotland) to yield hepatic nonparenchymal cells (NPC). LSEC were purified from NPC via negative selection using anti-CD45 biotinylated antibodies (eBioscience, San Diego, CA) in conjunction with MACS cell separation columns (Miltenyi Biotec**,** Auburn, CA).

eNOS mRNA and activity measurement

Male Balb/cJ mice were treated with APAP/PBS or APAP/Lac as described above. Mice were sacrificed 5 h after APAP challenge and liver tissues harvested. NPC were isolated and LSEC purified. Total RNA was isolated from liver tissue and LSEC, using RNeasy Mini Kits (Qiagen, Valencia, CA), for RT-PCR analysis. Primers for eNOS include: sense 5′-TAC CAG CTG GCC AAA GTG ACC ATA-′, and antisense ′-CAG AAT GGT TGC CTT CAC ACG CTT-′. The activity of eNOS in NPC was determined by using NOS activity Assay Kit (Cayman Chemical). Briefly, cells were disrupted and protein extracted before reacting with [^{3}H]-arginine (20 nCi/μl) in the presence of NADPH for 60 min. The reaction mixtures were subjected to resin columns, and [^{3}H]-citrulline was collected by centrifugation. Radioactivities were measured using a liquid scintillation counter.

Statistical analysis

Data are presented as mean ± SEM. Two-tailed Student's t-test was used to compare two groups. Comparisons among multiple groups were performed using one-way analysis of

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variance (ANOVA) with a post-hoc test of significance between individual groups. Differences were considered significant when $p < 0.05$.

Results

Lac attenuates AILI in mice

To examine whether Lac treatment could affect AILI, male Balb/cJ mice were i.v. administered bovine Lac concurrent with or following i.p. injection of APAP. Protection by Lac was observed in mice treated simultaneously with APAP and Lac, as demonstrated by the significant decrease in serum ALT levels at 24 h from 12400 ± 2333 IU/L (APAP alone-treated mice) to 5740 ± 1223 IU/L (APAP and Lac-treated mice). To investigate the potential of Lac as a rescue therapy after APAP challenge, Lac was administered 1 and 4 h following APAP administration (APAP/Lac), while control mice were treated with PBS (APAP/PBS). The data showed that post-treatment with Lac was able to dramatically reduce serum ALT levels (Fig. 1A). Histological evaluation further revealed that the APAP-induced massive bridging necrosis within the centralobular region of the livers was significantly ameliorated in mice treated with APAP/Lac (Fig. 1B). Moreover, when mice in the APAP/Lac-treated group were administered 3 doses of Lac (at 1, 4, and 8 h post-APAP), a more dramatic protection was observed at 24 h following APAP challenge (Fig. 1C).

Lac does not directly protect hepatocytes against APAP-induced toxicity

AILI is initiated by the formation of NAPQI, which binds to cellular proteins and causes mitochondrial damage and oxidative stress, culminating in hepatocyte death.[7] To examine whether the inhibition of NAPQI generation could account for Lac's protective effect, immunoblot analysis was performed to detect NAPQI-protein adduct formation in the liver. The data showed no significant differences in either the pattern or level of NAPQI-protein adducts in mice treated with APAP/PBS or APAP/Lac (Fig. 2).

As Lac has the ability to directly bind to hepatocytes, [23] it is possible that the Lac-induced amelioration of AILI is due to a direct protective effect on hepatocytes. To examine this hypothesis, primary hepatocytes were isolated, as described previously, [24] from naïve male Balb/cJ mice following overnight starving, in order to mimic the *in vivo* experimental conditions. Hepatocytes were treated *in vitro* with APAP in the presence or absence of Lac for 6 or 12 h and the release of aspartate aminotransferase (AST) into the supernatants was measured. Compared with naïve hepatocytes, APAP-treated hepatocytes released significantly higher levels of AST, which was unaffected by co-treatment with Lac (Fig. 3A). Further analysis using an MTT assay showed that co-treatment with Lac did not rescue against the significant decrease in hepatocyte viability following APAP challenge (Fig. 3B). These data suggest that Lac did not directly protect hepatocytes against APAP-induced cytotoxicity.

Based on the high affinity of Lac for iron and reports that high doses of iron chelators mitigate AILI in rats and mice, [25,26] we examined whether the hepato-protective effect of Lac was dependent on its iron-binding ability. Due to the unavailability of bovine holo-Lac (iron-saturated), human holo-Lac and non-holo-Lac (non-iron saturated) were used in these experiments. The holo-and non-holo-Lac exhibited similar degrees of protection against AILI (Fig. 1S), suggesting that the hepato-protective effect of Lac was independent of its iron-binding capacity as well as its origin, as both the human and bovine proteins exhibited similar degrees of protection.

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Lac protects LSEC and attenuates hepatic microcirculation dysfunction

Previous studies have demonstrated that APAP-induced LSEC damage leads to hepatic microcirculation disorder, which plays an important role in the pathogenesis of AILI. [8–15] To examine whether Lac treatment could ameliorate APAP-induced impairment to the hepatic microvasculature, we used non-targeting quantum dots (QD705), which are auto-fluorescent semiconductor nanocrystals, to visualize hepatic sinusoidal perfusion via multiphoton microscopy. The use of Quantum dots, which remain in the circulation, has been employed for *in vivo* biomedical imaging and to demarcate the hepatic sinusoids in mice. [27,28] Male Balb/cJ mice were treated with APAP/PBS or APAP/Lac as described above and naïve mice treated with PBS were used as controls. Five h after APAP challenge, the mice were i.v. injected with QD705 immediately prior to liver sinusoidal imaging. The data demonstrated that in control and APAP/Lac-treated mice, QD705 distributed within the sinusoids throughout the liver (Fig. 4A). In contrast, in APAP/PBS-treated mice, the perfusion of QD705 within the sinusoids was impaired, resulting in the accumulation of QD705 clusters (Fig. 4A). As a result of APAP-induced LSEC damage, several reports have described the accumulation of RBC within the liver as early as 2 – 6 h following APAP challenge. [8,9] Our data confirmed this finding and demonstrated that post-treatment with Lac drastically reduced hepatic accumulation of RBC at both 3 and 5 h after APAP challenge (Fig. 4B). To substantiate the finding that Lac treatment could attenuate the APAP-induced loss of endothelial integrity, we administered Evans blue dye 4 h after APAP challenge. The amount of Evans blue retained within the liver was quantified to determine the integrity of the sinusoidal endothelium, loss of which would enable penetration of the dye into the tissue. The data showed that Evans blue accumulation was significantly higher in mice treated with APAP/PBS compared with that in naïve and APAP/Lac-treated mice (Fig. 4C). This finding suggested that APAP challenge caused a loss of endothelial integrity, which was restored upon Lac treatment.

It has been reported that sinusoidal perfusion is significantly reduced following APAP challenge, with a nadir at 4 to 6 h after treatment. [8] To examine whether hypoxia is induced in the liver following APAP challenge and whether Lac treatment can inhibit this process, we assessed hepatic hypoxia levels by IHC. Our data showed that the hypoxyprobe staining was much stronger in the liver of APAP/PBS-treated mice compared to that in APAP/Lac-treated mice, with no significant staining in PBS-treated control mice (Fig. 5A). The degree of hypoxia was further assessed in liver homogenate by immunoblot analysis (Fig. 5B). The data confirmed the IHC findings and demonstrated that post-treatment with Lac could inhibit APAP-induced hypoxia in the liver.

We hypothesized that the Lac-mediated amelioration of impaired hepatic microcirculation during AILI was due to its protection against APAP-induced LSEC cytotoxicty. Mice were treated with either APAP/PBS or APAP/Lac, as described in Fig 1A. LSEC were purified from NPC isolated at 3 or 5 h after APAP challenge. The data revealed a significant decrease in the number of LSEC at both time points after APAP challenge (Fig. 6A), compared with those in PBS-treated control mice. This decline in LSEC number was reversed upon Lac treatment, suggesting that Lac inhibits APAP-induced LSEC cytotoxicity. To determine whether Lac exerts direct cytoprotection on LSEC, the cells were purified and treated *in vitro* with APAP alone or APAP in the presence of Lac. Analysis of cell viability by the MTT assay revealed that Lac was unable to improve the APAP-induced LSEC damage (Fig. 6B).

A previous study demonstrated that APAP could cause direct toxicity and GSH depletion in LSEC isolated from C3H, but not from Swiss Webster mice, [29] suggesting a strain dependent variation in SEC susceptibility to APAP-induced cytotoxicity. Our data showed that the CYP2E1 expression level was lower in SEC isolated from C57Bl/6J than in those

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from Balb/cJ mice (Fig. 2S). Furthermore, measurement of hepatic RBC accumulation revealed a 7-fold increase in the number of RBC above the baseline in C57Bl/6J mice (Fig. 2S), as opposed to a 17-fold increase in Balb/cJ mice (Fig. 4B). Consistent with the lower degree of hepatic RBC congestion, we also found that Lac treatment had less protective effect in C57BI/6J mice (Fig. 2S). These results suggest that Lac exerts greater protective effects in mouse strains with more severe LSEC damage and hepatic congestion.

The hepato-protective effect of Lac is dependent on hepatic KC

The lack of a direct protection of LSEC by Lac in conjunction with a previous report that Lac can bind to and activate macrophages [17,30] led to our hypothesis that Lac-induced LSEC protection may be mediated via hepatic KC. To examine this possibility, male Balb/cJ mice were i.v. injected with liposome/clodronate to deplete KC or empty liposomes as control. After 2 days, each group of mice were further treated with either APAP/PBS or APAP/Lac as described above. The data revealed that the APAP-induced decrease in LSEC number was reversed by Lac in empty liposome-treated KC-intact mice, but not in liposome/clodronate-treated KC-depleted mice (Fig. 7A). The role of KC in the Lac-induced protection of endothelial integrity was evaluated by the accumulation of RBC within the liver. Lac treatment was effective in reverse the hepatic congestion caused by APAP challenge in the presence of KC but not in their absence (Fig. 7B). These results indicate an important role for hepatic KC in mediating the protective effect of Lac against APAP-induced LSEC injury. Furthermore, the data revealed that the hepato-protective role of Lac in the attenuation of AILI was only possible in the presence of KC (Fig. 7C).

Consistent with previous reports demonstrating a protective effect of KC in AILI, [22] our data showed that KC-depleted mice were much more susceptible to APAP-induced hepatotoxicity compared to KC-intact mice (data not shown). Therefore, in an attempt to achieve similar levels of toxicity in both groups of mice, we decreased the dose of APAP from 300mg/kg in KC-intact mice to 250mg/kg in KC-depleted mice. These results indicated a crucial role of KC in mediating the hepato-protective effect of Lac during AILI.

Lac treatment induces up-regulation of eNOS

It has been demonstrated that eNOS-derived NO regulates hepatic perfusion and is protective to the liver. [31,32] Evidence also suggests that eNOS-derived NO play a protective role in liver microcirculation and inhibit AILI; on the other hand, iNOS, which catalyzes a much greater amount of NO, contributes to AILI.[33] To determine whether Lac treatment may affect eNOS expression and activity, male Balb/cJ mice were treated with either APAP/PBS or APAP/Lac, as described in Fig 1A. Liver tissues were harvested, NPC were isolated and LSEC were purified at 5 h after APAP challenge. The data demonstrated that eNOS activities in NPC, as well as the mRNA expression levels of eNOS in both liver tissues and purified LSEC were significantly higher in APAP/Lac-treated than APAP/PBS-treated mice (Fig. 8).

Discussion

APAP overdose, following either accidental overdose or attempted suicide, can cause severe liver damage. [2] The proportion of acute liver failure cases attributed to APAP rose from 28% in 1998 to 51% in 2003, [34] making APAP the most frequent cause of drug-induced liver failure in the U.S. Current treatment for APAP-induced liver failure is administration of NAC, as it replenishes GSH levels and detoxifies NAPQI. However, the effectiveness of NAC declines sharply over time following APAP overdose, with the potential to cause adverse reactions. [3,4] Therefore, the identification of additional therapeutic agents targeting unique molecular and cellular pathways remains a critical research endeavor. The present

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report describes the hepato-protective effect of Lac in the mouse model of AILI. Our data demonstrated that Lac treatment concurrent with APAP administration could prevent AILI. Furthermore, the use of Lac as a rescue therapy, when administered following APAP overdose was also able to significantly decrease APAP-induced hepatotoxicity (Fig. 1).

AILI is believed to be caused by the metabolic generation of NAPQI. Therefore, we first examined whether the hepato-protective effect of Lac could be due to the inhibition of NAPQI formation. Immunoblot analysis of NAPQI-protein adducts demonstrated that Lac did not alter APAP metabolism (Fig. 2). This result is not surprising, as Lac was not administered until 1 h post-APAP challenge, a time point at which the generation of NAPQI has already peaked. [35] Due to the high affinity of Lac for iron, we examined whether the hepato-protective effect of Lac was dependent on its iron-binding functionality. Our data revealed that the iron-saturated holo-Lac was as effective as the non-holo protein in protection against AILI (Fig. 1S), which would argue against a role of iron-chelating in the mechanism of Lac-mediated hepato-protection. Several studies evaluating the effect of iron-chelating agents, such as deferoxamine (DFO), on AILI failed to demonstrate a clear effectiveness of iron-chelation in protecting against AILI, [25,26,36] negating the importance of an iron-catalyzed oxidative reaction in the pathogenesis of AILI. Furthermore, accumulating evidence suggests that many antimicrobial, anti-viral and anti-inflammatory effects of Lac are independent of its iron-binding capacity. [17,37,38] Our data and these studies collectively support that the iron-binding function of Lac dose not attribute to its hepato-protective function.

Lac has been reported to have a variety of cytoprotective functions, including the ability to reduce oxidative stress. [18] APAP induces oxidative stress, whose mechanistic role in APAP hepatotoxicity has been supported by *in vitro* studies using cultured hepatocytes. [39] Therefore, we examined whether Lac could directly protect hepatocytes against APAP-induced cytotoxicity. The data revealed that Lac was unable to rescue primary hepatocytes from APAP-induced toxicity, as evaluated by both AST release and MTT cell viability assay (Fig. 3). This result argues against a direct effect of Lac on hepatocytes; however, it is possible that Lac protects hepatocytes *in vivo* through stimulating KC to produce protective mediators. The amounts of APAP (10 mM) used in the *in vitro* experiments are comparable to, if not even higher than the *in vivo* dose of APAP (300 mg/kg, equates to 10 mM, assuming 4 mL total blood volume in mice). However, results from both the AST measurement and MTT assay revealed that although 10 mM APAP caused direct cytotoxicity to hepatocytes *in vitro*, this toxicity was not substantial, therefore, suggesting that additional mechanism(s) besides direct hepatocyte injury may play an important role in the pathogenesis of AILI *in vivo*.

The maintenance of hepatic microcirculation in the presence of a toxic insult is critical to halt the progression of tissue damage. The adequate delivery of oxygen, amino acids, cofactors, and substrates to parenchymal cells can aid GSH replenishment, maintain mitochondrial function and energy production, and facilitate the repair and regeneration of damaged hepatocytes. An accumulating body of evidence suggests that LSEC damage and disruption of the hepatic microcirculation represent key factors in the mechanisms of several models of liver injury. [31,40,41] In the pathogenesis of AILI, it has been demonstrated that damage to LSEC and impairment of hepatic microcirculation precede NAPQI-induced direct hepatocellular damage, [8,9] and ample evidence suggests that the amelioration of LSEC injury can reduce the extent of AILI. [11–14] Consistent with these findings, we observed that, compared with Balb/cJ, C57Bl/6J mice exhibited a smaller extent of hepatic RBC congestion after APAP challenge and were protected by Lac to a lesser degree (Fig. 2S). This strain-dependent variation may be explained by the differential expression levels of CYP2E1 by LSEC (Fig. 2S), resulting in variable susceptibility to APAP-induced

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cytotoxicity. Collectively, these reports suggest that APAP-induced LSEC toxicity, resulting in microcirculation disorder, plays a key role in the pathogenesis of AILI and represents a potential therapeutic target in the treatment of AILI. Data presented in this study clearly demonstrated that the post-APAP treatment of mice with Lac attenuated APAP-induced LSEC damage and ameliorated microcirculatory impairment. The number of viable LSEC isolated from the liver increased significantly in mice treated with APAP/Lac compared with that in mice treated with APAP/PBS (Fig. 6A). The protection of LSEC by Lac resulted in an inhibition in the loss of sinusoidal endothelial integrity caused by APAP challenge. Our data revealed that both the resultant hemorrhage and Evans blue dye accumulation within the liver following APAP challenge were significantly alleviated by Lac treatment (Fig. 4B&C). APAP-induced impairment of sinusoidal perfusion was also mitigated by Lac, as demonstrated by the more homogeneous distribution of QD705 within the sinusoids (Fig. 4A), as well as the significant decrease in the extent of hepatic hypoxia (Fig. 5) within the liver of mice treated with APAP/Lac, compared to those treated with APAP/PBS.

Our data confirmed that the *in vitro* treatment of LSEC, purified from naïve mice, with APAP, can induce cytotoxicity (Fig. 6B), which is consistent with previous reports. [29] However, we did not observe a direct protection of LSEC by Lac in our *in vitro* experiments (Fig. 6B), suggesting that LSEC protection by Lac may be mediated by hepatic KC. Our data demonstrated that in the absence of KC, Lac no longer provided a protective effect of LSEC (Fig. 7A), nor attenuation of AILI (Fig. 7C). These results strongly indicate a crucial role for KC in mediating the hepato-protective function of Lac. Furthermore, our data demonstrated that Lac treatment caused up-regulation of eNOS mRNA expression as well as eNOS activities (Fig. 8). These data and literature reports support three possible mechanisms by which Lac could positively regulate eNOS-derived NO levels. First, eNOS expression and activity are preserved in APAP/Lac-treated mice compared with APAP/PBS-treated mice due to Lac-mediated LSEC protection through the activation of KC. Second, KC activation by Lac may induce or up-regulate eNOS activity in these macrophages. Although it is not known whether KC express eNOS, it has been reported that alveolar macrophages constitutively express eNOS, which produces low levels of NO, and as such activation of these cells by lung surfactant could enhance eNOS activity and NO production. [42] Third, it is possible that Lac-activated KC up-regulate the expression and activity of eNOS in LSEC. It has been demonstrated that injection of adenoviral vectors to mice causes activation of hepatic KC, which in turn release soluble factors to activate endothelial cells and promote an increase in eNOS activity. [43] A study of microvascular perfusion following hepatectomy in mice also revealed an important role for KC in the up-regulation of eNOS protein expression and down-regulation of the eNOS inhibitory protein caveolin-1. [44]

In summary, the present study is the first to demonstrate a significant protective effect of Lac against AILI in mice. The data showed that Lac did not appear to directly protect hepatocytes from APAP-induced cytotoxicity. The post-treatment of Lac inhibited the loss of LSEC due to APAP challenge *in vivo*, up-regulated eNOS expression and activity, and significantly attenuated APAP-induced vascular leakage and impairment of sinusoidal perfusion. Moreover, these effects of Lac, as well as the Lac-induced mitigation of AILI were abrogated in KC-depleted mice, suggesting a critical role of KC in mediating the hepato-protective function of Lac. Although Lac is currently in clinical trials for the treatment of infection, cancer and autoimmune diseases, the therapeutic potential of Lac in the treatment of AILI has not been investigated. Our finding of the hepato-protective effect of Lac, with its known favorable toxicity profile, points to a great potential for Lac in the treatment of AILI, as well as in the prevention of AILI due to the addition of Lac in the formulation of APAP. Findings from the current study also suggest that targeting hepatic microcirculation disorder is a valid therapeutic strategy in treating AILI and potentially other types of liver diseases.

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Supplementary Material

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Abbreviations

APAP	acetaminophen
NAC	N-acetylcysteine
AILI	acetaminophen-induced liver injury
NAPQI	N-acetyl-*p*-benzoquinone imine
GSH	glutathione
LSEC	liver sinusoidal endothelial cells
RBC	red blood cell
HA	hyaluronic acid
MMP	matrix metalloproteinase
NO	nitric oxide
Lac	Lactoferrin
DILI	drug-induced liver injury
i.p	intraperitoneally
i.v	intravenously
KC	Kupffer cells
liposome/clodronate	liposome-entrapped clodronate
ALT	alanine transaminase
H&E	Hematoxylin and eosin
IHC	immunohistochemical
NPC	nonparenchymal cell
AST	aspartate aminotransferase
holo-Lac	iron-saturated Lactoferrin
non-holo-Lac	non-iron saturated Lactoferrin
DFO	deferoxamine
SOS	sinusoidal obstruction syndrome
TIMP-1	tissue inhibitor of metalloproteinases-1

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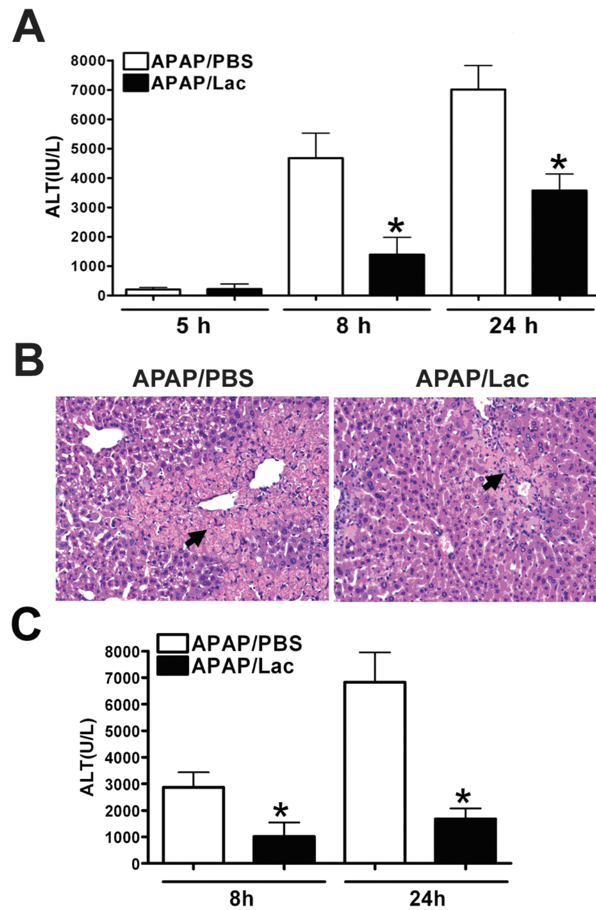

Figure 1. Lac post-treatment protected mice against AILI

Male Balb/cJ mice were i.v. injected with Lac (50 mg/kg, APAP/Lac) or PBS (APAP/PBS) at 1 and 4 h after APAP challenge (300 mg/kg). (A) Blood was collected at 5, 8 and 24 h after APAP injection for serum ALT measurement. (*) $P < 0.05$, compared with APAP/PBS-treated mice. The results shown represent mean ± SEM of more than 7 mice per group. (B) Liver tissues were collected at 24 h after APAP treatment. Photomicrographs (200X, final magnification) of H&E stained liver sections from APAP/PBS-and APAP/Lac-treated mice are shown. Arrows indicate necrotic areas. (C) Male Balb/cJ mice were i.v. injected with Lac (50 mg/kg, APAP/Lac) or PBS (APAP/PBS) at 1, 4 and 8 h after APAP challenge (300 mg/kg). Blood was collected at 8 and 24 h after APAP injection for serum ALT measurement. (*) $P < 0.05$, compared with APAP/PBS-treated mice. The results shown represent mean ± SEM of 8 mice per group.

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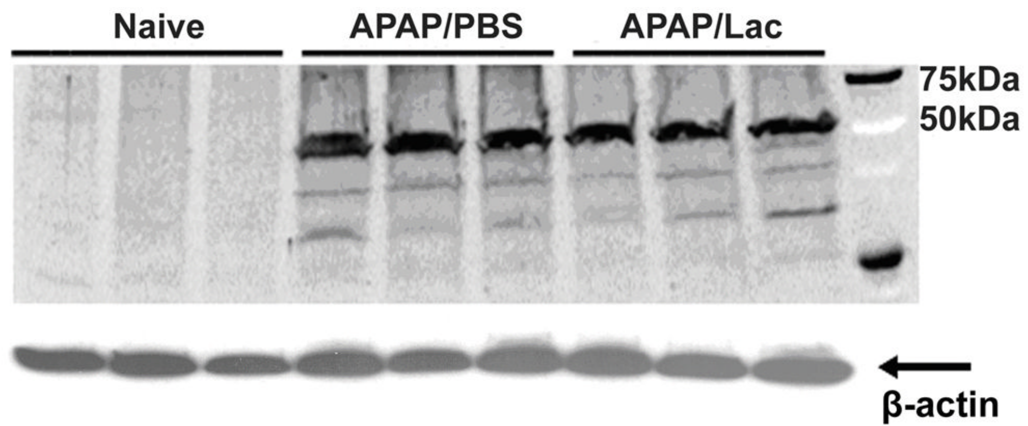

Figure 2. Lac did not affect APAP metabolism

Male Balb/cJ mice were treated with APAP/Lac or APAP/PBS as described in Fig. 1A. Naive mice (treated with PBS) were used as a control. Liver tissues were collected 5 h after APAP challenge. NAPQI-protein adducts were detected by immunoblot analysis, using a rabbit polyclonal anti-APAP antisera (1:500 dilution; gift from Dr. Lance Pohl, NIH, Bethesda, MD). Molecular mass markers (kDa) are indicated on the right. β-actin served as a loading control, as indicated by arrow.

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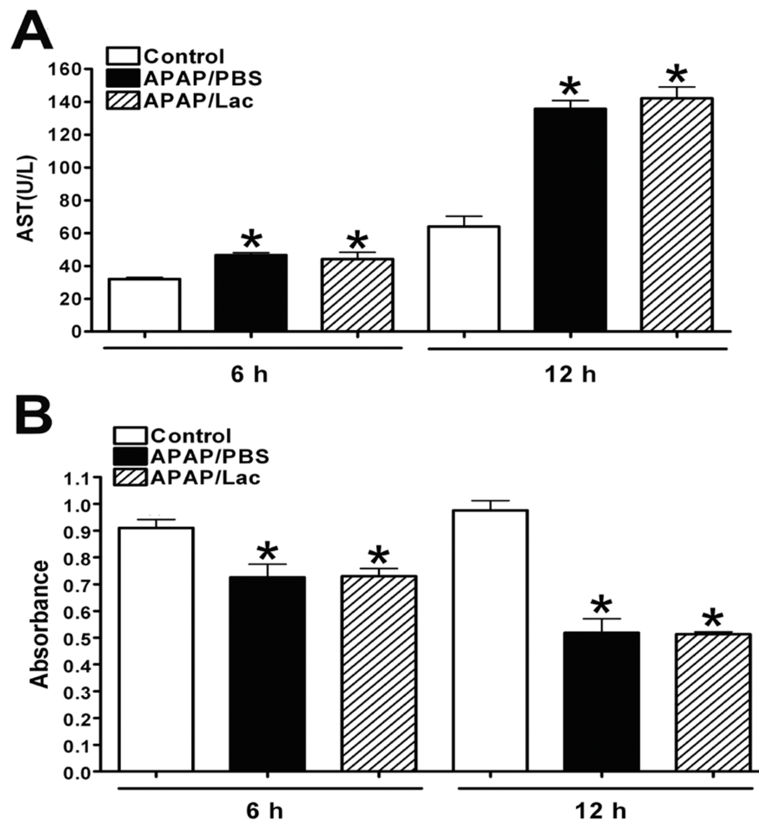

Figure 3. Lac did not directly protect against APAP-induced hepatocyte toxicity *in vitro*
Hepatocytes were isolated from naïve male Balb/cJ mice and cultured in 96-well plates (3×10^4 cells/well). The cells were treated with 10 mM of APAP in the absence (APAP/PBS) or presence of Lac (200 μg/ml, APAP/Lac) for 6 or 12 h. Non-treated cells serve as control. (A) AST levels were measured from culture supernatants. (B) Cell viability was determined by MTT assay. (*) $P < 0.05$, compared with control cells. There was no significant difference between APAP/PBS-and APAP/Lac-treated cells. The results shown represent mean ± SEM of more than 3 samples per group.

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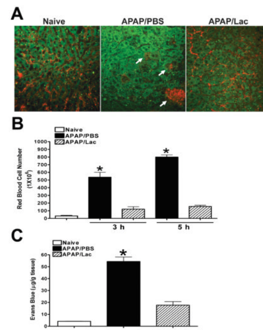

Figure 4. Lac attenuated the APAP-induced impairment of hepatic sinusoid perfusion and hepatic accumulation of RBC and Evans blue

Male Balb/cJ mice were treated with APAP/PBS or APAP/Lac, as described in Fig 1A. Naïve mice (PBS-treated) were used as a control. (A) Five hours after APAP challenge, the mice were i.v. injected with 100 µL QD705 (1:5 dilution in PBS) immediately prior to two-photon imaging. The livers were gently exteriorized onto a glass plate. Liver sinusoids were visualized using an inverted LSM 510 NLO multiphoton microscope (400 x, final magnification). Arrows in APAP/PBS panel indicate clusters of QD705. The data shown are representative of 3 mice per group. (B) Three or five hours after APAP challenge, the livers were perfused for 1 min to remove blood within the circulation. Liver tissues were disrupted, and single cell suspensions filtered through a 100 µm cell strainer. Dead cells and hepatocytes were removed using 34% Percoll. The remaining RBC were counted using light microscopy. (*) $P < 0.05$ compared with naïve and APAP/Lac-treated mice. The results shown represent mean ± SEM of 3 mice per group. (C) Four hours after APAP challenge, mice were i.v. injected with 20 mg/kg Evans blue dye 1 h prior to sacrifice. The amount of the Evans blue retained within the liver was determined. (*) $P < 0.05$ compared with naïve and APAP/Lac-treated mice. The results shown represent mean ± SEM of 4 mice per group.

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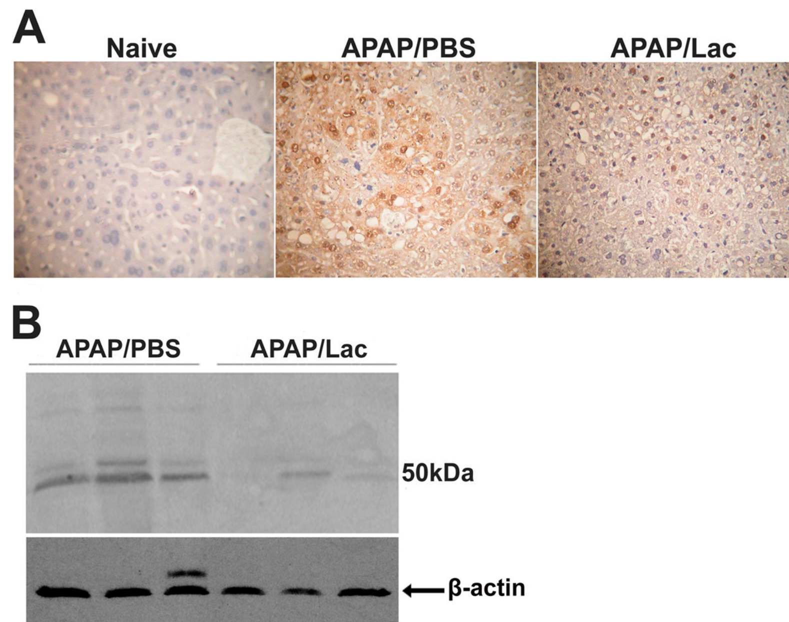

Figure 5. Lac decreased the APAP-induced hypoxia within the liver

Male Balb/cJ mice were treated with APAP/PBS or APAP/Lac as described in Fig. 1A. Naïve mice (PBS-treated) were used as a control. Liver tissues were collected at 5 h after APAP challenge. (A) Formalin-fixed, paraffin-embedded tissue sections were stained with Hypoxyprobe™-1 to evaluate hypoxia. Photomicrographs (400X, final magnification) of liver sections from PBS-treated (naïve), APAP/PBS-, and APAP/Lac-treated mice are shown. The data shown are representative from 4 mice per group. (B) Tissue homogenates were examined for hypoxia by immunoblot analysis, using Hypoxyprobe™-1. β-actin served as a loading control, as shown by arrow. The data shown are representative of 3 mice per group.

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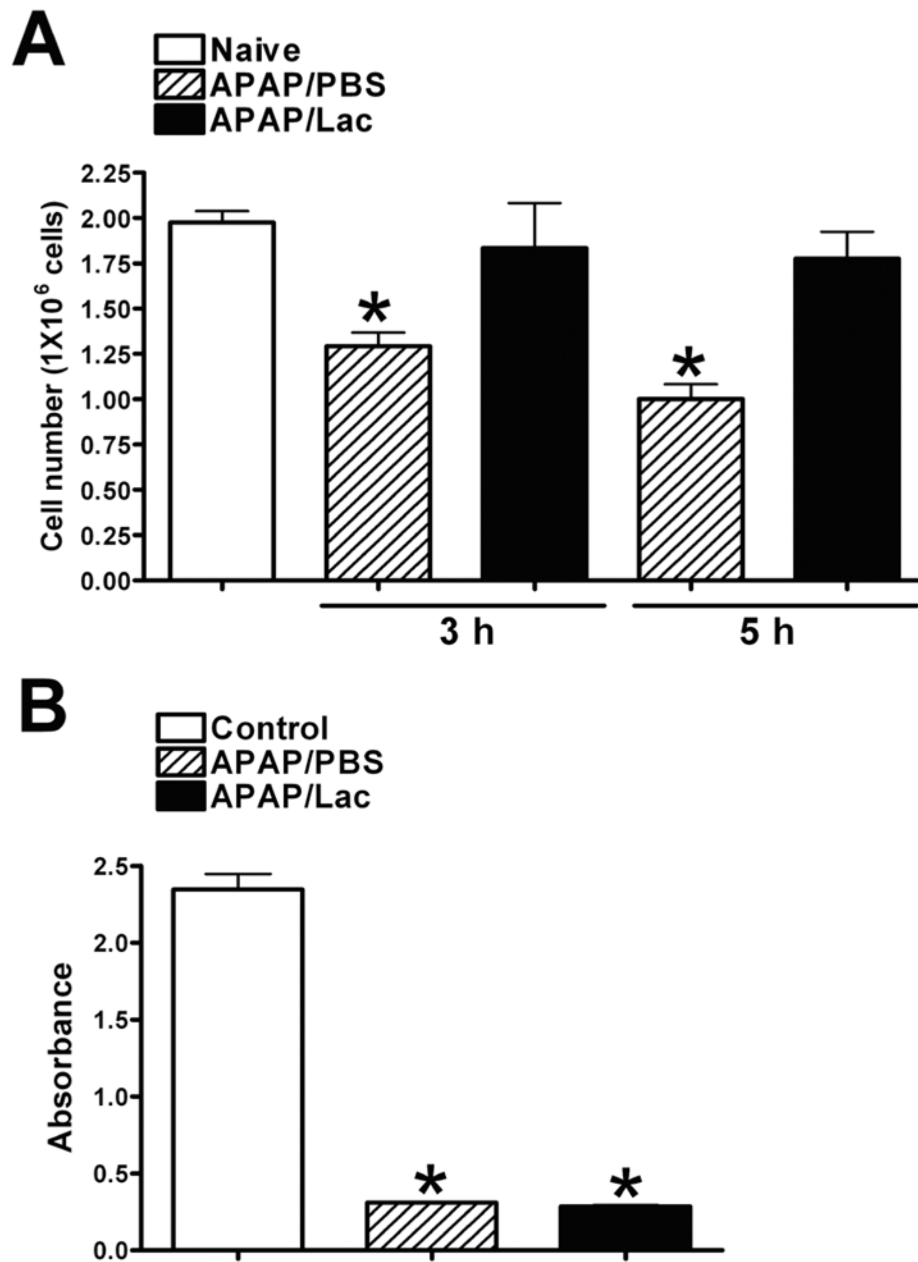

Figure 6. Lac inhibited the APAP-induced loss of LSEC *in vivo* but did not directly protect LSEC against APAP-induced cytotoxicity *in vitro*

(A) Male Balb/cJ mice were treated with APAP/Lac or APAP/PBS as described in Fig 1A. LSEC were isolated at 3 or 5 h after APAP challenge or from PBS-treated mice (naïve) as a control. The total numbers of LSEC within each group was determined. (*) $P < 0.05$, compared with APAP/Lac-treated and naïve mice. The results shown represent mean ± SEM of more than 3 mice per group. (B) LSEC were isolated from naïve Balb/cJ mice. Cells were treated with APAP (10mM) in the presence or absence of Lac (200μg) for 10 h, and cell viability was determined by the MTT assay. (*) $P < 0.05$, compared with control cells. There was no significant difference between APAP/PBS-and APAP/Lac-treated cells. The results shown represent mean ± SEM of 4 samples per group.

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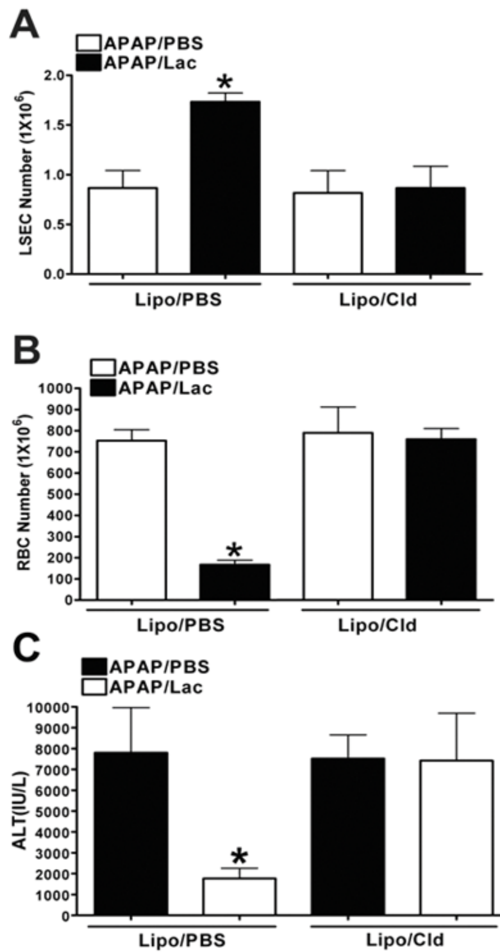

Figure 7. Lac inhibited the APAP-induced hepatic congestion, loss of LSEC and liver injury in KC-intact, but not KC-depleted mice

Male Balb/cJ mice were i.v. injected with liposome/clodronate (Lipo/Cld) or empty liposomes (Lipo/PBS). After 2 days, mice were i.v. injected with Lac (50 mg/kg, APAP/Lac) or PBS (APAP/PBS) at 1 and 4 h after APAP challenge (250 mg/kg for Lipo/Cld-treated group, 300mg/kg for Lipo/PBS-treated group). (A & B) Mice were sacrificed 5 h after APAP challenge. The total numbers of LSEC (A) and RBC (B) within the livers of each group were counted. (*) $P < 0.05$, compared with APAP/PBS-treated mice in Lipo/PBS-treated groups. The results shown represent mean ± SEM of 3 mice per group. (C) Blood was collected at 24 h after APAP challenge for serum ALT measurement. The results shown represent mean ± SEM of 5 mice per group. (*) $P < 0.05$, compared with APAP/PBS-treated mice in Lipo/PBS-treated groups.

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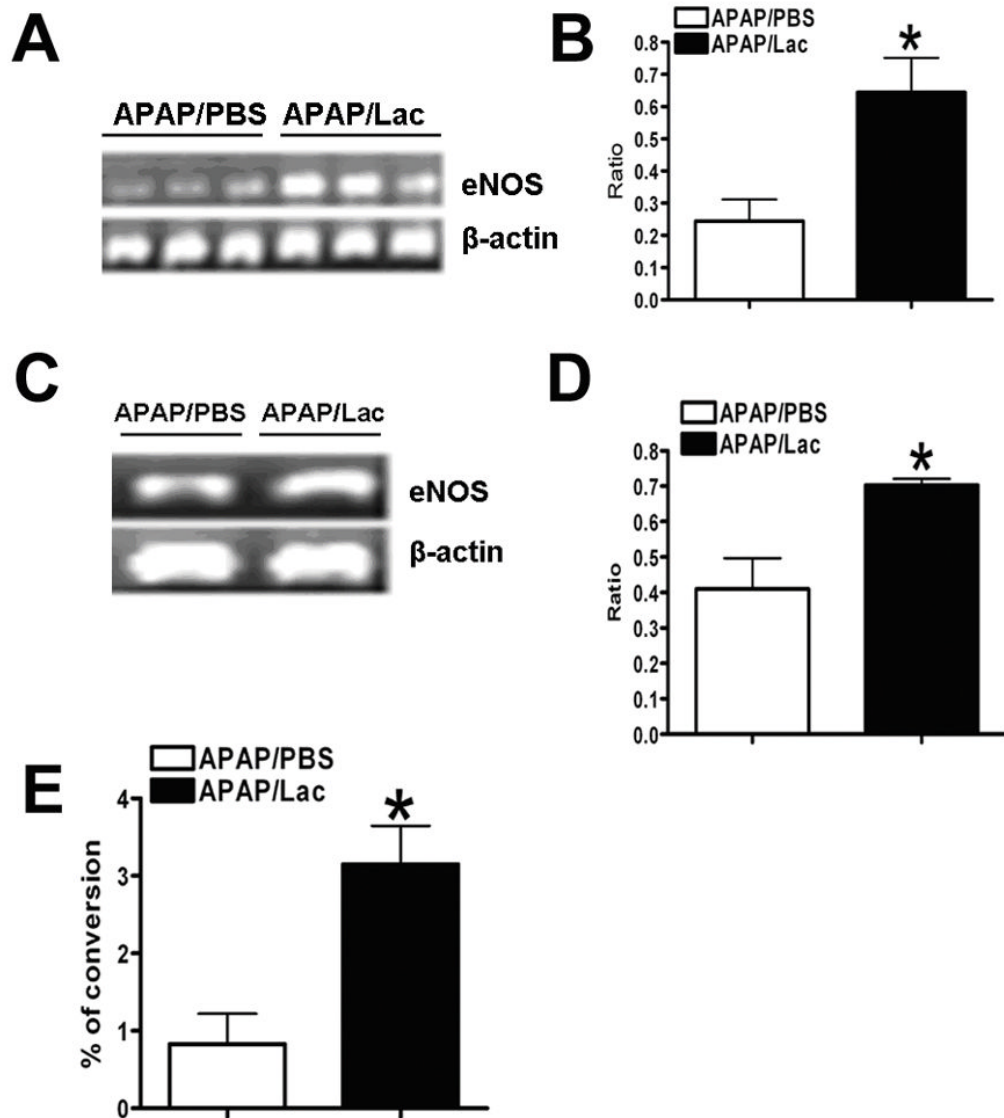

Figure 8. Lac up-regulated eNOS expression and activity

Male Balb/cJ mice were treated with APAP/Lac or APAP/PBS as described in Fig. 1A. Liver tissues were harvested, NPC were isolated and LSEC were purified at 5 h after APAP challenge. (A and B) The mRNA expression levels of eNOS in liver tissues. Results represent mean ± SEM of 6 mice in each group. * $P < 0.05$, compared with liver tissues from APAP/PBS-treated mice. (C and D) Liver NPC were isolated and pooled from 3 mice per group. LSEC were further purified, and RNA was extracted from the cells. Data shown in panel C represent three independent experiments producing similar results. Data from all three independent experiments were combined and relative eNOS expression levels were quantified by densitometry. * $P < 0.05$, compared with LSEC isolated from APAP/PBS-treated mice. (E) eNOS activities in NPC were determined by measuring the conversion from [^{3}H]-arginine to [^{3}H]-citrulline using an NOS activity assay kit. The results shown represent mean ± SEM of 6 mice per group. (*) $P < 0.05$, compared with NPC obtained from APAP/PBS-treated mice.