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Liver X receptor α activation with the synthetic ligand T0901317 reduces lung injury and inflammation after hemorrhage and resuscitation via inhibition of the NF- κ B pathway

Patrick D. Solan¹, Giovanna Piraino², Paul W. Hake², Alvin Denenberg², Michael O'Connor², Alex Lentsch¹, and Basilia Zingarelli²

¹Department of Surgery, University of Cincinnati, Cincinnati USA

²Division of Critical Care Medicine, Cincinnati Children's Hospital Medical Center, Cincinnati USA

Abstract

Liver X Receptor α is a nuclear transcription factor that regulates lipid metabolism. Recently, it has been shown that activation of LXR α with synthetic ligands has anti-inflammatory effects in atherosclerosis and chemical-induced dermatitis. Here, we investigated the effect of the LXR α agonist T0901317 on lung inflammation in a rodent model of hemorrhagic shock. Hemorrhagic shock was induced in male rats by withdrawing blood to a goal mean arterial blood pressure of 50 mmHg. Blood pressure was maintained at this level for 3 hours, at which point rats were rapidly resuscitated with shed blood. Animals were then treated with T0901317 (50 mg/kg) or vehicle intraperitoneally and sacrificed at 1, 2 and 3 hours after resuscitation. Treatment with T0901317 significantly improved the cardiac and stroke volume indices as well as heart rate of rats during the resuscitation period as compared to vehicle-treated rats. T0901317-treated animals showed significant improvement in the plasma level of lactate, while base deficit and bicarbonate levels both trended towards improvement. T0901317-treated animals also showed lower levels of the plasma cytokines and chemokines MCP-1, MIP-1 α , TNF- α , KC and IL-6. Lung injury and neutrophil infiltration were reduced by treatment with T0901317 as evaluated by histology and myeloperoxidase assay. At molecular analysis, treatment with T0901317 increased nuclear LXR α expression and DNA binding while also inhibiting activation of NF- κ B, a pro-inflammatory transcription factor, in the lung. Thus, our data suggest that LXR α is an important modulator of the inflammatory response and lung injury after severe hemorrhagic shock, likely through the inhibition of the NF- κ B pathway.

Keywords

Hemorrhage; liver X receptor α ; nuclear factor- κ B; acute lung injury; T0901317

INTRODUCTION

Traumatic injury and subsequent hemorrhage continue to be a major worldwide health concern. Hemorrhage is the second-leading cause of mortality amongst all trauma victims, leading to 30–40% of all deaths in injured patients (1). While many deaths occur in the pre-hospital phase, some patients reach the hospital and enter the resuscitative phase of care. As a result of injury and hemorrhage, these patients show a massive inflammatory response. This inflammatory response results in the release of many cytokines and chemokines as well

as activation of the innate immune system. The initiation of this cascade can quickly change from adaptive to maladaptive and lead to further injury including multi-organ failure (MOF) and death (2–4). Given the severity of the response and its consequences, down-regulation of this inflammatory cascade has been a focus of research in attempts to avoid the MOF and death often encountered after hemorrhage and resuscitation.

The Liver X Receptor (LXR) is a ligand-dependent nuclear transcription factor that is a regulator at the intersection between metabolism and inflammation (5–9). There are two sub-types of LXR, α and β . While LXR β is expressed ubiquitously, LXR α expression is confined to select organs including the spleen, liver, intestine, adipose and lung. LXR α can be activated by natural ligands, mainly oxysterols, or synthetic ligands such as T0901317 or GW3965. Once activated, the nuclear receptor forms a heterodimer with Retinoid X Receptor (RXR). This heterodimer then binds to the LXR response element (LXRE) on DNA, leading to transcription of various genes important in cholesterol metabolism. (5–9).

Several in vitro studies have shown that LXR agonism not only regulates cholesterol metabolism, but also exerts anti-inflammatory effects. It has been shown that activation of LXR α decreases pro-inflammatory cytokine levels following endotoxin exposure. These cytokines include tumor necrosis factor α (TNF α), inducible nitric oxide synthase (iNOS), interleukin-6 (IL-6), cyclooxygenase-2 (COX-2), interleukin-1 β (IL-1 β), macrophage inflammatory protein 1 α (MIP-1 α), matrix metalloproteinase-9 (MMP-9) and monocyte chemoattractant protein 1 (MCP-1) (10–13). In addition to these in vitro anti-inflammatory effects, LXR α agonism has also been shown to decrease inflammation in vivo. In models of contact dermatitis, tissue inflammation was markedly reduced with LXR α activation (11, 14). Further studies have also shown in vivo reduction of lung neutrophil infiltration after exposure to irritants and LPS (15, 16).

The exact mechanism through which LXR α activation reduces inflammation remains unclear, but many studies point to a regulation of the Nuclear Factor- κ B (NF- κ B) pathway as a possible mechanism. NF- κ B is a pro-inflammatory transcription factor that initiates transcription of a multitude of pro-inflammatory mediators, including cytokines, chemokines and adhesion molecules (17). It has long been acknowledged that NF- κ B is a key regulator in the inflammatory response and would be an ideal target for therapeutic intervention aimed at preventing the overwhelming inflammation seen after insults such as hemorrhagic shock (18). We have previously shown that some of the anti-inflammatory properties of LXR α activation may be secondary to reduction of NF- κ B activity. Our in vitro study showed that LXR agonism prevented the production of pro-inflammatory cytokines by reducing I κ B alpha degradation and subsequent NF- κ B activation in macrophages (19).

These promising results suggest that LXR α activation could be a key component in reduction and control of the overwhelming inflammatory response following hemorrhagic shock. Our aim in this study was to explore the anti-inflammatory effects of the LXR α agonist T0901317 in a rodent model of hemorrhagic shock. We hypothesized that activation of LXR α by T0901317 would stem the systemic inflammatory response via inhibition of the NF- κ B pathway and subsequently reduce hemodynamic abnormalities and hemorrhage-induced lung injury.

MATERIALS AND METHODS

Measurement of hemodynamic parameters

All aspects of this study complied with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23 revised 1996) and met approval of the Institutional Animal Care and Use Committee. Male

Wistar rats (Charles River Laboratories, Wilmington MA) weighing between 240–310 grams were subjected to hemorrhagic shock. Each animal was anesthetized using intraperitoneal pentobarbital (80 mg/kg). Tracheostomy was then performed and the animal was ventilated at a respiratory rate of 60 breaths per minute, tidal volume of 7 mL/kg and FiO_2 of 0.4 using a rodent ventilator (Harvard Apparatus, Holliston MA). Temperature was maintained at 37° C using a homeothermic blanket. The left carotid artery and right femoral artery were then cannulated with Polyethylene-50 tubing. For cardiac output measurement, polyethylene-10 tubing was inserted into the right internal jugular vein as well. Cardiac output (mL/min) was measured using a thermodilution technique (20). A thermistor was passed into the left carotid artery to the carotid arch. 0.15 mL of normal saline at room temperature was then rapidly injected into the right internal jugular vein. Heart rate (HR), mean arterial blood pressure (MABP) and cardiac output were measured using a Maclab A/D Converter and cardiac output pod (AD Instruments, Milford MA). The cardiac index (CI, mL/min/100g), total peripheral resistance index (TPRI, mmHg/mL/min/100g) and stroke volume index (SVI, mL/100g) were then calculated from computed integral values of thermodilution curves using standard arithmetic formulae.

Hemorrhagic shock model

After completion of the surgical procedure, rats were dosed with intravenous heparin to facilitate hemorrhage (100 IU/kg). Hemorrhagic shock was then induced using a pressure-controlled model as previously described (21). Blood was steadily withdrawn from the femoral arterial catheter until a MABP of 50 mmHg was obtained. This MABP was then maintained for a period of three hours by withdrawing or re-instilling small volumes of shed blood. After three hours of shock state, shed blood was rapidly re-infused over 5 minutes to resuscitate the animal. If re-transfusion of small volumes of blood were needed during the hypoperfusion period to maintain MABP at 50 mmHg, rapid resuscitation at the conclusion of hemorrhage was performed by transfusing the remaining shed blood supplemented with Ringer Lactate solution to a final volume of fluids equal to the initial total shed blood.

Animals were then randomly divided into three groups: 1) Rats in the vehicle hemorrhagic shock group received vehicle (100% dimethyl sulfoxide) instead of T0901317 (N=18). 2) Rats in the treatment group received T0901317 at a 50 mg/kg dose (N=16). 3) Sham operated animals served as control at time=0 and underwent the same surgical procedure but were not bled (N=4). T0901317 and vehicle were delivered intraperitoneally (i.p.) as a bolus at the beginning of resuscitation (180 minutes) and every hour thereafter for a maximum of three doses. Rats were sacrificed at 1, 2 and 3 hours post-resuscitation. Plasma and lung samples were collected for histologic and biochemical studies.

Plasma lactate, base deficit and bicarbonate levels

Plasma levels of lactate, base deficit and bicarbonate were measured at times 0, 3 and 6 hours using a commercially available i-Stat system (Abbott Point of Care, Princeton, NJ).

Plasma levels of cytokines and chemokines

Plasma levels of MIP-1 α , TNF α , IL-6, interleukin –10 (IL-10), KC, and MCP-1 were analyzed using a luminex multiplex system (Luminex Corporation, Austin TX) according to instructions from the manufacturer.

Plasma Cholesterol

Plasma levels of total cholesterol were measured by enzymatic procedures using a commercially available kit (Wako Diagnostics, Richmond VA).

Histology

Lung tissue was harvested and placed immediately in 10% neutral buffered formalin. The tissue was then embedded in formalin, sectioned and stained with hematoxylin and eosin. Light microscopy was used to evaluate cross-sections for tissue damage and inflammation.

Myeloperoxidase assay

Myeloperoxidase was measured as an indication of neutrophil infiltration in lung tissue following hemorrhagic shock. Lung tissue was homogenized in a buffer containing 0.5% HTAB in 10 mM MOPS. After homogenization, tissue was centrifuged for 30 min at 4,000 rpm at 4° C and supernatant was collected. Supernatant was then mixed with 3,3,5,5-tetramethylbenzidine and sodium phosphate buffer pH 5.5 in a 1:20 dilution. After 5 minutes of incubation, 0.1 mM H₂O₂ was added and the reaction was halted 3 minutes later by addition of 2 M acetic acid. Spectrophotometry was used to assess for rate of change of absorbance at 650nm. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol hydrogen peroxide/min at 37°C and was expressed in units per 100 mg of tissue.

Subcellular fractionation and nuclear protein extraction

Lung tissue was homogenized using a polytron homogenizer (Brinkman Instruments, West Orange NY) in a buffer containing 0.32 M Sucrose, 10 mM Tris HCl, 1 mM EGTA, 2 mM EDTA, 5 mM NaN₃, 50 mM NaF, 10 mM Mercaptoethanol, 20 μM Leupeptin, 0.15 μM Pepstatin A, 0.2 mM PMSF, 1 mM sodium orthovanadate and 0.4 nM microcystin. Samples were then centrifuged at 1,000g for 10 min at 4°C. The supernatant was collected as cytosol extract. The pellet was washed in buffer and centrifuged at 3500 rpm for 10 min at 4°C. The supernatant was removed and the remaining pellet was washed in a buffer containing 50 mM Tris-HCl pH 7.5, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1% Triton X-100, 0.5% NP40, 10% Glycerol, dH₂O, 0.2 mM PMSF, 0.1 mM sodium orthovanadate and aprotinin. This solution was then centrifuged at 15,000 g for 30 min at 4°C. The supernatant was then collected as nuclear extract.

Western blot analysis

The nuclear content of LXRα was measured using immunoblot analysis on nitrocellulose membranes using primary antibodies against LXRα and secondary peroxidase-conjugated antibody. Membranes were also probed with primary antibody for β-Actin to ensure equal loading of samples. Immunoreaction was visualized via chemiluminescence on a photographic film. Densitometric analysis was performed using ImageQuant software (Molecular Dynamics, Sunnyvale CA.).

Electrophoretic mobility shift assay

Oligonucleotide probes corresponding to the NF-κB consensus sequence (5'-AGT TGA GGG GAC TTT CCC AGG C-3') or LXRα sequence (5'-GGC AAG AAG TAA CTG TCG GTC AAA TCC T-3') were labeled with γ-[³²P]ATP using T4 polynucleotide kinase and purified in Bio-Spin chromatography columns (BioRad, Hercules CA). A standard amount of nuclear extract protein was incubated in EMSA buffer (12 mM HEPES (pH 7.9), 4 mM Tris-HCl (pH 7.9), 25 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 50 ng/ml poly(d(I-C)), 12% glycerol (v/v), and 0.2 mM PMSF) for 10 minutes. The radio-labeled probe was then added and incubated on ice for another 30 minutes. Samples were loaded into a polyacrylamide gel consisting of 5% acrylamide (29:1 ratio of acrylamide:bisacrylamide) and protein/nucleic acid complexes were resolved using electrophoresis at a constant current of 30 mA for 60 minutes in a buffer of 0.5X TBE (45 mM Tris-HCl, 45 mM Boric Acid, 1 mM EDTA). Gels were then transferred to Whatman

3M paper and dried under a vacuum at 80° C for one hour and exposed to photographic film at -70° C with an intensifying screen. Densitometric analysis was performed using ImageQuant software (molecular dynamics, Sunnyvale CA) (22, 23).

Materials

Primary antibodies directed against LXR α were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The primary anti-body directed against β -Actin was obtained from Abcam (Cambridge MA). The LXR α agonist T0901317 was obtained from Cayman Chemical Company (Ann Arbor MI). The oligonucleotide probes for NF- κ B were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The oligonucleotide probe for LXR α corresponded to a previously published LXR consensus sequence (24) and was synthesized by Invitrogen Corporation (Carlsbad, CA).

Data analysis

Statistical analysis was performed using SigmaStat for Windows Version 3.10 (SysStat Software, San Jose CA). Data are represented as mean $\pm$ SEM of n observations, where n represents the number of animals in each group. Hemodynamic parameters were assessed using a general linear model for repeated measures allowing for missing data. For the remainder of the data, a two-way analysis of variance with Bonferroni or Holm-Sidak correction was used.

RESULTS

Effect of T0901317 on hemodynamics

Rats selected for treatment with vehicle or T0901317 had similar heart rate (HR) (427 ± 9 bpm and 421.9 ± 10 bpm, respectively) and mean arterial blood pressure (MABP) (120.69 ± 3.3 mmHg and 117.7 ± 5 mmHg respectively) at baseline and had similar maximum volumes of blood and percentage of total blood volume withdrawn during the hemorrhagic shock phase (Fig 1, Table 1). The volume of Ringer Lactate solution delivered during resuscitation was also similar between the two groups (Table 1). Following resuscitation, MABP was similar in both vehicle and T0901317-treated groups (Fig. 1A). However, treatment with T0901317 significantly blunted hemorrhage-induced tachycardia at all measured time points when compared to vehicle treated animals ($P < 0.001$) (Fig. 1B). In addition, cardiac index (CI) and stroke volume index (SVI) were all increased in the T0901317-treated animals as compared to the vehicle group. While only certain time points showed significantly higher CI or SVI in T0901317-treated rats *versus* vehicle-treated rats, both CI and SVI were significantly higher in T0901317-treated rats as compared to vehicle-treated rats during the cumulative resuscitative period ($P < 0.005$ and $P < 0.001$, respectively) (Fig. 2).

Effect of T0901317 on total metabolic acidosis

T0901317-treated animals showed fewer signs of metabolic acidosis compared to vehicle-treated animals at 180 minutes post-resuscitation. Although not statistically significant, plasma bicarbonate levels were higher (22.23 ± 1.66 μ g/mL vs 18.9 ± 2.42 μ g/mL) and base deficit was less negative (-3.5 ± 1.67 *versus* -7 ± 3.62); while plasma lactate levels were significantly lower (2.5 ± 0.4 μ mol/mL vs. 4.4 ± 0.94 μ mol/mL, $P < 0.05$) in T0901317-treated animals compared to vehicle-treated animals (Fig. 3).

Effect of T0901317 on lung injury and neutrophil infiltration

At histological examination, hemorrhagic shock resulted in severe lung injury characterized by reduced alveolar space and accumulation of inflammatory cells in vehicle-treated rats. On

the contrary, histological images of rats treated with T0901317 (Fig. 4C) showed preservation of lung architecture and a reduction of neutrophil infiltration when compared to vehicle treated rats at 180 minutes post-resuscitation (Fig. 4B). The degree of neutrophil infiltration in the lungs was further confirmed by measurement of myeloperoxidase activity. At 180 minutes post-resuscitation, there was significantly less myeloperoxidase activity in the lungs of rats treated with T0901317 as compared to vehicle-treated rats (613 ± 34 U/100mg tissue *versus* 728 ± 35 U/100mg tissue, respectively; $P < 0.05$) (Fig. 4D).

Effect of T0901317 on plasma cytokine and chemokine levels

Plasma levels of cytokines and chemokines were measured at 60 and 180 minutes post-resuscitation. There was a significant decrease in plasma concentrations of MCP-1, MIP-1 α , TNF α and KC in T0901317-treated animals when compared to vehicle-treated rats. Although lower than vehicle-treated rats, plasma levels of IL-6 were not significantly different in T0901317-treated rats. Levels of IL-10 were similar in both groups (Fig. 5).

Effect of T0901317 on plasma cholesterol

Since LXR α is a crucial regulator of cholesterol metabolism, we also measured plasma levels of cholesterol. There was a significant increase of plasma total cholesterol concentration in vehicle-treated rats (74.1 ± 6 mg/dL) at 180 minutes post-resuscitation when compared to basal levels of sham control rats (55.2 ± 4.8 mg/dL). Treatment with T0901317 did not significantly change plasma concentration of total cholesterol (77.8 ± 5.1 mg/dL) at 180 minutes post-resuscitation when compared to treatment with vehicle alone ($P = 0.65$).

Effect of T0901317 on LXR α expression and activation

To confirm the mechanism of action of T0901317 on the LXR α pathway, we evaluated both the expression of the receptor as well as its DNA binding at 180 minutes post-resuscitation in lung nuclear extracts. At basal condition, a faint constitutive band was observed in sham animals. After resuscitation, there was an increase in the nuclear expression and DNA binding of LXR α in both vehicle-treated and T0901317-treated rats. However, treatment with T0901317 significantly increased both the nuclear expression and activity of the receptor when compared to vehicle-treated rats ($P < 0.05$) (Fig. 6).

Effect of T0901317 on NF- κ B activation

To explore the role of LXR α activation in the NF- κ B pathway, we investigated the amount of NF- κ B bound to the DNA in rat lung nuclear extracts after hemorrhage and resuscitation. Sham animals showed a faint constitutive band at basal condition. Hemorrhage and resuscitation increased DNA-binding in both vehicle and T0901317-treated animals. However, at 60 minutes post-resuscitation, we found that T0901317-treated rats had significantly decreased binding of NF- κ B in lung nuclear extracts as compared to vehicle-treated rats at the same time point (Fig. 7).

DISCUSSION

Our study has shown that activation of LXR α with the synthetic ligand T0901317 improves the hemodynamic profile of rats subjected to severe hemorrhage and resuscitation. Furthermore, lung injury and neutrophil infiltration are reduced with LXR α activation. This pulmonary protection is seen in conjunction with a decrease in plasma cytokine and chemokines levels. These anti-inflammatory effects are likely due to an inhibition of the NF- κ B pathway. These findings all suggest that LXR α regulates inflammation after a

hemorrhagic insult on a systemic scale, though its effects on organs other than the lungs remains to be explored.

In our study, we used a pressure-controlled model of hemorrhagic shock to standardize the level of hypoperfusion during the hemorrhage phase. After resuscitation, LXR α activation significantly decreased heart rate while also increasing stroke volume and cardiac index. Interestingly, there was no significant change in the MABP post-resuscitation despite these positive findings. One possible explanation for this is the interaction between LXR α and the Renin-Angiotensin System (RAS). Several studies have shown that activated LXR α interacts with the RAS, leading to decreased renin levels and a decreased response to Angiotensin II (25–27). This interaction would limit the ability of animals to vasoconstrict and increase their blood pressure. Concordant with this hypothesis, we did not observe any changes in total peripheral resistance (data not shown) in rats treated with T0901317, despite improvements in other hemodynamic parameters.

Improvement of hemodynamic parameters such as CI and SVI implies that systemic oxygen demand has decreased. However, systemic markers of acidosis are used in conjunction with hemodynamic parameters to clinically assess cellular hypoxia (28). Among the values used are plasma lactate, base deficit and bicarbonate. In our study, we found that activation of LXR α significantly improved plasma lactate and showed a trend towards improvement of bicarbonate and base deficit as compared to vehicle-treated animals. This indicates that therapy with T0901317 improved end organ perfusion and abrogated cellular hypoxia, leading to less metabolic acidosis. However, the role of LXR α activation in glucose utilization cannot be ignored. Several studies have shown that activation of LXR α leads to increased pancreatic insulin secretion, decreased hepatic gluconeogenesis and improved peripheral glucose uptake (7). This improved glucose utilization could lead to improved cellular metabolism and improvement in metabolic parameters of acidosis, especially lactate. In our study, we monitored glucose levels during hemorrhage and resuscitation and found elevated plasma glucose levels after hemorrhage, with reduction of these levels to baseline 180 minutes after resuscitation. However, there was no significant difference in these plasma glucose levels at any time point in rats treated with T0901317 *versus* vehicle-treated rats (data not shown). This implies that LXR α activation did not significantly change peripheral glucose utilization and the improvement in lactate was indeed a reflection of reduced cellular hypoxia in those rats treated with T0901317.

Decreased concentrations of total cholesterol occur early in course of critical illness, including major trauma and hemorrhage. Low cholesterol levels correlate with the severity of the systemic inflammatory response and high concentrations of pro-inflammatory cytokines (29, 30). Since LXR α is a master regulator of cholesterol synthesis, transport and catabolism (31), we also assessed plasma total cholesterol levels in rats subjected to severe hemorrhage and resuscitation. In contrast to clinical data, which report occurrence of hypocholesterolemia in critically ill patients, we observed that there was an increase of total cholesterol after hemorrhage and resuscitation as compared to basal levels of sham control rats. However, we found no significant impact on total cholesterol levels as a result of treatment with T0901317. This suggests that the anti-inflammatory effects of T0901317 are independent of its cholesterol regulating properties. The increase in total plasma cholesterol seen after hemorrhage and resuscitation in vehicle-treated rats is likely a stress response to severe hemorrhage, aimed at increasing the pre-cursors of cortisol biosynthesis. In support of this hypothesis, a previous study by Daull et al (32) showed a similar elevation of plasma cholesterol during the immediate resuscitative period following autologous blood transfusion amongst rats subjected to hemorrhage and resuscitation. Furthermore, a study by Abarca et al. (33) also showed an increase in plasma cholesterol levels of rats during the early phase of reversible circulatory shock induced by endotoxin injection.

Hemorrhage and resuscitation induces an inflammatory response that includes the infiltration of neutrophils into tissue (2). This infiltration is followed by the release of reactive oxygen species and subsequent tissue damage, which can ultimately lead to acute lung injury and even death (34, 35). Reduction of leukocyte adherence and infiltration into lung tissue has been found to improve survival after severe hemorrhage and resuscitation (36). In our study, we have shown a reduction in hemorrhage-induced neutrophil infiltration and lung injury as assessed by both histology and myeloperoxidase assay. Multiple studies have previously shown LXR α activation to reduce lung injury and neutrophil infiltration following infectious, LPS, or chemical insult to rodent lung (15, 16, 37, 38). Our study is the first report that LXR α activation affords lung protection following hemorrhagic shock. Thus, our findings suggest that LXR α activation not only limits inflammation in lung-specific models, but also exerts pulmonary protective properties during sterile non-infectious systemic inflammation.

Pro-inflammatory cytokine and chemokine release is a hallmark of the inflammatory response after hemorrhage and resuscitation (2, 4). This elevation leads to a systemic inflammatory response and organ dysfunction, including acute lung injury (34). Reduction of cytokine levels are therefore a therapeutic goal aimed at reduction of organ injury, especially pulmonary injury. Several previous studies have shown that LXR α activation reduces levels of these pro-inflammatory cytokines and chemokines after LPS or irritant insult both in vivo and in vitro. A recent study by Crisafulli et al. also shows the same reduction after splanchnic ischemia and reperfusion (11, 16, 19, 39). Our study confirms these results in a model of rodent hemorrhagic shock, with significant reductions in plasma levels of MCP-1, MIP-1 α , TNF- α and KC. High levels of TNF- α have been associated with increased acute lung injury (34, 35). MCP-1, MIP-1 α and KC are chemokines that induce chemotaxis of inflammatory cells (40–42). In our study, treatment with T0901317 induced a significant decrease of TNF α and several chemokines, in particular KC, which correlates with the observed reduction of lung neutrophilia and injury. Therefore, we hypothesize that LXR α activation abrogates neutrophil infiltration into the lung and subsequently limits parenchymal damage following hemorrhagic shock by limiting the production of crucial pro-inflammatory cytokines and chemokines.

NF- κ B is a nuclear transcription factor that regulates the expression of a multitude of pro-inflammatory genes and has been implicated as a factor in the development of acute lung injury (17, 35). Amongst the pro-inflammatory cytokines up-regulated by NF- κ B activation are TNF- α , KC, MIP-1 α , MCP-1 and IL-6. In a previous study, we demonstrated that LXR α activation inhibits the LPS-induced inflammatory response of murine macrophages by inhibiting the NF- κ B pathway (19). Recent in vivo models of spinal cord trauma and splanchnic ischemia and reperfusion have also shown a reduction in NF- κ B activation after treatment with a synthetic LXR α agonist (39, 43). In our current model of hemorrhagic shock and resuscitation, we have demonstrated that treatment with T0901317 inhibits the early onset (i.e. at 60 minutes post-resuscitation) of NF- κ B binding to DNA in the lung. The exact mechanism by which LXR α inhibits NF- κ B activation remains unclear and appears complex. Activated LXR α seems to inhibit NF- κ B/DNA binding while also inhibiting the transactivation of NF- κ B already bound to DNA. For example, earlier in vitro studies have shown that activation of the NF- κ B pathway in LPS-challenged macrophages is inhibited by LXR α activation, but failed to show a decrease in NF- κ B binding to DNA (10, 11, 15, 44). Ghisletti et al. support this finding in their study, showing that LXR α activation of macrophages prevents the clearance of co-repressors in a SUMOylation dependent manner, thus preventing the transcription of several pro-inflammatory genes (45). This trans-repression would decrease the transcriptional activity of NF- κ B without altering its binding profile. However, in vivo studies of splanchnic ischemia and reperfusion did show a reduction of NF- κ B binding to DNA, similar to the findings we report in this study (39).

This suggests that LXR α does block NF- κ B binding to DNA and subsequent transcription of pro-inflammatory genes in in vivo models or in models of ischemia and reperfusion. However, the exact mechanism remains unclear and further investigation is needed to define the precise role activated LXR α plays in inhibition of the NF- κ B pathway in vivo.

In conclusion, we have shown that activation of LXR α with the synthetic agonist T0901317 improves the hemodynamic profile and reduces lung inflammation after hemorrhage and resuscitation in a rodent model. The reduction of inflammation is consistent with inhibition of NF- κ B binding during the early phase of shock. Further studies using inhibitors of LXR α , such as GSK 2033 (46), or transgenic mice deprived of the functional gene for LXR α (14) will establish the precise mechanisms of LXR α and NF- κ B interaction.

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FIG. 1. Effect of in vivo treatment with vehicle or T0901317 on the (A) mean arterial blood pressure and (B) heart rate of rats subjected to severe hemorrhage and resuscitation. Each data point is represented as mean $\pm$ SEM of N = 12–18 in vehicle-treated rats or N = 10–16 in T0901317-treated rats. Asterisk (*) indicates P < 0.001 versus vehicle-treated rats.

FIG. 2. Effect of in vivo treatment with vehicle or T0901317 on (A) Cardiac Index and (B) Stroke Volume Index of rats subjected to severe hemorrhage and resuscitation

Data are represented as mean $\pm$ SEM of N= 3,4 rats for vehicle and T0901317-treated rats, respectively. Asterisk (*) indicates $P < 0.05$ versus vehicle-treated rats.

FIG. 3. Effect of in vivo treatment with vehicle or T0901317 on the plasma levels of (A) lactate, (B) bicarbonate and (C) base deficit in rats subjected to severe hemorrhage and resuscitation All values are displayed as mean $\pm$ SEM for N= 6, 5 for T0901317-treated animals and vehicle-treated animals, respectively. Asterisk (*) indicates $P < 0.05$ versus vehicle-treated rats.

FIG. 4. Effect of in vivo treatment with vehicle or T0901317 on lung injury and neutrophil infiltration in rats subjected to severe hemorrhage and resuscitation

A-C are representative histological images of rat lung tissue after staining with hematoxylin and eosin. (A) Cross section of lung tissue from a control rat; (B) cross section of lung tissue from a vehicle-treated rat at 180 minutes post-resuscitation; (C) cross section of lung tissue from a T0901317-treated rat at 180 minutes post-resuscitation. All images are shown at 100x magnification. (D) Lung myeloperoxidase activity in vehicle and T0901317-treated rats at 180 minutes post-resuscitation. Data are represented as mean $\pm$ SEM for N = 4–7 animals for each group. Asterisk (*) indicates $P < 0.05$ versus vehicle-treated rats, # indicates $P < 0.05$ versus sham rats.

FIG. 5. Effect of in vivo treatment with vehicle or T0901317 on plasma levels of (A) MCP-1, (B) MIP-1α, (C) TNF-α, (D) KC, (E) IL-6 and (F) IL-10 (pg/mL) in rats subjected to severe hemorrhage and resuscitation

Data are represented as mean value $\pm$ SEM for N= 3 (60 minutes) and 10 (180 minutes) in T0901317 treated animals; N = 3 (60 Minutes) and 11 (180 minutes) for vehicle-treated animals. Asterisk (*) indicates $P < 0.05$ versus vehicle-treated animals.

FIG. 6. Effect of treatment with vehicle or T0901317 on the expression and DNA binding of LXRα in rodent lung after hemorrhage and resuscitation
 (A) Representative western blot of LXRα and β-Actin from nuclear extract of rodent lung tissue at 180 minutes post-resuscitation. (B) Image analysis of representative western blot (Fig 6A) of LXRα content in nuclear extract of rodent lung tissue 180 minutes post-resuscitation as evaluated by western blot. N= 2, 4, 5 for sham, vehicle and T0901317 treated animals, respectively. (C) Representative EMSA probed for LXRα in nuclear extract of rodent lung tissue at 180 minutes post-resuscitation. (D) Image analysis of representative EMSA (Fig. 6C) showing LXRα binding to DNA in nuclear extract of rodent lung tissue at 180 minutes post-resuscitation. N= 2, 5, 5 for sham, vehicle and T0901317 treated animals, respectively. Asterisk (*) indicates $P < 0.05$ versus sham rats; pound (#) indicates $P < 0.05$ versus vehicle-treated rats.

FIG. 7. Effect of in vivo treatment with vehicle or T0901317 on DNA binding of NF- κ B in rodent lung after hemorrhage and resuscitation

(A) Representative EMSA probed for NF- κ B in nuclear extract of rodent lung tissue at 60, 120 and 180 minutes post-resuscitation in rats treated with T0901317 or vehicle. (B) Image analysis of representative EMSA (Fig. 7A) showing NF- κ B binding to DNA in nuclear extract of rodent lung tissue at 60, 120 and 180 minutes post-resuscitation. N= 1 for sham and N= 2 for both vehicle and T0901317 treated animals at each respective time point. Asterisk (*) indicates $P < 0.05$ versus vehicle-treated animals.

Table 1

Body weight, total and percent blood removed, and Ringer Lactate solution supplementation.

Parameter	Vehicle	T0901317	P Value
Body Weight (g)	274.8±5.8	283±6.4	0.349
Total Blood Removed (mL)	7.9±0.3	8.8±0.3	0.056
Percent Blood Removed (% of estimated circulating blood volume)*	41.6±1.7	44.4±1.3	0.202
Ringer Lactate Solution Supplementation (mL)	1.0±0.2	0.9±0.2	0.637

Values are means ± SEM of n = 18 rats in the vehicle-treated group and n = 16 in the T0901317-treated group.

* Calculation of % blood removed = (blood removed (mL)/(weight (kg) × 0.7)) × 100