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BASIC AND TRANSLATIONAL—LIVER

Simvastatin Prevents Progression of Acute on Chronic Liver Failure in Rats With Cirrhosis and Portal Hypertension

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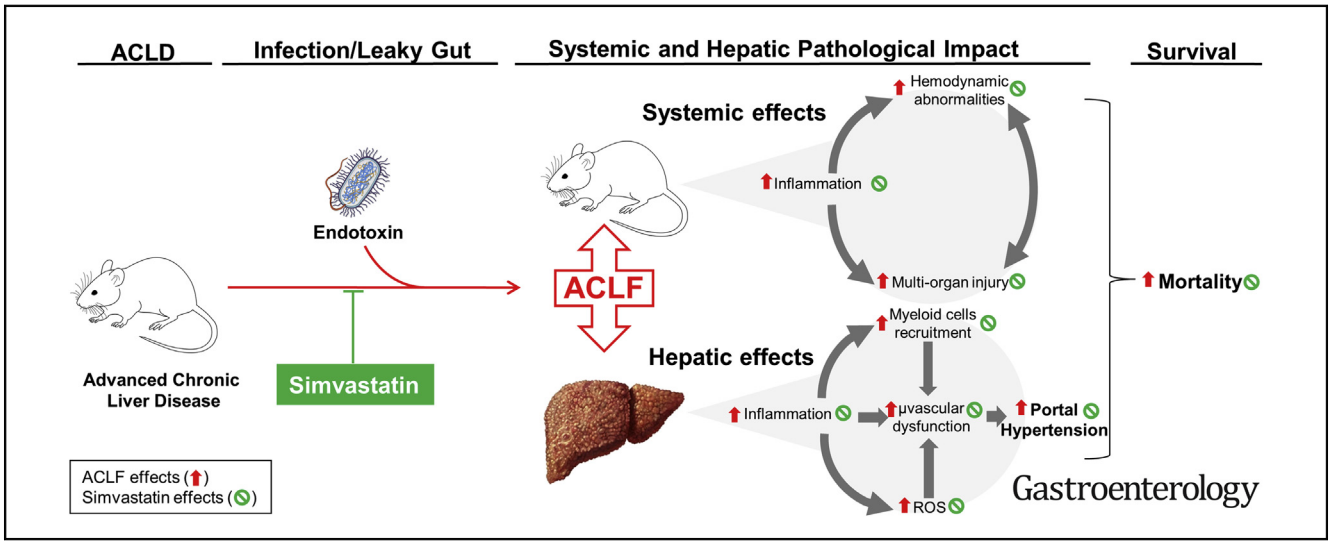

BACKGROUND & AIMS: Cirrhosis and its clinical consequences can be aggravated by bacterial infections, ultimately leading to the development of acute on chronic liver failure (ACLF), characterized by acute decompensation, organ failure, and high mortality within 28 days. Little is known about cellular and molecular mechanisms of ACLF in patients with cirrhosis, so no therapeutic options are available. We developed a sepsis-associated preclinical model of ACLF to facilitate studies of pathogenesis and evaluate the protective effects of simvastatin. **METHODS:** Male Wistar rats inhaled CCl_4 until they developed cirrhosis (at 10 weeks) or cirrhosis with ascites (at 15–16 weeks). Male Sprague-Dawley rats received bile-duct ligation for 28 days or intraperitoneal thioacetamide for 10 weeks to induce cirrhosis. After induction of cirrhosis, some rats received a single injection of lipopolysaccharide (LPS) to induce ACLF; some were given simvastatin or vehicle (control) 4 hours or 24 hours before induction of ACLF. We collected data on changes in hepatic and systemic hemodynamics, hepatic microvascular phenotype and function, and survival times. Liver tissues and plasma were collected and analyzed by immunoblots, quantitative polymerase chain reaction, immuno(fluoro)histochemistry and immunoassays. **RESULTS:** Administration of LPS aggravated portal hypertension in rats with cirrhosis by increasing the severity of intrahepatic microvascular dysfunction, exacerbating hepatic inflammation, increasing oxidative stress, and recruiting hepatic stellate cells and neutrophils. Rats with cirrhosis given LPS had significantly shorter survival times than rats with cirrhosis given the control. Simvastatin prevented most of ACLF-derived complications and increased survival times. Simvastatin appeared to increase hepatic sinusoidal function and reduce portal hypertension and markers of inflammation and oxidation. The drug significantly reduced levels of transaminases, total bilirubin, and ammonia, as well as LPS-mediated activation of hepatic stellate cells in liver tissues of rats with cirrhosis. **CONCLUSIONS:** In studies of rats with cirrhosis, we found administration of LPS to promote development of ACLF, aggravating the complications of chronic liver disease and decreasing survival times. Simvastatin reduced LPS-induced inflammation and liver damage in rats with ACLF, supporting its use in treatment of patients with advanced chronic liver disease.

Keywords: ACLF; Animal Model; Cirrhosis; Decompensated; *E coli*; Hepatic Microvascular Dysfunction; Hepatocytes; Portal Hypertension.

WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Acute on chronic liver failure (ACLF) leads to acute decompensation, organ failure and high mortality in cirrhotic patients. However, its cellular and molecular mechanism are unknown.

NEW FINDINGS

The authors developed and characterized a new pre-clinical model of ACLF, evaluated the molecular and cellular mechanisms involved, and tested simvastatin as a potential therapeutic approach to treat ACLF.

LIMITATIONS

High mortality due to ACLF in decompensated cirrhotic animals results in only a partial understanding of the extrahepatic effects in this pre-clinical model.

IMPACT

The development of a new ACLF pre-clinical model represents an important tool that may lead to a better understanding of its pathophysiology and to generate new therapeutics for patients suffering ACLF. Simvastatin may be a novel and efficient therapy in this clinical scenario.

Liver cirrhosis is a pathological condition characterized by extracellular matrix deposition leading to fibrous septa, formation of regenerative nodules, sinusoidal microcirculatory dysfunction, and portal hypertension, altogether ultimately leading to liver failure.[1,2] Clinically, cirrhosis progression follows 2 stages: a long compensated one, usually asymptomatic and with good prognosis, followed by a decompensated stage with poor prognosis, heralded by the occurrence of complications such as ascites, variceal bleeding, jaundice, and hepatic encephalopathy.[3,4] At this stage, patients' conditions can acutely deteriorate as they rapidly develop hepatic encephalopathy, coagulopathy, acute kidney injury, and multiorgan failure, configuring the syndrome of acute on chronic liver failure (ACLF).[5,6] ACLF is defined as an acute hepatic insult manifesting as jaundice and coagulopathy, complicated within 4 weeks by ascites and/or encephalopathy with high 28-day mortality, and it is frequently precipitated by bacterial infections and sepsis.[5,7,8] To our knowledge, no experimental model of ACLF has been developed and well characterized, thus limiting the preclinical research in the field.

Among the different therapeutic approaches proposed for liver cirrhosis,[9,10] recent studies have shown that statins are among the most promising drugs for improving portal hypertension and its complications.[11,12] Statins are lipid-lowering agents with immumodulatory, anti-inflammatory, and vasoprotective effects.[9] In particular, simvastatin is able to improve the phenotype of sinusoidal cells, inhibiting the activation and proliferation of hepatic stellate cells (HSCs) and ensuing liver fibrosis, ameliorating liver sinusoidal endothelial cells functionality, and reducing inflammation and angiogenesis.[13–16]

Beneficial effects of simvastatin have been recently shown in double-blind randomized clinical trials to translate into a decrease in portal pressure, improved quantitative liver function test results, and increased survival in patients with cirrhosis and portal hypertension.[17,18] Moreover, simvastatin has beneficial effects on liver perfusion and microvascular functionality in healthy rodents with bacterial infection, suggesting a possible protective role during endotoxemia,[19] a concept that is further supported by clinical observations of decreased prevalence of septic shock and death in subjects receiving statins.[20] Nevertheless, the effect of simvastatin preventing ACLF syndrome in compensated and decompensated cirrhotic rat models has not been yet studied.

The current study aimed at developing a sepsis-related, preclinical model of ACLF in rats with advanced chronic liver disease (ACLD) and evaluating the possible protective effects of acute simvastatin treatment on hepatic hemodynamic, microvascular dysfunction, inflammation, and underlying mechanisms involved.

Experimental Procedures

Animal Models and Treatments

Preclinical Rat Models of ACLD. *Carbon Tetrachloride (CCl_4) Inhalation.* Male Wistar rats (50–75 g) underwent CCl_4 (Sigma-Aldrich, St. Louis, MO) inhalation 3 times a week and received phenobarbital (0.3 g/L, Kern Pharma, Barcelona, Spain) in the drinking water as previously described.[13,21] A high yield of micronodular cirrhosis with ascites was obtained after 15 to 16 weeks. Administration of toxicants was stopped, and treatments were started 1 week later.

A group of animals received CCl_4 for only 10 weeks, and animal treatments started 1 week later; this shorter CCl_4 inhalation period caused cirrhosis but no animal developed ascites.

Common Bile Duct Ligation (BDL). Secondary biliary cirrhosis was induced in male Sprague-Dawley rats (200–225 g) by ligation of common bile duct for a period of 28 days.[22] Treatments started on the 25th day when cirrhosis and ascites had already developed.

Thioacetamide Administration (TAA). Male Sprague-Dawley rats (150–200 g) received TAA (Sigma-Aldrich) dissolved in saline intraperitoneally at a dosage of 250 mg/kg twice a week for 10 weeks without developing ascites.[23] Treatments started 1 week later, after TAA administration was stopped.

Preclinical Rat Model of Prehepatic Portal Hypertension. Prehepatic portal hypertension was induced

*Authors share co-first authorship; § Authors share co-senior authorship.

Abbreviations used in this paper: ACLD, advanced chronic liver disease; ACLF, acute-on-chronic liver failure; BDL, bile duct ligation; CCl_4, carbon tetrachloride; eNOS, endothelial nitric oxide synthase; HSC, hepatic stellate cells; HVR, hepatic vascular resistance; LPS, lipopolysaccharide; NET, neutrophil extracellular trap; PBF, portal blood flow; P-eNOS, phosphorylated endothelial nitric oxide synthase; PP, portal pressure; PPVL, partial portal vein ligation; TAA, thioacetamide.

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in male Sprague-Dawley rats (300 g) by partial portal vein ligation (PPVL) as described.[24] Briefly, under isoflurane anesthesia, a calibrated constriction was performed using a single ligature of 3-0 silk tied around the portal vein and a 19-gauge blunt-tipped needle. The needle was then removed, leaving a calibrated constriction of the portal vein.

Induction of Endotoxemia by Lipopolysaccharide (LPS) Administration. Acute endotoxemia induced by intraperitoneal or intravenous administration of LPS (from *Escherichia coli* O111:B4, Sigma-Aldrich) was used to cause an acute deterioration of cirrhosis, mimicking ACLF. This was done at a dose of 1 mg/kg 4 hours before the hemodynamic study in decompensated CCl_4 and BDL animals, and compensated animals that received CCl_4 or TAA for 10 weeks were treated with LPS (1 mg/kg) 24 hours before the study. LPS doses and incubation times were based on previous bibliography[25,26] and our own preliminary research.

Animals were caged in pairs on a 12:12-hour light-dark cycle, in environmentally controlled animal facilities at the Institut d'Investigacions Biomèdiques August Pi i Sunyer. All experiments were approved by the Laboratory Animal Care and Use Committee of the University of Barcelona and were conducted in accordance with the European Community guidelines for the protection of animals used for experimental and other scientific purposes (EEC Directive 86/609).

Simvastatin Treatment. Rats were randomized to receive simvastatin (Normon Laboratories, Madrid, Spain) (25mg/kg/day in CCl_4- and TAA-induced ACLD animals, 5 mg/kg/day in BDL-induced animals) or water as vehicle compound, administered orally by gavage once a day for 3 days, followed by a fourth dose 30 minutes before the study. Nontoxic simvastatin doses were based on previous studies and preliminary data from our team[27,28] and were prepared by a third person, and experimental studies were realized blindly. Supplementary Figure 1 summarizes the experimental groups included in the study.

Hemodynamic Assessment and Biochemical Analysis

Rats were anesthetized with ketamine hydrochloride (60 mg/kg; Merial Laboratories, Barcelona, Spain) plus midazolam (3mg/kg; Laboratorios Reig Jofre, Barcelona, Spain) intraperitoneally, fastened to a surgical board, and maintained a constant temperature of 37°C ± 0.5°C. Tracheotomy and endotracheal cannulation (PE-240 catheter; Portex, Minneapolis, MN) was performed to maintain adequate respiration during anesthesia. The femoral artery and the ileocolic vein were cannulated with PE-50 catheters to measure mean arterial pressure (mm Hg) and portal pressure (PP) (mm Hg), respectively. A nonconstrictive perivascular ultrasonic transit time flow probe (2PR, 2-mm diameter; Transonic Systems Inc., Ithaca, NY) was placed around the portal vein as close as possible to the liver to measure portal blood flow (PBF) (mL/min).[29] Hepatic vascular resistance (HVR) (mm $Hg \cdot mL \cdot min^{-1} \cdot g^{-1}$) was calculated as PP/PBF. Blood pressures and flows were registered on a multichannel, computer-based recorder using Chart, version 5.0.1, for Windows software (PowerLab; AD Instruments, Colorado Springs, CO). Hemodynamic data were collected after a 20-minute stabilization period. At the end of the hemodynamic study, serum samples were collected to evaluate alanine aminotransferase, aspartate aminotransferase, total bilirubin, albumin, creatinine, sodium, and ammonia levels, all by standard protocols.

Hepatic Vascular Functionality Analysis

After in vivo hemodynamic assessment, livers were isolated and perfused by a flow-controlled perfusion system as previously described.[30,31] The perfused rat liver preparation was allowed to stabilize for 20 minutes, and portal perfusion pressure was analyzed before vasoactive substances were added. Precontraction was performed with the α-adrenergic agonist methoxamine (10^{-4} mol/L, Sigma-Aldrich). After 5 minutes, increasing concentrations of acetylcholine (10^{-7}, 10^{-6} and 10^{-5} mol/L; Sigma-Aldrich) were added to the system to test the vasodilatory capacity of the liver circulation to ascertain the sinusoidal microcirculatory function.[14] Gross appearance of the liver, stable perfusion pressure, bile production over 0.4 μL/min/g of liver, and stable buffer pH (7.4 ± 0.1) were monitored during the entire study.

Endotoxemia Quantification

Endotoxemia was quantified using LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific, Waltham, MA), following manufacturer's instructions.

Protein Expression Analysis

Protein expression was determined by Western blot in all hepatic and cell samples as previously described[13] using antibodies shown in Supplementary Table 1. Blots were visualized by chemiluminescence, and digital images were taken using a luminescent image analyzer LAS-4000 (General Electric, Little Chalfont, Buckinghamshire, UK). Protein expression was determined by densitometric analysis using the Science Lab 2001, Multi Gauge V2.1 (Fuji Photo Film, Düsseldorf, Germany). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for the normalization of quantitative densitometry values. The ratio between the densitometry readings of phosphorylated endothelial nitric oxide synthase (P-eNOS) and endothelial nitric oxide synthase (eNOS) blots was calculated to evaluate the degree of eNOS phosphorylation at Ser^{1176}.

Circulating cytokines were determined in plasma samples using a cytokine array (R&D Systems, Minneapolis, MN), following the manufacturer's instructions.

Neutrophil gelatinase–associated lipocalin was quantified in serum samples using a rat neutrophil gelatinase–associated lipocalin ELISA kit (Cusabio Technology, Houston, TX), following the manufacturer's instructions.

Messenger RNA Expression Analysis

Gene expression was analyzed in total RNA from all hepatic and cell samples according to the manufacturer's protocol using primers described in Supplementary Table 2. Values were reported relative to the endogenous control glyceraldehyde-3-phosphate dehydrogenase. All amplification reactions were performed in duplicate, and nuclease-free water was used as no-template control in the reaction sets.

Immunostaining and Microscopy

Immunostaining of paraffin-embedded liver sections was performed with antibodies shown in Supplementary Table 1

or with phosphate-buffered saline as a negative control. Ten fields from each slide at ×400 magnification were randomly selected, and photographs were taken using a fluorescent microscope (Olympus, Tokyo, Japan) and quantified with Image J 1.33u software (National Institutes of Health, Bethesda, MD). Infiltrated neutrophils were characterized as myeloperoxidase-positive cells, and their activity was characterized as presence of neutrophil extracellular traps (NETs) (structures double-positive for myeloperoxidase and histone 2B).[32] Two independent researchers performed immunostaining quantifications blindly.

Measurement of Superoxide (O_2^-) Levels in Liver Tissue

Oxidative stress in liver tissue was evaluated with the fluorescent dye dihydroethidium (Molecular Probes, Eugene, OR).[33] Six fields from each slide at ×200 magnification were randomly selected. Fluorescent images were obtained with a fluorescent microscope, and quantitative analysis was performed with Image J 1.33u software.[34]

Quantification of Hepatic Fibrosis

Fixed livers were stained with 0.1% Sirius Red (Sigma-Aldrich). Slices were photographed at ×50 magnification using a microscope equipped with a digital camera. Red-stained area was measured using AxioVision software[35] and divided by total photographed area. Values were expressed as the mean percentage of 8 fields per sample.

In Vitro Experiments

Immortalized HSCs (LX2 cells, kindly provided by Dr Bataller) were maintained at 37°C under an atmosphere of 5% CO_2 in complete Dulbecco's modified Eagle medium (Gibco, Thermo Fisher Scientific) containing 10% fetal bovine serum (Biological industries, Cromwell, CT) and 1% penicillin/streptomycin (Biological Industries).[15]

Neutrophils were isolated from human healthy volunteers' peripheral blood by density centrifugation using a Histopaque-1077 (Sigma-Aldrich) gradient.[36] Briefly, blood samples were collected in tubes containing heparin (Laboratorios Rovi, Barcelona, Spain). Plasma was discarded, and blood was diluted in saline solution. Histopaque-1077 was added and centrifuged at 700*g* at room temperature for 20 minutes. After centrifugation, the supernatant was removed, and the residual erythrocytes were eliminated by hypotonic lysis buffer, containing 0.83% NH_4Cl (Sigma-Aldrich) and washed in phosphate-buffered saline. Neutrophils were maintained in RPMI 1640 medium (Biological Industries) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.[36,37]

Neutrophils were pretreated with vehicle (0.001% dimethyl sulfoxide, Sigma-Aldrich) or simvastatin (1–5 μmol/L) for 1 hour, treated with LPS (100 ng/mL) or vehicle for 4 hours, and afterwards co-cultured with HSC for 16 hours. Direct co-culture assays were performed by adding 2×10^4 neutrophils to 6×10^4 LX2 cells. In indirect co-culture experiments, LX2 cells were co-cultured with neutrophils in 12-well Transwell plates (0.4 μm/pore; Corning Inc, Corning, NY).[38]

Statistical Analysis

Statistical analysis was performed using the SPSS 19.0 statistical package (IBM, Armonk, NY). Data are reported as mean ± standard error of the mean. Survival analyses were done using the log-rank (Mantel-Cox) test comparing groups. Analysis of variance test and Tukey post hoc test were used for analyzing differences between groups. Differences were considered significant at $P < .05$.

Results

Systemic Effects of LPS Administration in Cirrhotic Rats

Exogenous administration of LPS to cirrhotic animals markedly elevated endotoxemia, which correlated with severe detrimental effects on systemic hemodynamic and renal function (Supplementary Figure 2). Effects of LPS on hepatic hemodynamic and function are described in the next section.

LPS Administration Aggravates Portal Hypertension and Liver Microcirculatory Dysfunction in Rats With Cirrhosis; Simvastatin Treatment Prevents ACLF Deleterious Effects

Intraperitoneal LPS administration markedly increased PP in decompensated and compensated CCl_4 cirrhotic rats (+11% and +28%, respectively), with the same trend in compensated TAA cirrhotic animals (+20%) ($P = .06$) (Table 1). No changes in PBF were observed, thus suggesting that portal hypertension aggravation in ACLF was derived from an increment in the HVR (decompensated cirrhotic rats: +94% in CCl_4, compensated: +57% in CCl_4 and +130% in TAA). Intravenous administration of LPS led to similar deleterious effects (+57% in PP and +64% in HVR) (Supplementary Table 3). No significant hemodynamic changes were observed in decompensated BDL rats. In addition, no aggravation in portal pressure was observed in a group of animals with prehepatic portal hypertension due to PPVL (14.6 ± 2.5 mmHg in PPVL + vehicle vs 15.1 ± 1.2 mm Hg in PPVL + LPS).

Analysis of hepatic microcirculation ex vivo further confirmed the aggravation in liver microvascular dysfunction during ACLF, as shown by marked increments in portal perfusion pressure (Figure 1, *top*) and the attenuation of the hepatic vasodilatory response to acetylcholine (Figure 1, *bottom*).

Scrutiny of a possible therapeutic intervention for ACLF showed that simvastatin prevented the LPS-induced aggravation in portal hypertension. Indeed, animals treated with simvastatin showed significantly lower PP after ACLF challenge than those receiving vehicle + LPS (decompensated cirrhotic rats: –21% in CCl_4 and –22% in BDL, compensated: –20% in TAA). No changes in PBF were observed, further suggesting a reduction in HVR as the cause for the improvement in PP. Indeed, HVR was reduced in response to simvastatin in decompensated CCl_4 rats (–35%), although this was not observed in BDL animals. Simvastatin treatment resulted in no significant changes in PBF or HVR in

Table 1. Effects of LPS and Simvastatin on Hepatic and Systemic Hemodynamic in Rats With Decompensated and Compensated ACLD

	Decompensated ACLD					
	CCl_4			BDL		
Parameters	Vehicle	Vehicle + LPS	Simvastatin + LPS	Vehicle	Vehicle + LPS	Simvastatin + LPS
Number of animals	**9**	**12**	**9**	**8**	**7**	**8**
MAP (*mm Hg*)	79 ± 16	100 ± 15[a]	112 ± 25[a]	74 ± 9	60 ± 10	71 ± 28
PP (*mm Hg*)	12.2 ± 1.9	13.5 ± 2.7[a]	10.7 ± 1.9[b]	15.2 ± 2.0	13.9 ± 2.2	10.8 ± 2.1[a,b]
PBF (*mL/min*)	18.5 ± 7.6	15.9 ± 8.7	16.2 ± 6.1	18.9 ± 8.6	20.6 ± 4.8	17.1 ± 7.2
HVR ($mm\ Hg/mL \cdot min^{-1} \cdot g^{-1}$)	6.8 ± 3.2	13.2 ± 6.8[a]	8.6 ± 2.6[b]	21.4 ± 10.4	20.2 ± 7.3	22.6 ± 13.0
HR (*beats/min*)	298 ± 51	368 ± 29[a]	382 ± 62[a]	345 ± 33	388 ± 46	426 ± 37[b]
Liver weight (*g*)	11.7 ± 1.8	11.8 ± 2.1	13.0 ± 2.5	25.0 ± 6.6	27.7 ± 8.4	27.4 ± 4.9
Body weight (*g*)	369 ± 49	367 ± 52	404 ± 70	413 ± 95	378 ± 134	394 ± 75
	Compensated ACLD					
	CCl_4			TAA		
	Vehicle	Vehicle + LPS	Simvastatin + LPS	Vehicle	Vehicle + LPS	Simvastatin + LPS
Number of animals	**5**	**6**	**6**	**4**	**3**	**5**
MAP (*mm Hg*)	97 ± 16	88 ± 21	116 ± 23[b]	89 ± 20	108 ± 27	110 ± 15
PP (*mm Hg*)	8.6 ± 1.5	11.0 ± 1.5[a]	10.4 ± 0.9[a]	10.2 ± 0.5	12.3 ± 1.3	9.8 ± 1.5[b]
PBF (*mL/min*)	13.4 ± 4.1	15.2 ± 4.7	10.9 ± 2.9	18.9 ± 1.9	13.9 ± 11.0	17.3 ± 4.0
HVR ($mm\ Hg/mL \cdot min^{-1} \cdot g^{-1}$)	6.3 ± 1.6	9.9 ± 4.8	11.2 ± 4.6	7.3 ± 0.6	16.8 ± 10.6	9.0 ± 2.7
HR (*beats/min*)	369 ± 24	384 ± 90	423 ± 50	352 ± 61	366 ± 24	354 ± 35
Liver weight (*g*)	9.4 ± 1.9	12.3 ± 2.1[a]	10.7 ± 2.2	13.9 ± 1.2	14.0 ± 1.8	14.9 ± 0.9
Body weight (*g*)	333 ± 27	382 ± 39[a]	365 ± 21	358 ± 32	332 ± 35	393 ± 31[b]

NOTE. Values represent mean ± standard deviation.
HVR, hepatic vascular resistance; MAP, mean arterial pressure; PBF, portal blood flow; PP, portal pressure; TAA, thioacetamide.
[a]$P < .05$ vs vehicle.
[b]$P < .05$ vs vehicle + LPS.

CCl_4-compensated cirrhotic animals. Mean arterial pressure was slightly ameliorated in rats treated with simvastatin (Table 1).

In agreement with the improvement observed in vivo, simvastatin also prevented the hemodynamic deterioration from LPS-induced ACLF in ex vivo portal perfusion pressure (Figure 1, *top*) and in the hepatic vasodilatory response to acetylcholine (Figure 1, *bottom*).

ACLF Exacerbates Inflammation and Oxidative Stress, Effects of Statins

LPS promoted a marked aggravation in systemic and hepatic inflammation in cirrhotic rats, as shown by significant changes in pro- and anti-inflammatory mediators (Figure 2 and Supplementary Figures 3 and 4). This inflammatory burst was associated with significant increments in hepatic neutrophil infiltration and neutrophil activity measured as formation of NETs (Figure 3 and Supplementary Figure 5). Simvastatin treatment prevented the inflammatory burst due to endotoxemia in decompensated cirrhotic rats, although its anti-inflammatory effects were less evident in the BDL preclinical model (Figures 2 and 3 and Supplementary Figures 3 and 4). A similar trend was observed in compensated CCl_4 rats (Supplementary Figure 5).

LPS exacerbated hepatic oxidative stress in decompensated cirrhotic rats, as shown by significantly elevated levels of superoxide and nonsignificant increment in peroxynitrite (Figure 4*A* and *B*). Oxidant deregulations were associated with a marked decrease in hepatic eNOS activation (Figure 4*C* and *D*). Simvastatin prevented the ACLF-derived increase in oxidative stress in both decompensated cirrhotic models (Figure 4*A* and *B*) and improved the P-eNOS/eNOS ratio in the CCl_4 model (Figure 4*C*) but not in the BDL model (Figure 4*D*).

Inflammatory and oxidative markers in livers from compensated cirrhotic rats receiving LPS exhibited the same trend observed in decompensated cirrhotic animals, although it did not reach statistical significance (data not shown).

LPS Administration Worsens the Phenotype of Hepatic Stellate Cells, Which Is Prevented by Simvastatin

Further analysis of the molecular and cellular mechanisms underlying the liver microcirculatory aggravation

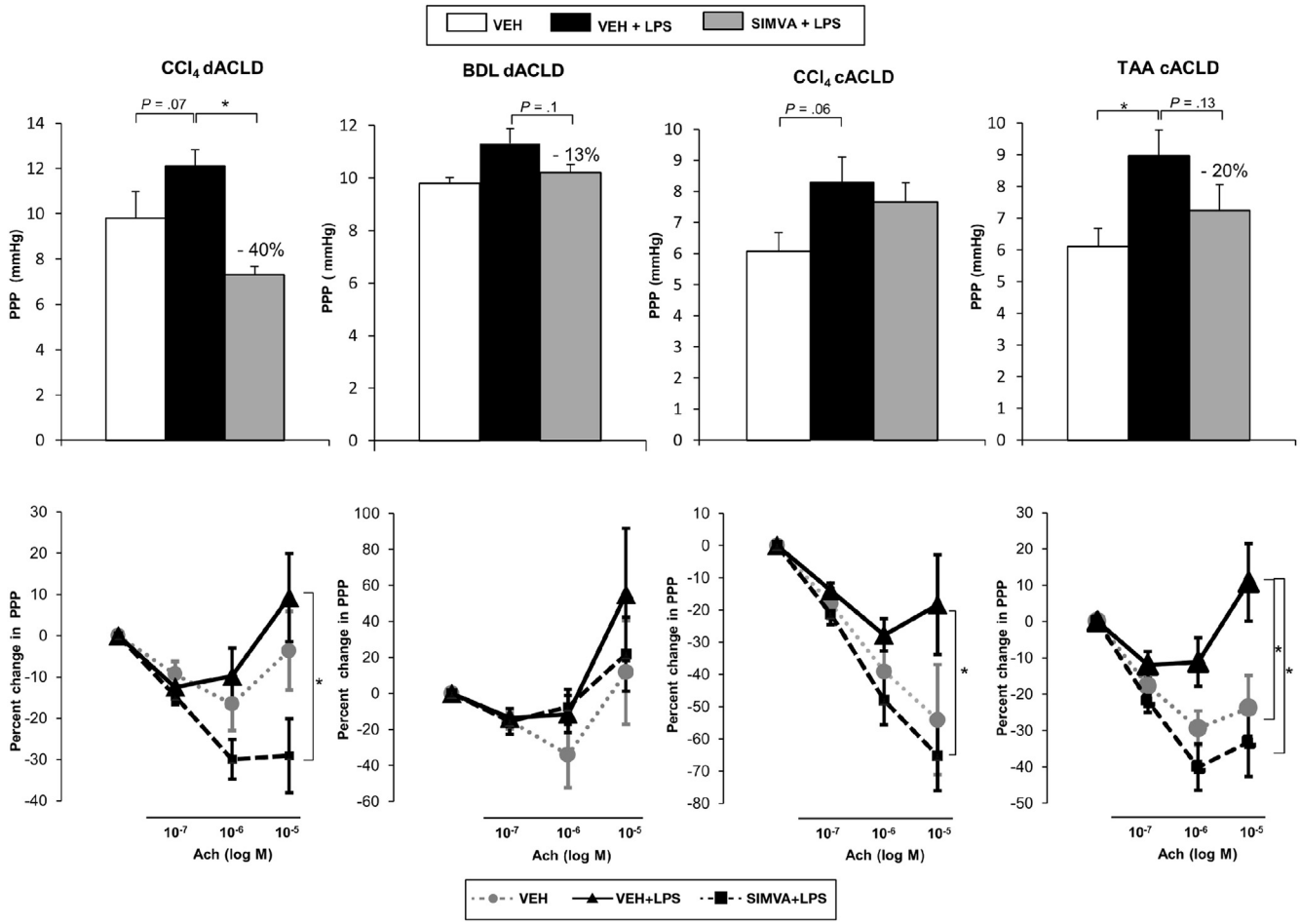

Figure 1. Effects of ACLF on hepatic microvascular function in rats with ACLD treated with simvastatin or vehicle. *Top*, portal perfusion pressure (PPP) was evaluated in rats with decompensated ACLD (dACLD) or compensated ACLD (cACLD) treated with vehicle (Veh), vehicle + LPS (Veh + LPS), or simvastatin + LPS (Simva + LPS). *Bottom*, liver vasodilatory response to incremental doses of acetylcholine was evaluated in animals described above. Values represent mean ± standard error of the mean. *$P < .05$. Number of animals included in each experimental group is defined in Table 1.

during ACLF showed that endotoxemia led to acute HSC activation in decompensated cirrhotic animals, shown by increased hepatic α-smooth muscle actin expression both at messenger RNA and protein levels (Figure 5), with no changes in liver fibrosis (data not shown). Simvastatin did not modify liver fibrosis but prevented the LPS-mediated HSC activation in both decompensated cirrhotic rat models (Figure 5).

In addition, and considering the increment in neutrophil infiltration during ACLF, we ascertained whether LPS-activated neutrophils might influence HSC phenotype. As shown in Supplementary Figure 6, direct co-culture of activated neutrophils with LX2 cells caused HSC over-activation, which was not observed when indirect co-culture (Transwell) was performed (data not shown). The detrimental effects of LPS-stimulated neutrophils on the LX2 phenotype were partly abrogated by simvastatin (Supplementary Figure 6), altogether suggesting a possible role of neutrophils partly mediating HSC activation during LPS-induced ACLF.

ACLF Negatively Affects Cirrhotic Animals' Survival and Is Prevented by Simvastatin

Rat survival was monitored during 4 or 24 hours (in decompensated or compensated cirrhotic animals, respectively) after LPS administration. No mortality was observed in decompensated and compensated cirrhotic animals without ACLF. On the contrary, significant mortality was observed in LPS-treated groups, especially in the decompensated cirrhotic models (66.6% survival in CCl_4, $P = .05$; 58.3% survival in BDL, $P < .05$) (Table 2). Corresponding data in compensated cirrhotic models were as follows: CCl_4: 75% survival after intraperitoneal LPS, $P = .48$; 66.7% survival after intravenous LPS, $P = .08$; TAA: 50% survival, $P = .13$) (Table 2 and Supplementary Table 3). Detrimental effects of LPS-induced ACLF were further confirmed by marked worsening of biochemical tests in surviving rats (Table 3).

Simvastatin pretreatment improved survival of LPS-induced ACLF in decompensated cirrhotic models (CCl_4: 100% survival, $P < .05$; BDL: 89% survival, $P = .07$)

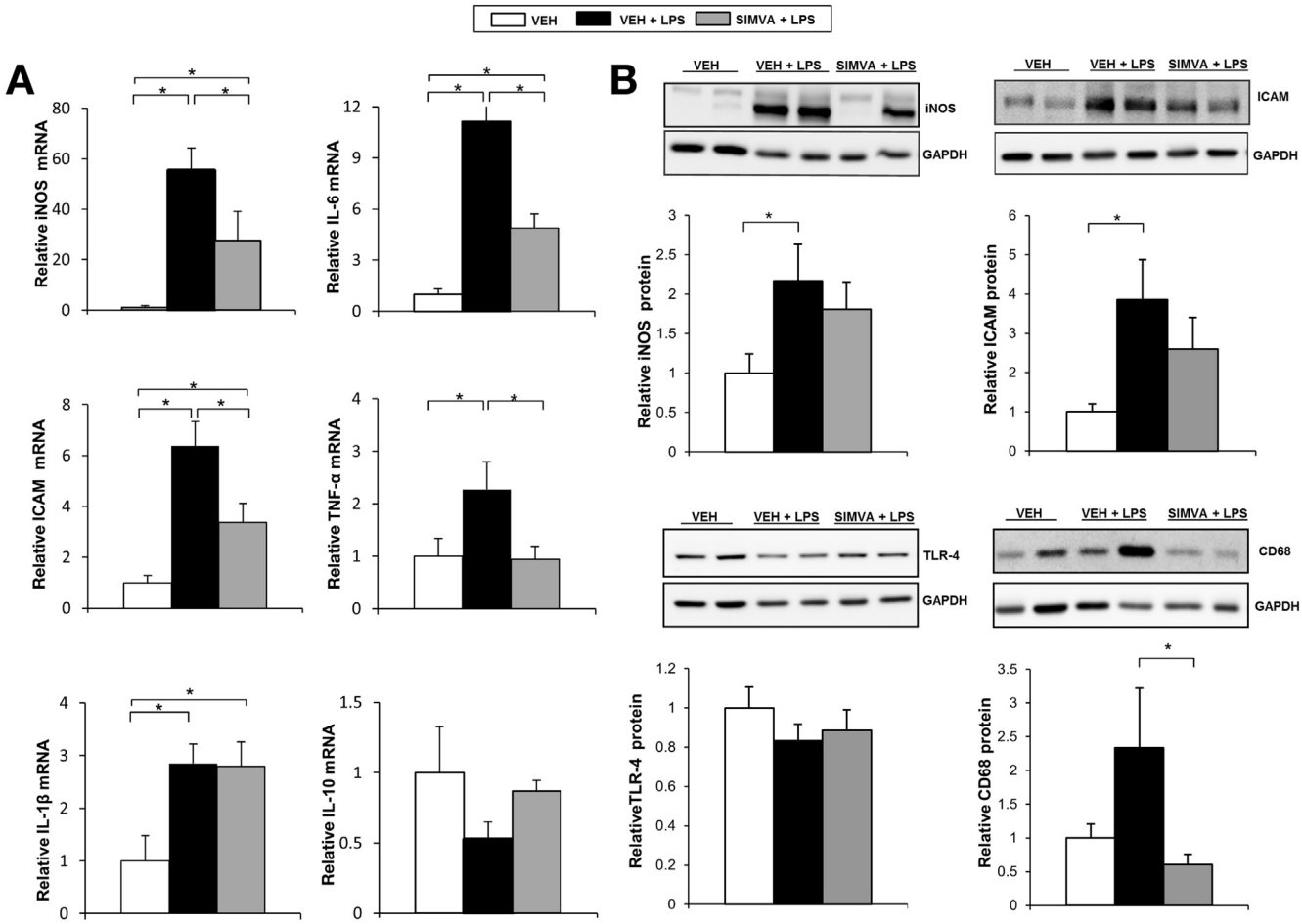

Figure 2. Hepatic inflammation in rats with decompensated ACLD suffering ACLF. Inflammatory markers were evaluated in rats treated with vehicle (Veh, n = 9), vehicle + LPS (Veh + LPS, n = 12), or simvastatin + LPS (Simva + LPS, n = 9) in a CCl_4 decompensated ACLD model at the (*A*) messenger RNA (mRNA) and (*B*) protein levels. Values represent mean ± standard error of the mean. *$P < .05$.

(Table 2) and significantly decreased transaminases, total bilirubin, and ammonia levels (Table 2). A similar trend was observed in compensated cirrhotic rats (CCl_4: 86% survival, $P = .43$; TAA: 83% survival, $P = .18$) (Tables 2 and 3).

Discussion

ACLD is classified in compensated and decompensated stages, with the presence of ascites, variceal hemorrhage, jaundice, and/or hepatic encephalopathy being the most common hallmarks of decompensation.[39,40] Approximately 24%–40% of patients with ACLD will eventually develop ACLF, which leads to a markedly decreased life expectancy. ACLF denotes an acute deterioration of liver failure leading to multiorgan failure, and it is thought to be due to increased systemic inflammation.[41] However, there is no specific treatment available for correcting or preventing ACLF.

The present study aimed at evaluating the possible efficacy of simvastatin, a compound with vasoprotective and anti-inflammatory activity, in preventing ACLF in experimental models of ACLD. Before that, and considering that to our knowledge no validated model of ACLF has been reported, we characterized the effects of ACLF on hepatic hemodynamic microcirculatory function and survival in validated models of compensated and decompensated chronic liver disease. To this end, we used LPS administration to precipitate ACLF in these experimental models, because sepsis/bacterial infections are clinically the most common precipitant of ACLF in human cirrhosis.

At the hemodynamic level, we observed that LPS administration significantly affected the intrahepatic sinusoidal function, leading to increased intrahepatic vascular resistance and consequently aggravated portal hypertension. This relevant effect of ACLF, which agrees with clinical observations,[42] was not seen in the BDL model, probably because this is a much more aggressive model of cirrhosis with endogenous altered gut permeability, and therefore LPS did not further aggravate portal hypertension due to preexisting endotoxemia. Indeed, in compensated cirrhotic models, with lower spontaneous bacterial translocation, LPS increased portal pressure, further suggesting a greater impact of endotoxemia when there is no previous bacterial translocation. LPS administration did not modify portal

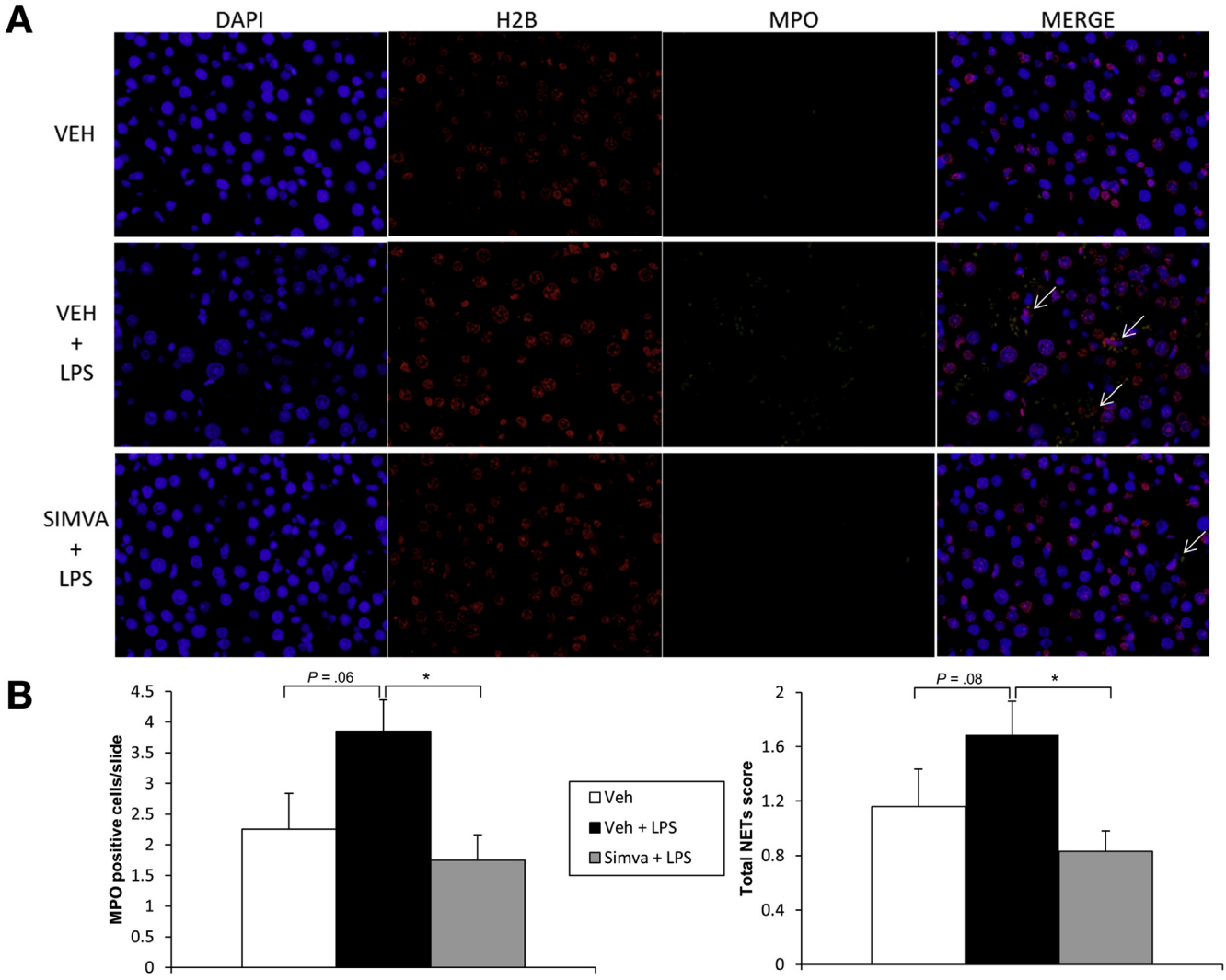

Figure 3. Detection of neutrophils and NETs in rats with decompensated ACLD suffering ACLF. Neutrophils (MPO-positive cells) and NETs (structures double-positive for MPO and histone 2B, *arrows*) were evaluated in liver tissue from CCl_4 decompensated ACLD rats treated with vehicle (Veh, n = 9), vehicle + LPS (Veh + LPS, n = 12) or simvastatin + LPS (Simva + LPS, n = 9). Representative image original magnification, ×400; (*A*) and (*B*) quantifications. Values represent mean ± standard error of the mean. *$P < .05$. H2B, histone-2B; MPO, myeloperoxidase.

pressure in a model of prehepatic portal hypertension, suggesting that endotoxemia per se does not influence portal pressure in the absence of liver injury.

In fact, we observed a global aggravation of hepatic microvascular function during the development of ACLF, with increased resistance and impaired endothelial-dependent vasodilatation, which is in keeping with previous studies reporting systemic hyporesponsiveness to vasoconstrictors and vascular damage during sepsis.[25,43] The aggravation of liver microvascular function triggered by LPS might partially be due to a combination of decreased production of the vasodilator nitric oxide, as shown by decreased P-eNOS/eNOS ratio, and increased oxidative stress, which profoundly affects the hepatic vascular resistance in cirrhotic livers.[9] Contrarily, we did not observe modulation of mesenteric vascular tone in response to LPS. This paradoxical effect may be due to the preexisting endogenous endotoxemia that would activate per se splanchnic vasodilatory pathways, including the nitric oxide machinery[44] and would not further respond to exogenous LPS challenge.

Additionally, it is well known that oxidative stress is also involved in HSC activation,[45] and therefore it may not only contribute to liver microvascular dysfunction but also enhance fibrosis. Indeed, we observed activation of HSC in LPS-treated rats, although no significant changes in liver fibrosis were seen, probably because of the short post-LPS observation period.

As hypothesized, we observed a marked increase in systemic and hepatic inflammation markers in this ACLF model, observations in agreement with previous studies showing systemic inflammation in ACLF patients.[6,46] In addition, increased hepatic neutrophil infiltration and NETs were found, which agrees with clinical observations

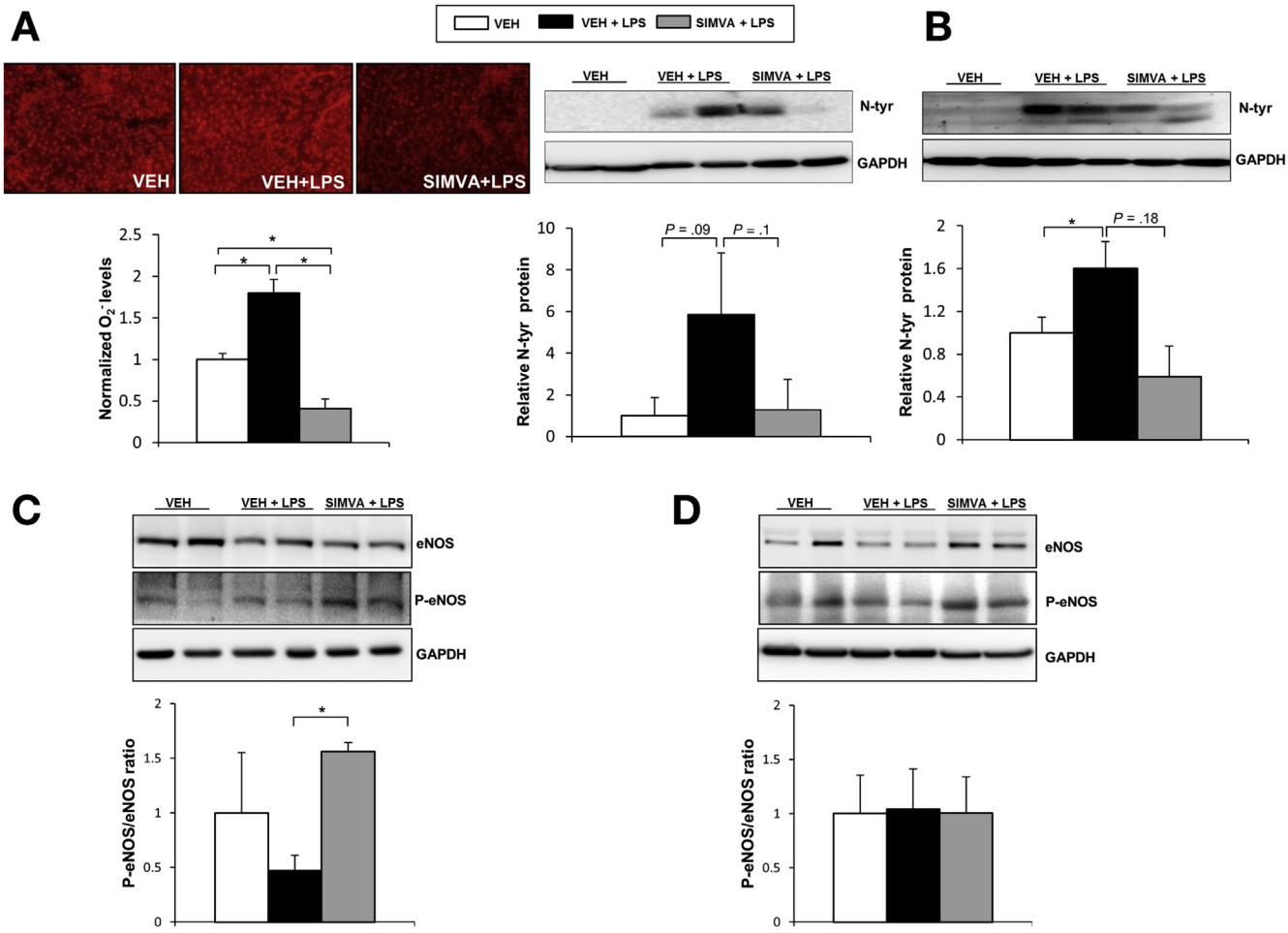

Figure 4. Analysis of oxidative stress and nitric oxide pathways in rats with decompensated cirrhosis suffering ACLF pretreated with simvastatin or vehicle. Hepatic oxidative stress was evaluated by DHE staining and nitrotyrosine protein expression in rats treated with vehicle (Veh, n = 9), vehicle + LPS (Veh + LPS, n = 12) or simvastatin + LPS (Simva + LPS, n = 9) in (*A*) CCl_4 and (*B*) BDL (Veh, n = 8; Veh + LPS, n = 7; Simva + LPS, n = 8) dACLD. P-eNOS/eNOS ratio was analyzed in the same groups described in (*C*) CCl_4 and (*D*) BDL dACLD. Values represent mean ± standard error of the mean. *$P < .05$. DHE, dihydroethidium.

describing that polymorphonuclear cells like neutrophils rapidly react by migrating to inflammation sites,[6] forming NETs, and contributing to the inflammation-associated liver damage by releasing reactive oxygen species, proteolytic enzymes, and inflammatory mediators.[47,48] Moreover, we describe a novel inflammatory mechanism involved in the pathophysiology of ACLF: activated neutrophils might per se contribute to HSC activation through an intercellular interaction, therefore representing a novel therapeutic target for this syndrome.

Finally, considering the severity of ACLF in humans, we evaluated short-term mortality in this preclinical model of ACLF. LPS administration negatively affected survival and biochemical parameters in all cirrhotic groups, although the effects were more evident in decompensated animals.

Altogether, these data suggest that the ACLF model presented herein appears to be a proper model mimicking the clinical ACLF syndrome observed in cirrhotic patients.[49]

As previously stated, no specific treatment is available for ACLF patients. However, different studies have shown that statins improve portal hypertension and survival in cirrhosis without ACLF[11,17,18,50–52] and ameliorate vascular function during sepsis.[20,26,53,54] In this study, we report for the first time to our knowledge that simvastatin prevents the endotoxemia-induced aggravation of chronic liver disease, with improvements in hepatic hemodynamic, inflammation, oxidative stress, and survival both in decompensated and compensated cirrhotic animals, although a greater protection was observed in decompensated CCl_4-cirrhotic rats.

Underlying mechanisms of simvastatin-derived protection may be multiple[9]: simvastatin confers vasoprotection in both normal and cirrhotic rat livers[13] and has a broad spectrum of anti-inflammatory and immunomodulatory effects. In fact, rats pretreated with simvastatin exhibited improved hepatic sinusoidal function, which prevented portal hypertension aggravation due to LPS and conferred both hepatic and systemic anti-inflammatory effects in cirrhotic rats with LPS-induced ACLF, thus suggesting possible extrahepatic protection in cirrhosis.[18]

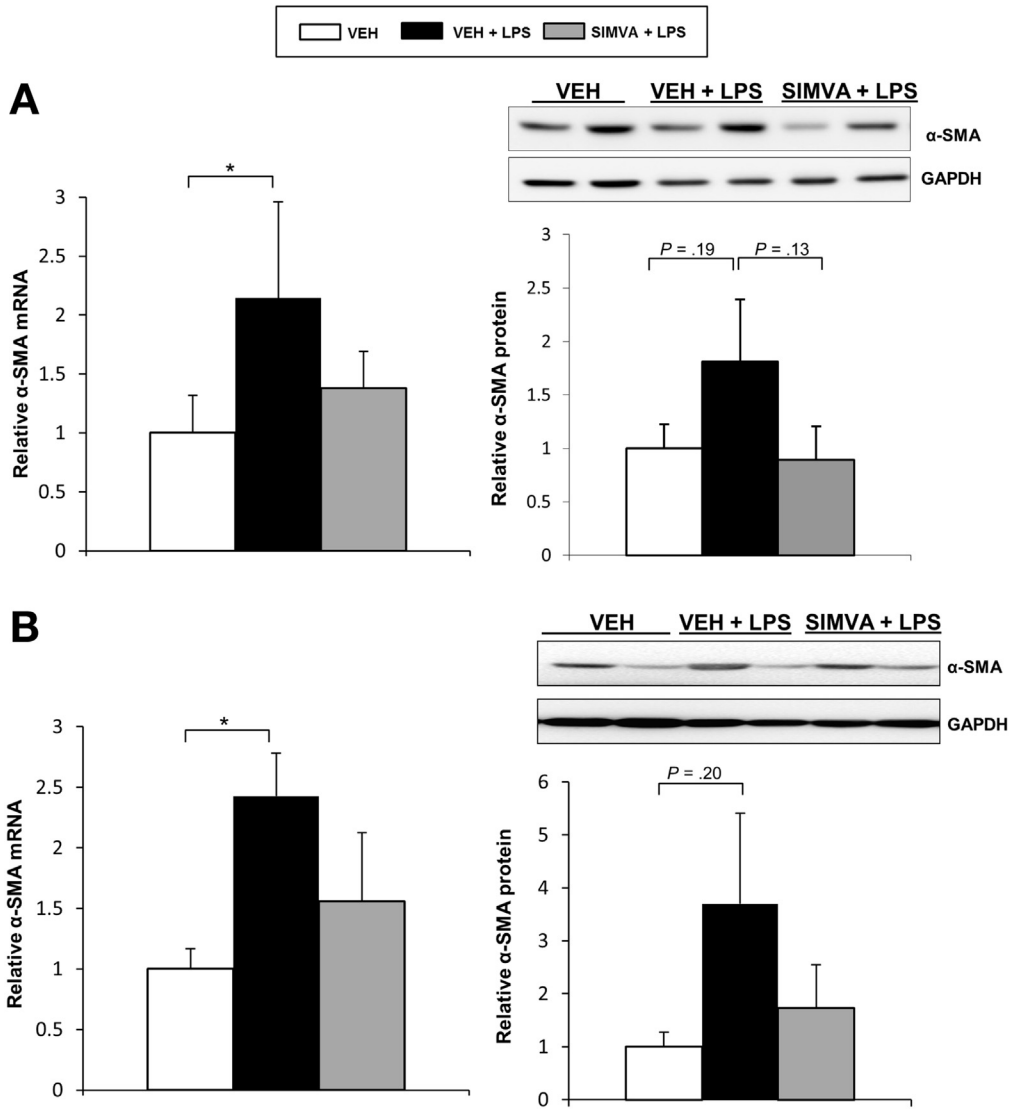

Figure 5. Effects of ACLF on the HSC phenotype in rats with ACLD treated with simvastatin or vehicle. HSC activation marker α-SMA was analyzed at the messenger RNA and protein expression levels in (*A*) CCl_4 and (*B*) BDL decompensated ACLD rats treated with vehicle (veh, n = 9 in CCl_4 and n = 8 in BDL), vehicle + LPS (veh + LPS, n = 12 in CCl_4 and n = 7 in BDL), or simvastatin + LPS (Simva + LPS; n = 9 in CCl_4 and n = 8 in BDL). Values represent mean ± standard error of the mean. *$P < .05$. α-SMA, α-smooth muscle actin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

We observed that simvastatin pretreatment profoundly decreased hepatic oxidative stress and improved nitric oxide production during ACLF. Such ameliorations were associated with significant improvement in HSC phenotype, which may also be partly due to reduced number of infiltrated neutrophils and NETs. However, we did not observe a reduction in collagen deposition in simvastatin-treated rats, probably because short-term simvastatin treatment may promote only HSC deactivation, whereas fibrosis reduction would require a much longer treatment period.

The described analysis is derived from survivors (those cirrhotic animals that resisted LPS challenge), and therefore it is plausible that the effects of ACLF on hepatic hemodynamics, inflammation, and liver cell phenotype, but also the protection conferred by simvastatin, in reality would be much more pronounced. In agreement with this, we did not observe evident aggravation in extrahepatic organs in cirrhotic animals with ACLF, which may be seen as a limitation of the model. Indeed, mild renal injury was suggested by elevated levels of plasma neutrophil gelatinase–associated lipocalin with no changes in creatinine levels. However, and as stated earlier, this may derive from the fact that we analyzed only those animals that survived to LPS administration, or multiorgan failure demonstration may require longer periods of LPS challenge.

In conclusion, development of ACLF due to LPS administration markedly exacerbated hepatic and systemic inflammatory pathways, leading to a profound aggravation of the already dysfunctional hepatic microvasculature and to decreased survival of cirrhotic animals. Simvastatin prevented these deregulations, mainly by avoiding the inflammatory burst, oxidative stress, and sinusoidal cell dysfunction. Considering our results reported here and previous studies,[17,19,28] we propose simvastatin as a safe and useful therapeutic strategy for cirrhotic patients undergoing ACLF. A large-scale clinical trial will evaluate the possible beneficial effects of this compound in patients with decompensated cirrhosis.[55]

Table 2. Initial and Final Number of Animals and Survival Rate in Rats With Decompensated and Compensated ACLD

	Decompensated ACLD					
	CCl_4			BDL		
Survival table	Initial No. of Animals	Final No. of Animals	% of Survival	Initial No. of Animals	Final No. of Animals	% of Survival
Vehicle	9	9	100	8	8	100
Vehicle + LPS	18	12	66.6[a]	12	7	58.3[a]
Simvastatin + LPS	9	9	100[b]	9	8	88.9[c]
	Compensated ACLD					
	CCl_4			TAA		
	Initial No. of Animals	Final No. of Animals	% of Survival	Initial No. of Animals	Final No. of Animals	% of Survival
Vehicle	5	5	100	4	4	100
Vehicle + LPS	8	6	75	6	3	50
Simvastatin + LPS	7	6	85.7	6	5	83.3

[a]$P < .05$ vs vehicle.
[b]$P < .05$ vehicle + LPS vs simvastatin + LPS.
[c]$P < .10$ vs vehicle + LPS.

Table 3. Effects of LPS and Simvastatin on Biochemical Parameters in Rats With Decompensated and Compensated ACLD

	Decompensated ACLD					
	CCl_4			BDL		
Parameters	Vehicle	Vehicle + LPS	Simvastatin + LPS	Vehicle	Vehicle + LPS	Simvastatin + LPS
Number of animals	**9**	**12**	**9**	**8**	**7**	**8**
AST (*U/L*)	193 ± 136	522 ± 399[a]	247 ± 161[d]	423 ± 156	763 ± 613	997 ± 571[c]
ALT (*U/L*)	84 ± 47	172 ± 125[c]	98 ± 32	68 ± 14	80 ± 52	122 ± 82
Total bilirubin (*mg/dL*)	0.27 ± 0.27	2.10 ± 0.76[a]	0.32 ± 0.27[b]	7.83 ± 2.07	6.94 ± 1.87	5.60 ± 1.66[a]
Albumin (*g/L*)	23 ± 5	23 ± 4	26 ± 2[d]	25 ± 4	20 ± 6	20 ± 7
Creatinine (*mg/dL*)	0.53 ± 0.19	0.65 ± 0.24	0.67 ± 0.18	0.80 ± 0.15	0.79 ± 0.24	0.70 ± 0.29
Sodium (*mEq/L*)	170 ± 18	176 ± 25	169 ± 21	187 ± 25	173 ± 26	160 ± 17[c]
Ammonia (*μmol/L*)	263 ± 153	392 ± 193	167 ± 127[b]	376 ± 135	514 ± 220	592 ± 213[c]
	Compensated ACLD					
	CCl_4			TAA		
Parameters	Vehicle	Vehicle + LPS	Simvastatin + LPS	Vehicle	Vehicle + LPS	Simvastatin + LPS
Number of animals	**5**	**6**	**6**	**4**	**3**	**5**
AST (*U/L*)	161 ± 139	135 ± 51	166 ± 66	149 ± 60	1060 ± 2200	156 ± 37
ALT (*U/L*)	78 ± 57	50 ± 18	72 ± 25	59 ± 7	381 ± 796	46 ± 15
Total bilirubin (*mg/dL*)	0.10 ± 0.00	0.86 ± 0.53[a]	0.13 ± 0.05[b]	0.12 ± 0.05	0.61 ± 0.27[a]	0.12 ± 0.04[b]
Albumin (*g/L*)	30 ± 2	28 ± 3	28 ± 4	32 ± 3	34 ± 3	32 ± 4
Creatinine (*mg/dL*)	0.65 ± 0.21	0.67 ± 0.12	0.59 ± 0.06	0.48 ± 0.09	0.85 ± 0.38	0.81 ± 0.29
Sodium (*mEq/L*)	172 ± 12	179 ± 5	178 ± 13	189 ± 5	176 ± 14	172 ± 24
Ammonia (*μmol/L*)	166 ± 127	166 ± 59	183 ± 140	255 ± 117	357 ± 127	297 ± 141

NOTE. Values represent mean ± standard deviation.
ALT, alanine aminotransferase; AST, aspartate aminotransferase.
[a]$P < .05$ vs vehicle.
[b]$P < .05$ vehicle + LPS vs simvastatin + LPS.
[c]$P < .10$ vs vehicle.
[d]$P < .10$ vs vehicle + LPS.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2018.07.022.

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The authors disclose no conflicts.

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