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The Protective Function of Neutrophil Elastase Inhibitor in Liver Ischemia and Reperfusion Injury

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Abstract

Background—A neutrophil elastase (NE) inhibitor, Sivelestat, has been approved for the treatment of acute lung injury associated with systemic inflammation in humans. Some reports have also shown its protective effects in liver inflammatory states. We have recently documented the importance of NE in the pathophysiology of liver ischemia and reperfusion injury (IRI), a local Ag-independent inflammation response. This study was designed to explore putative cytoprotective functions of clinically available Sivelestat in liver IRI.

Methods—Partial warm ischemia was produced in the left and middle hepatic lobes of C57BL/6 mice for 90 min, followed by 6 or 24 h of reperfusion. Mice were given Sivelestat (100 mg/kg, s.c.) at 10 min prior to ischemia, 10 min prior to reperfusion, and at 1 h and 3 h of reperfusion thereafter.

Results—Sivelestat treatment significantly reduced serum ALT levels and NE activity, as compared with controls. Histological liver examination has revealed that unlike in controls, Sivelestat ameliorated the hepatocellular damage and decreased local neutrophil activity and infiltration. The expression of pro-inflammatory cytokines (TNF-α, IL-6), chemokines (CXCL-1, CXCL-2, CXCL-10) and TLR4 was significantly reduced in the treatment group, along with diminished apoptosis via caspase-3 pathway. Moreover, in vitro studies confirmed downregulation of pro-inflammatory cytokine and chemokine programs in mouse macrophage cell cultures, along with depression of innate TLR4 signaling.

Conclusion—Sivelestat-mediated NE inhibition may represent an effective therapeutic option in liver transplantation and other inflammation disease states.

Keywords

Liver; Neutrophil Elastase; Ischemia/Reperfusion Injury; Toll-like Receptor-4

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INTRODUCTION

Ischemia/reperfusion injury (IRI), an exogenous Ag-independent inflammatory event, remains the major problem in clinical transplantation. In the liver case, IRI causes up to 10% of early transplant failures and can lead to a higher incidence of both acute and chronic rejection (1). The mechanisms underlying liver IRI involve leukocyte accumulation and activation (neutrophils, Kupffer cells, and T cells), pro-inflammatory cytokine and chemokine secretion, complement activation, and vascular cell adhesion molecule activation (2,3).

The activation of sentinel Toll-like receptor (TLR) system plays an important role in the infectious and inflammatory disease states (4,5). We have reported (6-9) that: (i) TLR4, but not TLR2 signaling plays a key role in initiating exogenous Ag-independent innate immunity-dominated liver IRI, (ii) the hepatocellular damage proceeds by down-stream interferon regulatory factor-3 (IRF-3)-dependent TLR4 activation pathway, and (iii) the endogenous TLR4 ligand network is required to facilitate IR-mediated inflammation. In addition, in agreement with others findings, we have reported on the crucial role of neutrophils, in particular neutrophil elastase (NE) in the pathophysiology of liver IRI (10). The NE is a 29-kDa glycoprotein chymotrypsin-like serine protease stored in azurophil granules in its active form until it is released following neutrophil exposure to the inflammatory stimuli. Once released, NE is potentially fully active because it functions optimally in a neutral environment (11,12). As a result, excessive release of NE degrades elastin, collagens, laminins, and other extracellular matrix components, thereby leading to subsequent organ damage through endothelial cell injury (13,14).

Sivelestat (ONO-5046) is a low molecular weight synthetic specific and competitive inhibitor of NE activity (15). It has been used clinically in Japan, and shown to attenuate acute lung injury associated with systemic inflammation response, which may occur after infection, surgical intervention, traumatic or burn injury (16). In addition, Sivelestat exerts potent cytoprotective functions in animal models of liver transplantation (17), hepatectomy (18,19), microvascular dysfunction (20) and IRI (21).

This study was designed to examine the efficacy and mechanism of Sivelestat in well-established mouse model of liver warm ischemia followed by reperfusion. Sivelestat profoundly attenuated otherwise fulminant liver damage and inflammatory cell recruitment. These cytoprotective effects were accompanied by downregulation of TLR4 innate signaling, data supported by in vitro cell culture system. Hence, NE inhibition by a clinically available drug, Sivelestat, represents an attractive new therapeutic option in liver transplantation and other inflammatory disease states.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (8-10 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in the University of California Los Angeles animal facility under specific pathogen-free conditions. All animals received human care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of sciences and published by the National Institute of Health (NIH publication 86-23, revised 1985).

Liver IRI Model

We used an established mouse model of partial warm hepatic IRI (22, 23). Briefly, mice were anesthetized with isoflurane, injected with heparin (100 U/kg), and an atraumatic clip

was used to interrupt the artery/portal venous blood supply to the left and middle liver lobes. After 90 min of partial warm ischemia, the clamp was removed, initiating hepatic reperfusion.

Based on our previous NE activity data (10), and $t^{1/2}$ of Sivelestat, NE inhibitor (ONO-5046; N-[2-(4-[2,2-dimethylpropionyloxy] phenylsulphonyl-amino) benzoyl] amino acetic acid; Ono Pharmaceuticals, Osaka, Japan) of 26.45±7.9 min, we used Sivelestat (100 mg/kg, s.c x4) 10 min prior to ischemia, 10 min prior to reperfusion, and then 1 h and 3 h of reperfusion (n=6/group). Animals were sacrificed at 6 h or 24 h after reperfusion for serum and liver sampling (24). Sham controls underwent the same procedure, but without vascular occlusion.

Hepatocellular function

Serum alanine aminotransferase (sALT) levels, an indicator of hepatocellular injury, were measured in peripheral blood samples at 6 or 24 h after reperfusion with an autoanalyzer (ANTECH Diagnostics, Los Angeles, CA).

Histology

Liver specimens were fixed in 10% buffered formalin solution and embedded in paraffin. Liver paraffin sections (5-μm thick) were stained with hematoxylin and eosin. The severity of liver IRI was blindly graded with modified Suzuki's criteria (25). In this classification, sinusoidal congestion, hepatocyte necrosis, and ballooning degeneration are graded from 0 to 4. The absence of necrosis, congestion/centrilobular ballooning is given a score of 0, whereas severe congestion/ballooning, as well as >60% lobular necrosis, is given a value of 4.

Serum NE Activity

Serum NE activity was determined using N-methoxysuccinyl-Ala-Ala -Pro-Val-p-nitroanilide, a highly specific synthetic substrate for NE according to the method of Yoshimura et al. (26, 27). Briefly, serum sample was incubated with 0.1 M Tris–HCl buffer (pH 8.0) containing 0.5 M NaCl and 1 mM substrate at 37°C for 24 h and the amount of p-nitroanilide (pNA) liberated was measured spectrophotometrically at 405 nm and was considered as NE activity.

Myeloperoxidase (MPO) assay

The MPO activity, an enzyme specific for polymorphonuclear cells, was used as an index of hepatic neutrophil accumulation (23). Briefly, the frozen tissue was thawed and placed in iced 0.5% hexadecyltrimethyl- ammonium (Sigma-Aldrich) and 50 mM potassium phosphate buffer solution (Sigma-Aldrich, pH = 5.0). Each sample was homogenized and centrifuged at 15,000 rpm for 15 min at 4°C. Supernatants were then mixed with hydrogen peroxide-sodium acetate and tetramethyl- benzidine solutions (Sigma-Aldrich). The change in absorbance was measured by spectrophotometry at 655 nm in 1 min. One unit of MPO activity was defined as the quantity of enzyme degrading 1 μM peroxide/min at 25°C/g of tissue.

Immunohistochemistry

Livers were snap frozen, and cryostat sections (5 μm) were fixed in acetone. Endogenous peroxidase activity was inhibited with 0.3% hydrogen peroxidase. Primary rat mAb against mouse polymorphonuclear cells (Ly-6G, 1A8; BD Pharmingen) was diluted (1/250 in 3% goat serum), and 100 μL was added to each section. The secondary Ab, a biotinylated goat anti-rat immunoglobulin G (Vector; diluted 1:200), was incubated for 40 min. Sections were

incubated with immunoperoxidase (ABC Kit, Vector), washed, and developed with a 3,3'-diaminobenzidine kit (Vector). Negative control was prepared by omission of primary Ab. Polymorphonuclear cells, identified by staining and morphology, were counted in 10 HPF/section under light microscopy (x400), and numbers of cells/HPF (mean±SD) are shown.

Apoptosis Assay

Apoptosis in 5-μm cryostat liver sections was detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method with an *in situ* cell death detection kit (Roche) according to the manufacturer's protocol. A negative control was prepared by omission of the terminal transferase. Positive controls were generated by treatment with DNase I (1μg/ml for 10 min). The peroxidase activity was visualized with a diaminobenzidine substrate, which yielded a brown oxidation product; methyl green was used for counterstaining. TUNEL-positive cells were counted in 10 HPF/section under light microscopy (X400), and the numbers of cells/HPF (mean±SD) are shown.

RNA Extraction and Quantitative RT-PCR

Total RNA was extracted from cells and liver tissue using the TRIzol reagent (Life Technologies, Inc., Grand Island, NY). Reverse transcription was performed using 5 μg of total RNA in a First-Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, Maryland). Primers used in PCR were as follows: TNF-α sense (5'-GCCTCTTCTCATTCCTGCTTGT-3') and TNF-α antisense (5'-GATGATCTGAGTGTGAGGGTCTG-3'); IL-6 sense (5'-GCTACCAAACTGGATATAATCAGGA-3') and IL-6 antisense (5'-CCAGGTAGCTATGGTACTCCAGAA-3'); CXCL-1 sense (5'-ACCCAAACCGAAGTCATAG-3') and CXCL-1 antisense (5'-TTGTATAGTGTTGTCAGAAGC-3'); CXCL-2 sense (5'-ACTTCAAGAACATCCAGAG-3') and CXCL-2 antisense (5'-CTTTCCAGGTCAGTTAGC-3'); TLR4 sense (5'-GGACTCTGATCATGGCACTG-3') and TLR4 antisense (5'-CTGATCCATGCATTGGTAGGT-3'); CXCL-10 sense (5'-GCTGCCGTCATTTTCTGC-3') and CXCL-10 antisense (5'-TCTCACTGGCCCGTCATC-3 ') and HPRT sense (5'-TCAACGGGGGACATAAAAGT-3') and HPRT antisense (5'-TGCATTGTTTTACCAGTGTCAA-3'). Quantitative PCR was performed using the DNA Engine with Chromo 4Detector (MJ Research, Waltham, MA). In a final reaction volume of 20 μL, the following were added: 1 X SuperMix (Platinum SYBR Green qPCR Kit, Invitrogen, Carlsbad, CA), complementary DNA, and 10 μM of each primer. Amplification conditions were: 50°C (2 min), 95°C (5 min), followed by 45 cycles of 95°C (15 sec), 60°C (30 sec).

Western Blot Assay

The extracted proteins (40 μg/sample) were separated by SDS - PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes. The membranes were probed with Ab against cleaved caspase-3 (Cell Signaling Technology, Danvers, MA) and β-actin (Abcam Inc., Cambridge, MA), and then incubated with HRP-conjugated secondary Ab (Cell Signaling Technology). Detection was performed using a SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL). Relative quantities of protein were determined using a densitometer.

Cell Cultures

The mouse macrophage cell line RAW 264.7 (TAB-71 ATCC, Manassas, VA) was maintained in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Inc. Manassas,

VA) supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine, penicillin, and streptomycin in a humidified 37°C/5% CO_2 incubator. Cells remained untreated or were pretreated with Sivelestat (100μM) 10 and 30 min prior to LPS adjunct (10ng/mL, Sigma, St. Louis, MO) and 1 and 2.5 h after the stimulation. Cells were then incubated for 4 h before harvesting for Quantitative Reverse Transcriptase PCR analysis as described.

Statistical Analysis

All data are expressed as means ± SD. Differences between experimental groups were analyzed using one-way analysis of variance or Student's *t* test for unpaired data. All differences were considered statistically significant at the P value of <0.05.

RESULTS

Sivelestat Inhibits NE Activity and Ameliorates Liver IRI

In our model of 90 min liver warm ischemia, Sivelestat pre-treatment significantly inhibited NE activity at both 6 and 24 h after reperfusion (Fig. 1a; [6 h] 6.81 ± 2.62 vs 3.02 ± 0.65 and [24 h] 3.44 ± 1.47 vs 1.25 ± 0.23; $p<0.01$). The inhibition of NE activity was accompanied by amelioration of IR-induced hepatocellular damage (Fig. 1b). Indeed, sALT levels remained diminished at both 6 h and 24 h of reperfusion in the treated mice, as compared with controls ([6 h] 25833 ± 1204 vs 11698 ± 3257 and [24 h] 6465 ± 1844 vs 2670 ± 1302; $p<0.01$). These data correlated with the histological criteria of hepatocellular damage, as defined by modified Suzuki's criteria (25). As shown in Fig. 1c, livers in untreated hosts showed severe lobular edema, congestion, ballooning and hepatocellular necrosis (score = [6 h] 11.33 ± 0.82 and [24 h] 8.83 ± 0.98). In contrast, livers in Sivelestat treated mice had largely preserved architecture and histological detail (score = [6 h] 9.17 ± 1.17 and [24 h] 6.50 ± 1.52; $p<0.01$).

Sivelestat Suppresses Neutrophil Infiltration

We analyzed MPO activity, an index of neutrophils infiltration, in livers at 6 h and 24 h of reperfusion after 90 min of warm ischemia (Figure 2a). The MPO activity (U/g) remained markedly suppressed after treatment with Sivelestat, as compared with controls ([6 h] 10.69 ± 2.07 vs 4.89 ± 0.50 and [24h] 8.94 ± 1.19 vs 2.19 ± 0.93; $p< 0.05$). These MPO activity data correlated with the number of Ly-6G positive cells sequestered in the liver (Figure 2b; [6 h] 29.97 ± 7.24 vs 13.17 ± 5.14 and [24h] 18.43 ± 4.65 vs 8.63 ± 4.02; $p< 0.05$).

Sivelestat Suppresses Pro-Inflammatory Cytokine/Chemokine Programs and TLR4 Expression

We used quantitative RT-PCR to analyze liver expression of pro-inflammatory cytokines (TNF-α and IL-6), chemokines (CXCL-1 [KC: a mouse homolog of human chemokine gro-α] and CXCL-2 [macrophage inflammatory protein-2: MIP-2]), CXCL-10 and TLR4 during IRI, and calculated the ratio between post-IR and basal mRNA levels in each animal (Fig. 3). Untreated mice showed increased induction ratios of TNF-α (TNF-α/HPRT mRNA: [6 h] 1.60±0.35 vs 0.79±0.20, p<0.05; [24 h] 0.86±0.47 vs 0.41±0.20) and IL-6 (IL-6/HPRT mRNA: [6 h] 3.08±0.51 vs 0.64±0.10, p<0.01; [24 h] 1.00±0.25 vs 0.30±0.13, p<0.05) as compared with the treated group (Figure 3a). The CXC chemokines are known to act predominantly on neutrophils (28), with CXCL-1 (KC) and CXCL-2 (MIP-2) being particularly relevant in the mechanism of liver IRI (29). The expression of both CXCL-1 and -2 was significantly increased in untreated vs Sivelestat treated group at reperfusion (CXCL-1/HPRT mRNA: [6 h] 3.09±0.45 vs 1.10±0.67 and [24 h] 0.95±0.15 vs 0.26±0.12, p<0.01, CXCL-2/HPRT mRNA: [6 h] 6.01±0.90 vs 2.80±1.04 and [24 h] 2.08±0.36 vs 0.73±0.17, p<0.01; Figure 3b). Moreover, the expression of mRNA coding for TLR4 and

CXCL-10 increased sharply in untreated group. Sivelestat treatment inhibited TLR4 expression (TLR4/HPRT mRNA: [6 h] 2.18±0.34 vs 1.02±0.21 and [24 h] 1.71±0.18 vs 0.46±0.37, p<0.01), which was accompanied by diminished CXCL-10 expression levels (CXCL-10/HPRT mRNA: [6 h] 1.64±0.15 vs 0.85±0.18, p<0.01; [24 h] 0.54±0.29 vs 0.25±0.13; Figure 3c).

Sivelestat Suppresses Apoptosis

Western blot analysis (Figure 4a) has revealed that treatment with Sivelestat inhibited the expression of cleaved caspase-3, as compared with untreated controls (cleaved caspase-3/β-actin: [6 h] 1.51 ± 0.05 vs 0.81 ± 0.13, $p<0.05$; [24 h] 0.44 ± 0.39 vs 0.10 ± 0.03). Furthermore, Sivelestat treatment decreased the number of TUNEL positive cells, as compared with the untreated controls ([6 h] 24.87 ± 5.93 vs 10.93 ± 4.86 and [24 h] 18.93 ± 6.73 vs 6.80 ± 5.10; $p<0.05$; Figure 4b).

Sivelestat Regulates Neutrophil - Macrophage Cross Talk

We performed in vitro experiments utilizing mouse macrophage RAW 264.7 cells, in an attempt to mimic our in vivo liver IRI setting. As shown in Fig. 5a/b, addition of Sivelestat significantly suppressed LPS-stimulated TNF-α, IL-6, CXCL-1 and CXCL-2 expression in vitro (TNF-α/HPRT mRNA: 2.07±0.26 vs 0.48±0.06 and IL-6/HPRT mRNA: 71.36±1.95 vs 34.40±0.55; p<0.05 , C X C L-1/HPRT mRNA: 0.62±0.007 vs 0.20±0.003; p<0.01, CXCL-2/HPRT mRNA: 2.56±0.30 vs 1.27±0.002; p<0.05). Moreover, consistent with our in vivo findings, Sivelestat supplement decreased LPS-induced TLR4 and CXCL-10 expression (TLR4/HPRT mRNA: 2.21±0.32 vs 0.63±0.47 and CXCL-10/HPRT mRNA: 13.77±0.44 vs 5.07±0.56; p<0.01; Figure 5c).

DISCUSSION

In the present study in a well-established model of mouse liver injury due to warm ischemia followed by reperfusion, we found that Sivelestat-mediated suppression of NE activity: i) ameliorated the hepatocellular damage; ii) reduced neutrophil activity/infiltration; iii) downregulated pro-inflammatory cytokine/chemokine and TLR4 gene induction programs; and iv) reduced apoptosis via caspase-3 pathway. Moreover, our concomitant macrophage (RAW 264.7) cell cultures have confirmed the efficacy of Sivelestat to downregulate not only pro-inflammatory cytokines, chemokines, but also TLR4 expression in vitro. Hence, our results highlight the role of NE - TLR4 cross talk in facilitating the liver damage, and suggest that NE inhibitors, such as clinically available Sivelestat, may represent an effective new therapeutic option to ameliorate the “harvesting” insult component in solid organ transplantation.

The present results are consistent with our previous findings (10) on a commercially available NE inhibitor (Sigma; GW311616A). However, unlike the latter, Sivelestat has been safely used in humans (16). Despite its half-life 10-fold shorter (0.12 h) and the efficacy to inhibit NE 15-fold weaker (Ki value 46 nM), the striking efficacy to suppress liver IRI damage, as shown here, was comparable with that of GW311616A (30). Unlike GW311616A, which required a single oral dose, a more frequent systemic Sivelestat regimen was needed to maintain adequate concentration of NEI before and during IR stress, consistent with the published reports (21). Moreover, our present in vitro culture data support the role of NE in the intricate IR-triggered innate immune cascade that leads to perfusion disturbances, which culminate in the liver damage.

The acute inflammation response during liver reperfusion consists of two phases. In the first, during Kupffer cell-mediated phase (0–6 h of reperfusion) the generation of reactive oxygen

species aggravates the organ damage. Activated Kupffer cells and infiltrating lymphocytes elaborate cytokines that further promote the inflammation response. In the second (6–24 h of reperfusion), the neutrophils become fully activated to express panels of mediators that dominate the liver injury process (31). These include ROS, complement components, proteases as well as CXCL-1 (KC) and CXCL-2 (MIP-2), neutrophil chemoattractants particularly important in liver IRI cascade (29,33). Murine CXCL-2, a chemokine functionally analogous to human IL-8 and rat neutrophil chemoattractant (34) is primarily induced by TNF-α (35). Here, we show that inhibition of NE suppressed the expression of TNF-α, IL-6, CXCL-1 and CXCL-2, in parallel with marked decrease in MPO activity and Ly-6G neutrophil infiltration in the ischemic liver lobes. Hence, consistent with previous reports (10), NE promotes local inflammation response, whereas treatment with NEI reduces the levels of inflammatory mediators by inhibiting NF-kB (9,36,37).

TNF-α, the central mediator in liver response to IR (38), has been also shown to initiate the apoptotic cascade, which represents a key event in IRI cascade, the severity of which correlates with the degree of hepatic injury (39). Consistent with our previous studies (10), we found significantly increased frequency of TUNEL-positive apoptotic cells during unmodified liver IRI. Indeed, neutralization of NE activity not only ameliorated hepatic apoptosis but also diminished the cleaved caspase-3 expression, one of putative molecular pathways through which the hepatocyte damage occurs in our model system.

TLR4 signaling on Kupffer cells is critical in the pathogenesis of liver IRI (40). This signaling pathway is triggered by endotoxin in the blood interacting with LPS-binding protein, which elicits LPS to anchor to the CD14 molecule and the extracellular TLR4 portion. This compound mediates LPS signaling, transducing extracellular inflammatory signals into cells and thus initiating the inflammation response (41). During liver IR, portal vein occlusion results in congestion of the intestinal wall, leading to the release of gut-derived molecules, including endotoxin, into the blood stream. Although LPS is an obvious candidate for TLR4 activation (42-44), our own data documents the functional importance of endogeneous TLR ligands as an initiating factor in liver IRI (7). Furthermore, we and others have reported that TLR4 is involved in the initiation of IRI, as evidenced by the full protection of TLR4-deficient livers (6,40,45,46). Activation of TLR4 is required for the development of liver IRI, and its downstream CXCL-10 mediator facilitatates IR-induced liver inflammation and hepatocellular damage (8). Although TLR4 downstream MyD88-independent signaling mediated by IRF-3 induces CXCL-10 (6), MyD88-dependent pathway leads to direct NF-kB activation and induction of pro-inflammatory cytokines (8). Our results show that NE inhibition after intermittent Sivelestat treatment up to 3 h after reperfusion, did suppress TLR4 expression at 6 h, with subsequent diminished CXCL10 levels at both 6 and 24 h. These correlated with LPS-stimulated mouse macrophage cell culture experiments in which NE inhibition suppressed both TLR4 and CXCL-10 expression in vitro. This is also consistent with previous reports, which have shown the efficacy of Sivelestat to prevent LPS-induced liver injury or to inhibit NF-kB activation in cell culture (19,36). As NF-kB inhibition profoundly (ca. 90%) suppresses CXCL-10 gene expression (47-49), Sivelestat might regulate MyD88-dependent signaling in TLR4 pathway through NF-kB inhibition.

Figure 6 depicts putative mechanisms underlying the protective effects of Sivelestat in the context of IR-triggered NE and the inflammatory cascade. Activated Kupffer cells produce inflammatory cytokines (TNF-α and IL-6) and chemokines (CXCL-1 and CXCL-2). Moreover, TLR4 activation on Kupffer cells and hepatocytes accelerates production of the key downstream mediator, CXCL-10. TNF-α affects the surrounding hepatocytes causing their apoptosis, and triggers neutrophil-attracting CXC chemokines to express adhesion molecules on vascular endothelial cells. Neutrophil adhesion to endothelial cells leads to

their transmigration into the liver parenchyma. Neutrophil-derived NE induces inflammatory chemokines (CXCL-1, CXCL-2), and accelerates IR-mediated damage via the feedback mechanism with recruited neutrophils, which results in the direct injury to membrane components (38). It has been documented that IL-8 up-regulation by NE occurs in part through the cell surface membrane–bound TLR4 in the bronchial epithelium (50). Although NE associates with increased expression of monocyte/macrophage TLR4 in sepsis (51), the direct effect of NE on TLR4 could not be discarded in *Leishmania* infection (52). As the mechanism by with IR insult activates TLR4 system remains to be elucidated (7), our results imply that a direct effect of NE on TLR4 complex cannot be discarded. Thus, NE may affect TLR4 by serving as its putative endogenous ligand, and thus causing TLR4 up-regulation. Sivelestat-mediated depression of chemokines prevents recruitment of neutrophils and macrophages, which in turn suppresses local inflammation. Moreover, Sivelestat may inhibit various inflammatory mediators through NF-kB inhibition on Kupffer cells and hepatocytes.

In conclusion, inhibition of NE activity in mouse livers subjected to IRI by a relatively high dose of clinically available Sivelestat, suppressed neutrophil activation / secretion of pro-inflammatory mediators. The most effective dose, timing and duration of Sivelestat treatment for clinical use in liver injury remains to be determined. However, this study provides evidence that by affecting cross talk between NE and TLR4 signaling, NE inhibitors may represent new therapeutic option for treatment of organ IRI, to increase the organ donor pool and the overall success of organ transplantation.

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Abbreviations

ALT	alanine aminotransferase
IRI	ischemia/reperfusion injury
LPS	lipopolysaccharide
MPO	myeloperoxidase
NE	neutrophil elastase
NEI	neutrophil elastase inhibitor
TLR4	toll-like receptor 4
TNF	tumor necrosis factor
ROS	reactive oxygen species

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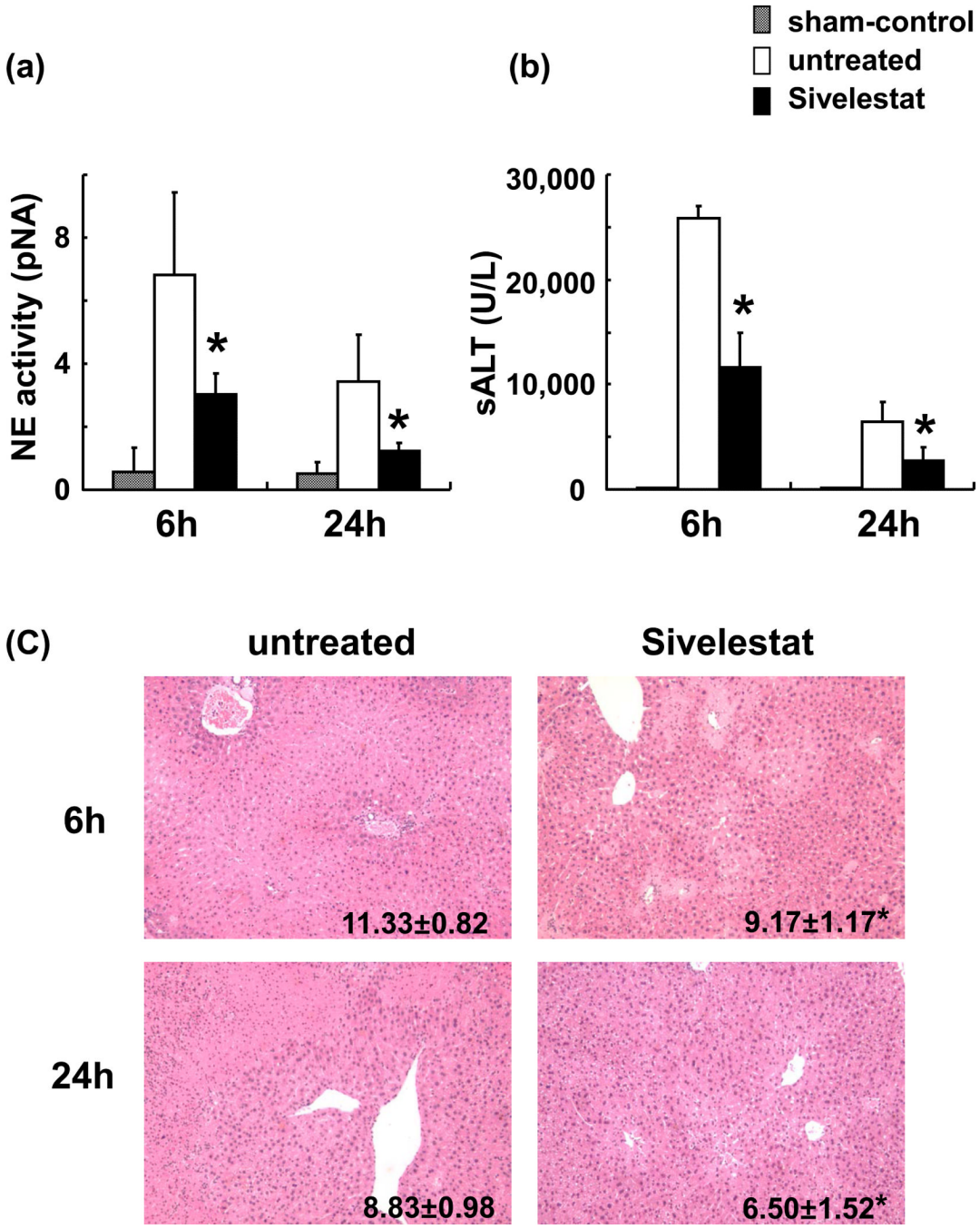

Figure 1.

The hepatocellular damage. (a) Sivelestat treatment inhibited NE activity in mouse serum after liver warm ischemia (90 min) followed by reperfusion (6 and 24 h) (*P<0.01; n=4-6/group). (b) Serum ALT levels at both 6 and 24 h after reperfusion were lower in Sivelestat treatment group, as compared with untreated controls (*P<0.01; n=6/group). Means and SD are shown. (c) Representative liver histology (HE staining; X100 magnification) of ischemic liver lobes harvested at 6 and 24 h after reperfusion (Upper panel: 6 h; Suzuki's score = 11.33 ± 0.82 vs 9.17 ± 1.17; p<0.01, Lower panel: 24 h; 8.83 ± 0.98 vs 6.50 ± 1.52; *P<0.01, n=6/group).

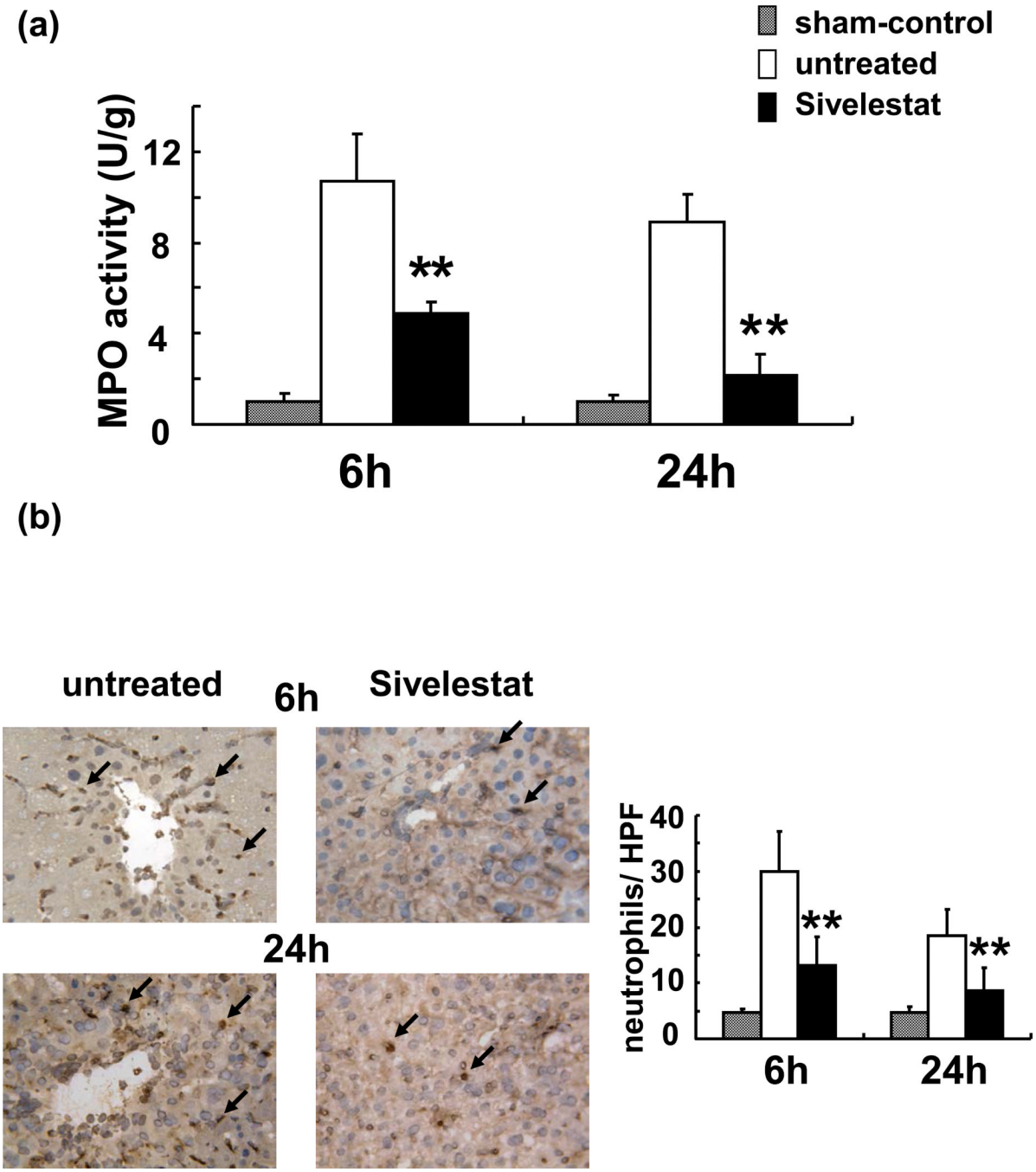

Figure 2.

Neutrophil accumulation. (a) MPO, an index of neutrophil infiltration, activity was markedly reduced in the treatment group (**P<0.05 ; n=3/group). (b) Sivelestat-treated livers showed significantly decreased numbers of infiltrated polymorphonuclear cells stained by Ly-6G (dark brown spots) as compared with untreated controls (X400 magnification **P < 0.05; n=3/group). Means and SD are shown.

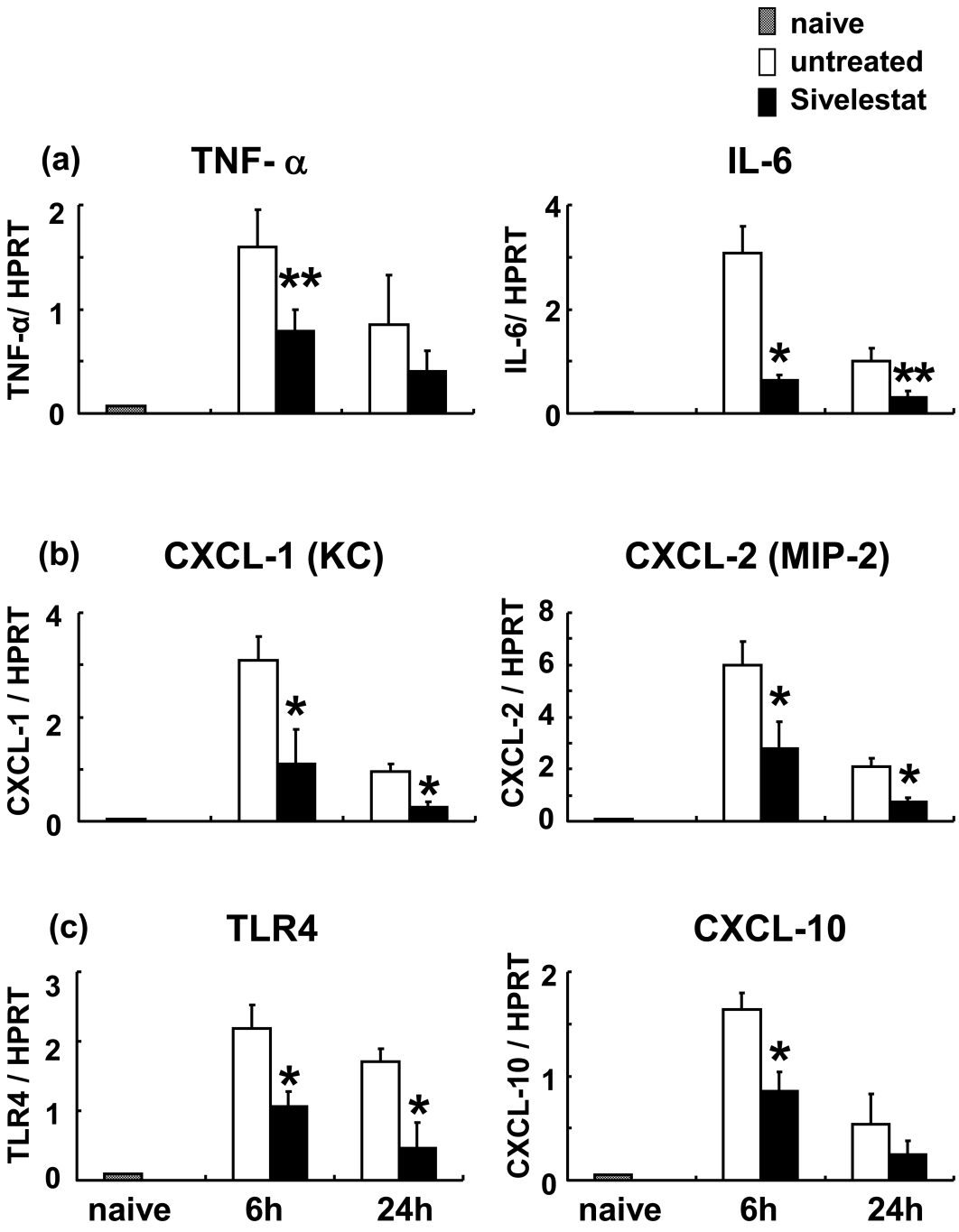

Figure 3.

RT-PCR-assisted detection of pro-inflammatory mediators in IRI livers. (a) Cytokine gene expression. (b) Chemokine gene expression. (c) TLR4 and CXCL-10 gene expression in untreated and treated livers. Data were normalized to HPRT gene expression (*$p < 0.01$, **$p < 0.05$; n =3/group). Means and SD are shown.

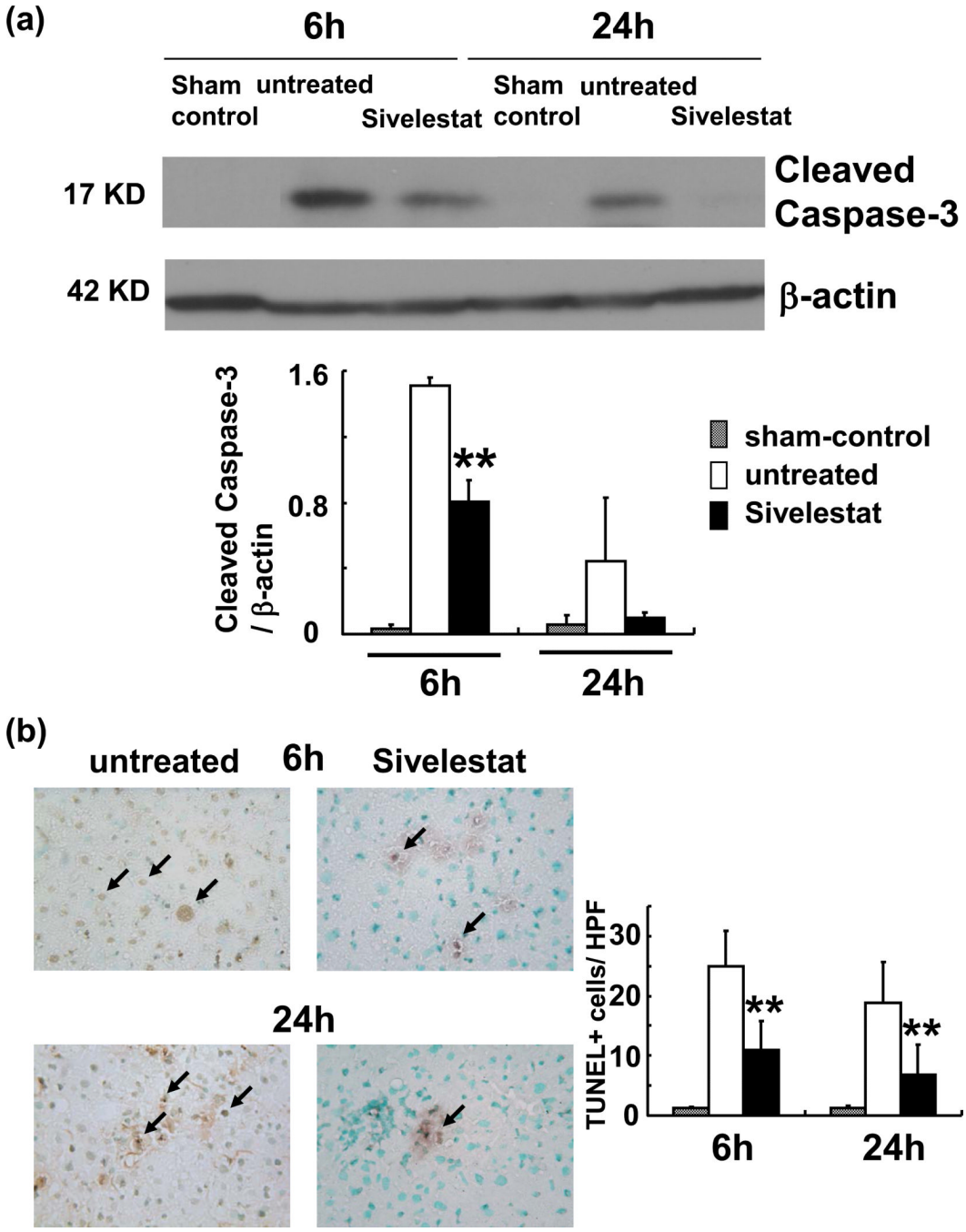

Figure 4.

Apoptosis in ischemic livers. (a) Treatment with Sivelestat significantly reduced cleaved Caspase-3 expression by Western blots (**P<0.05; n=2-3/group). (b) Sivelestat-treated livers showed significantly decreased numbers of TUNEL positive cells (dark brown spots) as compared with untreated controls (X400 magnification, **P<0.05; n=3/group). Means and SD are shown.

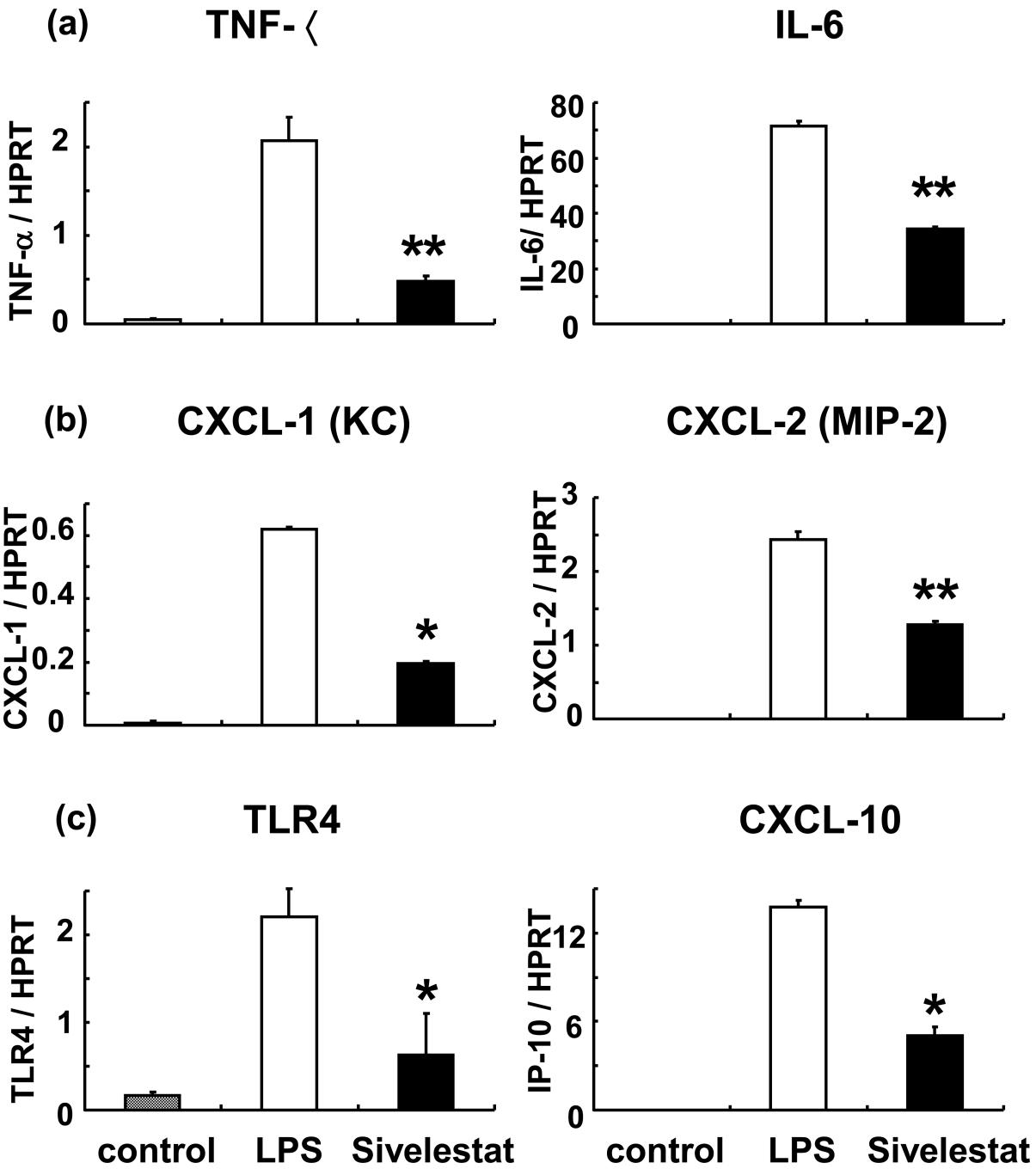

Figure 5.

RT-PCR-assisted detection of pro-inflammatory mediators in mouse macrophage (RAW 264.7) cell cultures. Cells were pretreated with Sivelestat (100 μM) 10 and 30 min prior to LPS (10 ng/ml), and 1 and 2.5 h after the stimulation. Cells were harvested at 4 h. (a) Cytokine gene expression. (b) Chemokine gene expression. (c) TLR4 and CXCL-10 gene expression, as compared with LPS alone. Data were normalized to HPRT gene expression (*$p < 0.01$, **$p < 0.05$). Means and SD are shown.

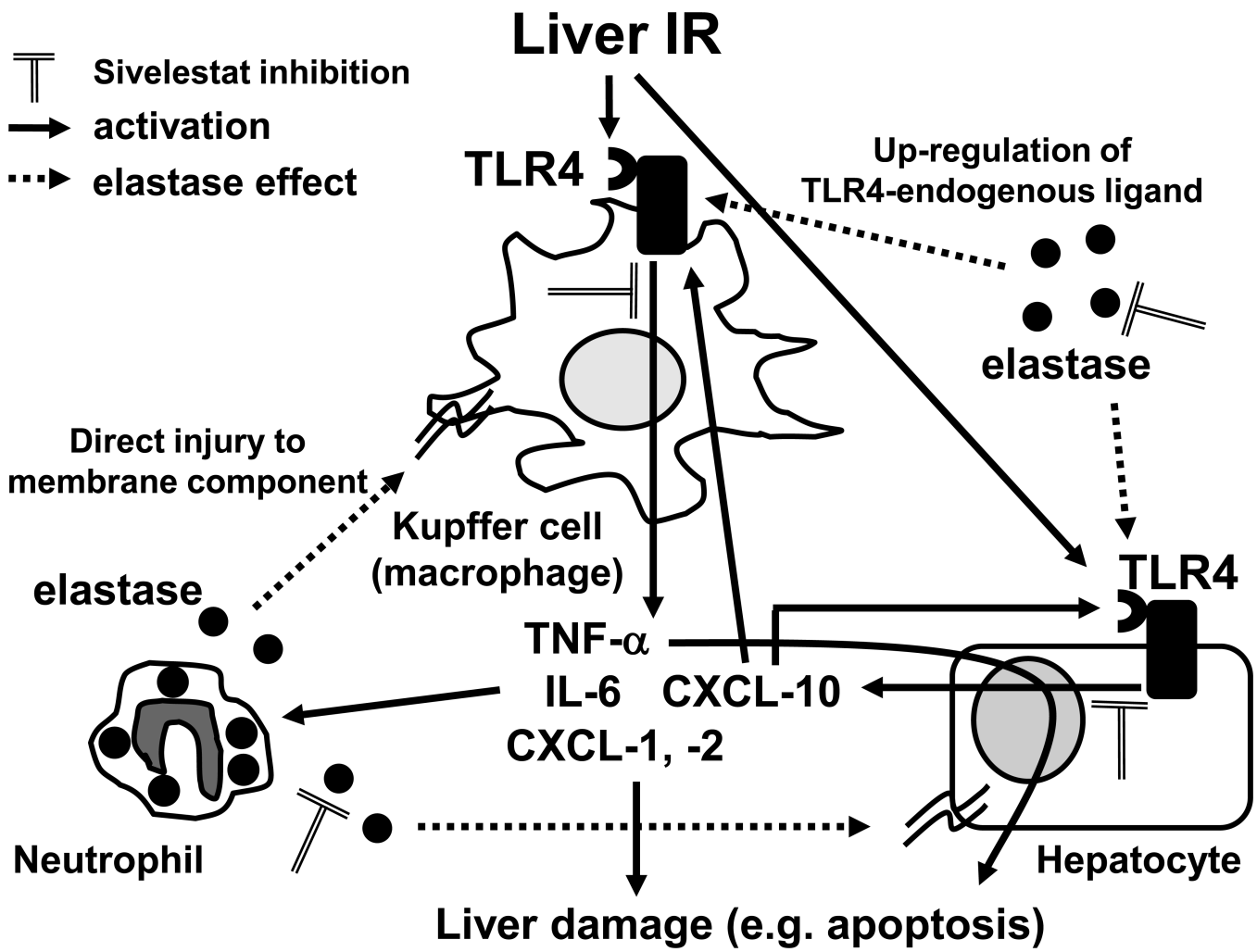

Figure 6.

Cross-talk between NE and inflammation responses in liver IRI. Activated Kupffer cells produce pro-inflammatory cytokines (TNF-α and IL-6) and chemokines (CXCL-1 and CXCL-2). Moreover, activation of TLR4 on Kupffer cells and hepatocytes accelerates the production of its downstream CXCL-10. TNF-α affects surrounding hepatocytes causing their apoptosis, and triggers neutrophil-attracting CXC chemokines. The activated neutrophil-derived NE induces inflammatory chemokine (CXCL-1, CXCL-2) expression by neutrophils and accelerates IR-mediated damage due to the feedback with recruited neutrophils, resulting in the direct injury to membrane components. NE may also serve as a putative endogenous TLR4 ligand, causing TLR4 up-regulation on Kupffer cells/ hepatocytes. Sivelestat may also inhibit the inflammatory mediators through NF-kB inhibition on Kupffer cells and hepatocytes.