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LPS-CHALLENGED MICE KIDNEY AND MODULATORY EFFECT OF SIMVASTATIN

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ABSTRACT

Background: Lipopolysaccharide (LPS) is recognized by the innate immune system. This study was designed to investigate the effects of simvastatin on LPS-induced renal oxidative and immunological changes of mice.
Methods: Male Swiss mice were injected with LPS (1 mg/kg; i.p.) and the effects of pretreatment with simvastatin (10 mg/kg; i.p.) on LPS-induced renal failure and kidney pathology were examined 3 hours post LPS injection. Plasma concentrations of urea, creatinine and lactate dehydrogenase (LDH) activity as well as kidney contents of interleukin-1β (IL-1β) and IL-10 were assessed. Oxidative stress as well as the RNA expression of *neutrophil gelatinase-associated lipocalin* (*NGAL*) and *inhibitor of nuclear factor-kappa B* (*NF-κB*) *alpha* (*IκBα*) in the kidney were also evaluated.
Results: LPS markedly increased plasma urea and creatinine levels as well as LDH activity. Furthermore, LPS augmented renal malondialdehyde and IL-10 levels as well as caspase-3 activity. However, it diminished the reduced glutathione and IL-1β levels; besides, it inhibited superoxide dismutase and catalase activities in the kidney. Histopathologic studies backed the previous observations. Simvastatin pretreatment significantly ameliorated LPS-induced alterations and suppressed acute kidney injury (AKI) by modulating *NGAL* and *IκB-α* mRNA levels.
Conclusion: The present study suggests that simvastatin has potential beneficial role in sepsis prevention and its associated renal derangements.

INTRODUCTION

Septic shock is one of the most common causes of acute kidney injury (AKI) (Parrillo, 1993). It was observed that acute renal failure resulted in 45% mortality compared with 70% mortality when it is combined with sepsis (Schrier and Wang, 2004). Therefore, it is crucial to reveal the precise mechanisms involved in development of AKI. Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria that is involved in the pathogenesis of sepsis-induced AKI (Doi *et al*., 2009). LPS is commonly employed for investigating mechanisms of sepsis-related conditions (Hollenberg *et al*., 2000), where alterations of immunity as well as proinflammatory conditions were suggested as possible culprits (Knotek *et al*., 2001). Toll like receptor 4 (TLR4) is considered as the critical component of the LPS receptor complex (Ahmad-Nejad *et al*., 2002).

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Furthermore, TLR4 was suggested to recognize endogenous molecules that are exposed during cellular injury and extracellular matrix remodeling (Ohashi *et al*., 2000). Consequently, TLR4 activation may also be involved in signaling during tissue injury. *In vivo* LPS can cause endotoxic shock by inducing massive release of proinflammatory cytokines and chemokines from immune and non-immune cells that may be entirely mediated by TLR4. Activation of TLR4 leads to the nuclear translocation and activation of *nuclear factor-kappa B* (*NF-κB*) (Vogel *et al*., 1999) that causes enhanced expression of inflammatory cytokines and chemokines important in the recruitment of neutrophils and macrophages (Becker *et al*., 2000). Studies have shown that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors have various nonlipid effects, including antiproliferative effects (Corsini *et al*., 1996), induction of apoptosis (Tan *et al*., 1999), suppression of lymphocyte functions (Cutts and Bankhurst, 1989), and anti-inflammatory effects (Pruefer *et al*., 1999). HMG CoA reductase inhibitors

could inhibit LPS-induced production of cytokines and NO in astrocytes, microglia, and macrophages in vitro (Pahan *et al*., 1997). The effect of in LPS-induced AKI is unknown. The aim of the current study was to examine whether pretreatment with simvastatin had a protective effect on LPS-induced AKI in mice and explore the possible underlying mechanisms.

MATERIALS AND METHODS

Drugs and chemicals

LPS from Escherichia coli 0111:B4, simvastatin, thiobarbituric acid, vanadium III chloride, Ellman's reagent, reduced glutathione (GSH) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of the highest analytical grades commercially available.

Animals

Male Swiss mice, weighing 20–30 g, were used in the study. Animals were maintained under controlled conditions (25 ± 1°C, 55% relative humidity, 12 h lighting cycle), and fed standard chow and water ad libitum throughout the experimental period. All procedures were approved by the Ethics Committee for Animal Experimentation and were carried out in accordance with the Guide for Care and Use of Laboratory Animals (US National Institutes of Health, Publication No. 85-23, revised 1996).

Experimental design

Mice were randomly allocated into five groups (n=8). Group 1 "normal": received the vehicle (10 % DMSO/saline, *ip*). Group 2 and 3 were sacrificed 3h post LPS administration; Group 2 "LPS-3h": received LPS (Huang *et al*., 2007), Group 3 "Simvastatin+LPS-3h": received simvastatin plus LPS; simvastatin was administered as three doses (10 mg/kg; *ip*) (Giusti-Paiva *et al*., 2004) at 48, 24 and 3h prior to LPS administration.

Analysis of blood samples

Blood samples were collected via retro-orbital sinus into heparinized tubes under mild ether anesthesia. Plasma was separated by centrifugation at 3000 rpm for 15 min at 4°C, divided into several aliquots and stored at -20ºC till determination of urea, creatinine and LDH using commercial kits (Stanbio, San Antonio, TX, USA).

Analysis of tissue samples

After blood collection, mice were immediately decapitated under mild ether anesthesia and kidneys were removed, rinsed in ice-cold saline, blot-dried and weighed. The left kidney was divided into two sections: one used for quantitative PCR analysis and the other used for histological investigation. The right kidney was homogenized in ice-cold saline to make 10% homogenate that was centrifuged at 12,000 rpm at 4°C for 30 min and used for measuring the rest of the biochemical parameters.

Estimation of oxidative stress markers

Lipid peroxidation was quantified as malondialdehyde (MDA) according to Uchiyama and Mihara (1978) and was expressed as nmol/g wet tissue. GSH content was quantified according to the method of Beutler *et al*. (1963) and expressed as µg/g wet tissue. Nitric oxide (NO) content was quantified indirectly as total nitrate/nitrite (NO_x) according to the method of Miranda *et al*. (2001) and expressed as µmol/g wet tissue. Superoxide dismutase (SOD) and catalase (CAT) activities were determined according to the method of Marklund and Marklund (1974) and Góth (1991), respectively and expressed as U/mg protein. Homogenate supernatant protein content was determined according to Lowry *et al*. (1951).

Estimation of caspase-3 activity

Caspase-3 activity (apoptosis marker) was determined using ApoAlert caspase-3 colorimetric assay kit (USA) and expressed as U/mg protein.

Estimation of inflammatory biomarkers

Kidney contents of IL-1β and IL-10 were assayed using mouse IL-1β and IL-10 ELISA kits (R&D Systems, Minneapolis, USA) and expressed as ng/g wet tissue.

Real-time quantitative PCR

Total RNA was isolated using RNeasy mini kit (Qiagen, CA, USA) and the purity of obtained RNA was evaluated by the 260/280 ratio. Equal amounts of RNA were used to prepare cDNA using QuantiTect Reverse Transcription kit (Qiagen, CA, USA). cDNA was used for quantifying the expression of *neutrophil gelatinase-associated lipocalin* (*NGAL*), *inhibitor of NF-κB alpha* (*IκBα*) and *high-mobility group protein B1* (*HMGB1*) genes by real-time PCR using Rotor-Gene SYBR Green kit (Qiagen, CA, USA) with Rotor-Gene Q system (Qiagen, CA, USA). β-actin was used as the housekeeping reference gene. Primers sequences are shown in Table 1. The relative expression of target genes was obtained using comparative CT (ΔΔCT) method and presented as fold change using the $2^{-\Delta\Delta CT}$ formula (Livak and Schmittgen, 2001).

Histopathological assessment

Kidney samples were kept in 10% formol saline for 24 h, dehydrated in ethanol and embedded in paraffin. Sections were cut at 4 µm thicknesses and H&E-stained. All processing and assessment of specimens were performed by an experienced pathologist blinded to the study groups. A semiquantitative scoring of tubular injury adopted from Nomura *et al*. (1995) was conducted for each of three variables: tubular dilatation/flattening, tubular casts, and tubular degeneration/vacuolization. For each animal, 4 high-power fields (HPF) were examined at random. A score of 0, < 5%; 1, 5–33%; 2, 34–66%, and 3, > 66% of the tubules were affected. The average score of all three variables were summed to generate a total injury score for each animal.

Statistical analysis

Data were presented as mean percentage. One way analysis of variance (ANOVA) was used for comparison different groups followed by Tukey–Kramer multiple comparisons test using GraphPad Prism 5. Differences were considered statistically significant at $p<0.05$ for all tests.

RESULTS

Renal function tests

LPS caused significant nephrotoxicity as indicated by significant increase in creatinine level to about 2 fold as compared to normal group. Pretreatment with simvastatin significantly decreased the elevated creatinine level by 38% as compared to LPS-control group. LPS caused significant nephrotoxicity as indicated by significant increase in urea level to about 1.6 fold as compared to normal group. Simvastatin pretreatment normalized the urea level (Figure 1a & b).

Tissue damage biomarkers

LPS showed a significant increase in plasma LDH activity to about 2 fold as compared to normal group. Simvastatin pretreatment normalized its activity (Figure 2a). The LPS-induced increase in LDH activity was reflected in the significantly increased kidney total cumulative histopathological score in the LPS-3h group by 4 fold as compared to normal group. Pretreatment with simvastatin normalized its level (Figure 2b). Consistently, LPS increased kidney *NGAL* gene expression in the LPS-3h group by 162 fold as compared to normal group. Pretreatment with simvastatin decreased kidney *NGAL* gene expression in the LPS-3h group by 26% as compared to LPS-control group (Figure 2c).

Oxidative stress biomarkers

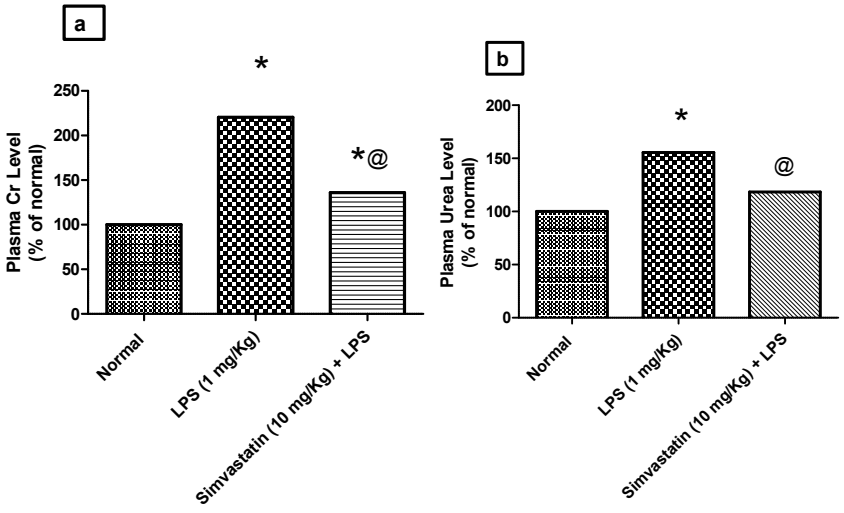
LPS significantly augmented kidney TBARS content of the LPS-3h group by 1.4 fold as compared to normal group. Meanwhile LPS significantly diminished kidney GSH by 33% as compared to normal group. Simvastatin pretreatment normalized TBARS and GSH contents (Figure 3a&b). In addition, NO_x content in the LPS-3h group increased by 1.7 fold as compared to normal group. Simvastatin pretreatment did not significantly change NO_x content (Figure 3c). LPS significantly diminished kidney SOD activity by 31% and CAT activity by 37%, respectively as compared to normal group. Simvastatin pretreatment normalized SOD and CAT activities (Figure 3d&e).

Cytokines and Inflammatory biomarkers

LPS significantly increased kidney IL-10 content in the LPS-3h group by 1.75 fold as compared to normal group. Pretreatment with simvastatin significantly increased kidney IL-10 content by 55% as compared to LPS-control group (Figure 4a). On the other hand, LPS significantly decreased kidney IL-1β content in LPS-3h group by 13% as compared to normal group. Pretreatment with simvastatin significantly decreased kidney IL-1β content by 31% as compared to LPS-control group (Figure 4b). LPS significantly increased kidney *IκB-α* gene expression in the LPS-3h group by 10 fold as compared to normal group.

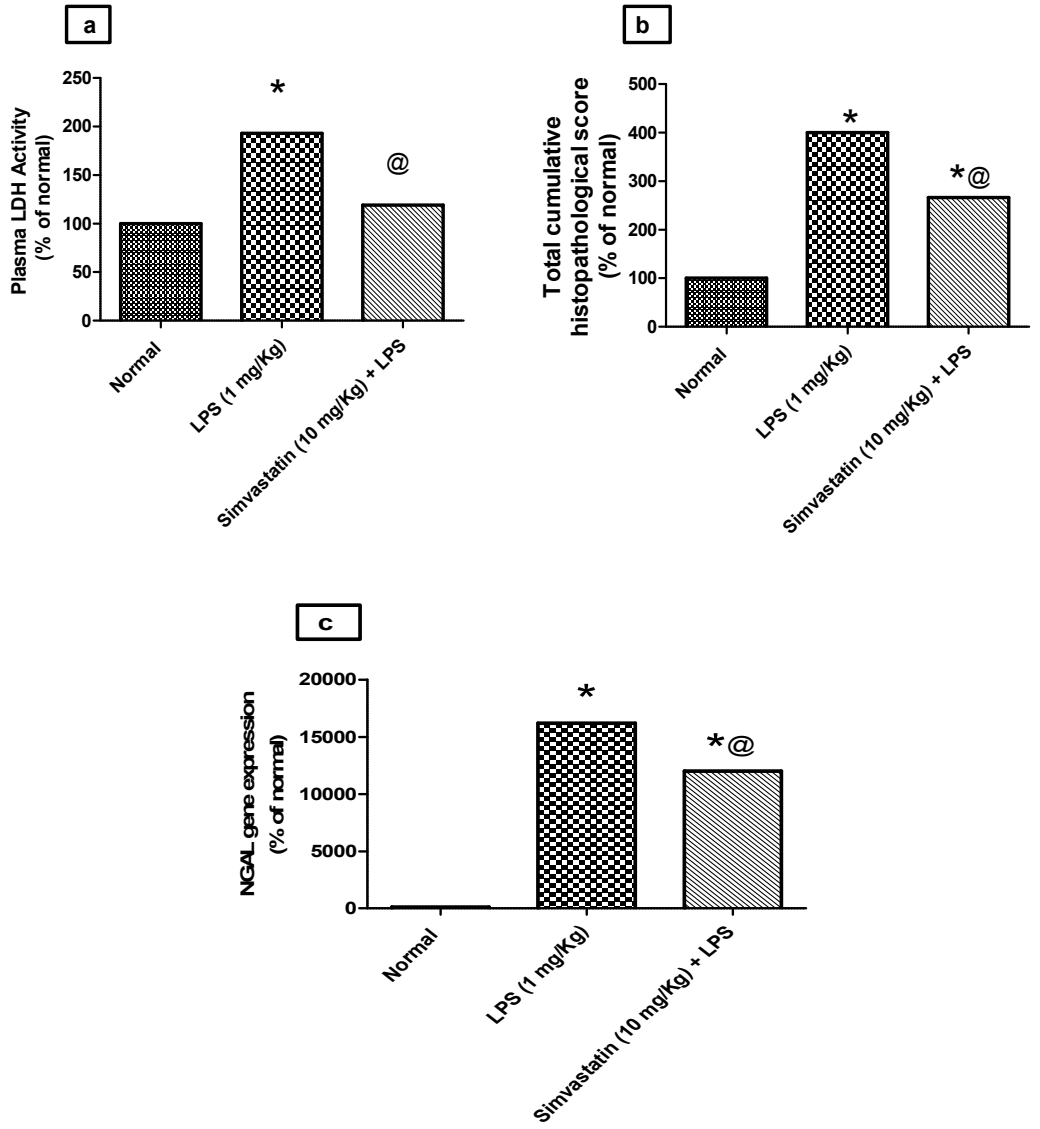
Table 1. Primers Sequences

Genes	Primers Sequences
β-actin	For 5'-CTAAGGCCAACCGTGAAAAG-3'
	Rev 5'-ACCAGAGGCATACAGGGACA-3'
NGAL	For 5'-CCATCTATGAGCTACAAGAGAACAAT-3'
	Rev 5'-TCTGATCCAGTAGCGACAGC-3'
IκBα	For 5'-ATGAAGGACGAGGAGTACGAGCAA-3'
	Rev 5'-TCTCTTCGTGGATGATTGCCAA-3'
HMGB1	For 5'-TGGGCGACTCTGTGCCTC-3'
	Rev 5'-GCCTCTCGGCTTTTTAGGATC-3'



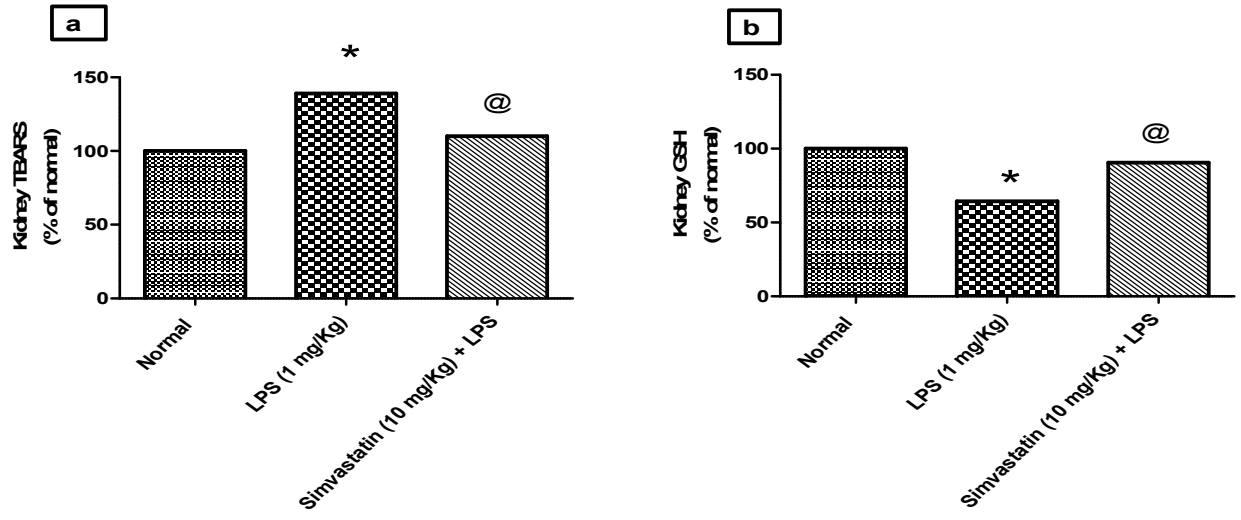
Data are expressed as mean percentage (n = 6-8).
* Significantly different from normal group at $p < 0.05$.
@ Significantly different from LPS group at $p < 0.05$.

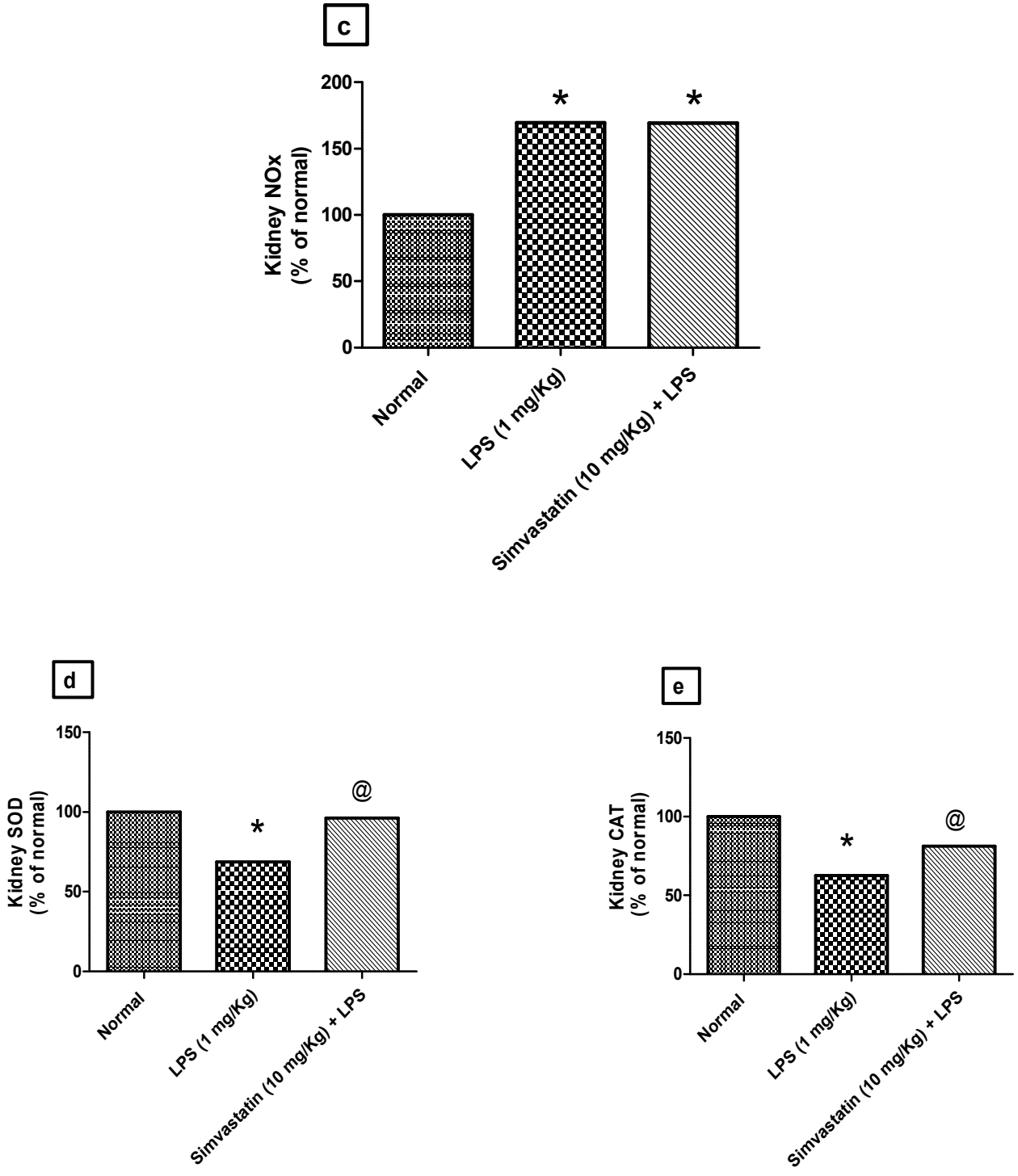
Figure 1. Effect of simvastatin pretreatment on plasma levels of (a) creatinine and (b) urea of LPS-treated mice



Data are expressed as mean percentage (n = 6-8).
* Significantly different from normal group at $p < 0.05$.
@ Significantly different from LPS group at $p < 0.05$.

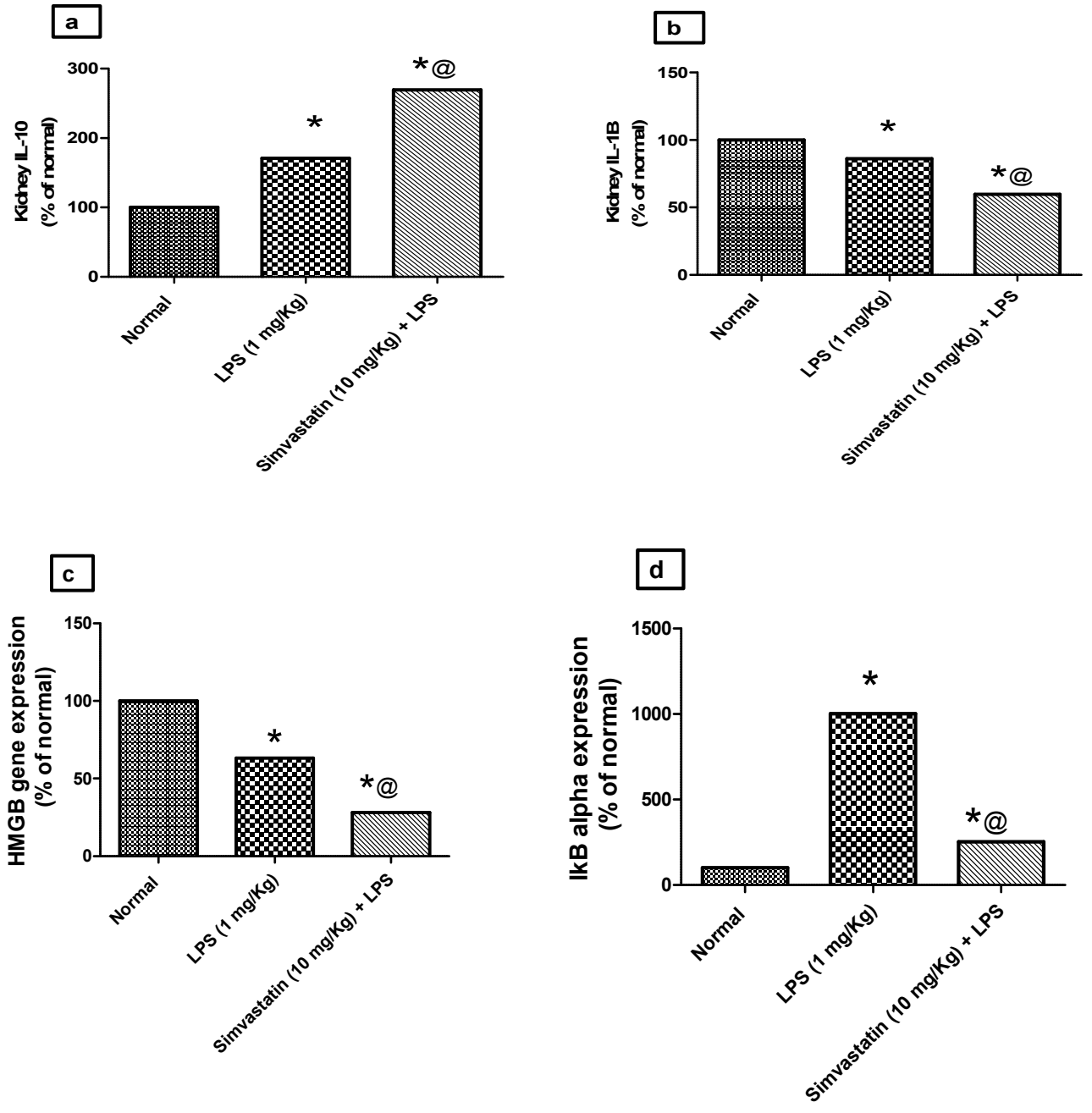
Figure 2. Effect of simvastatin pretreatment on (a) plasma LDH activity, (b) total cumulative histopathological score and (c) gene expression of NGAL of LPS-treated mice





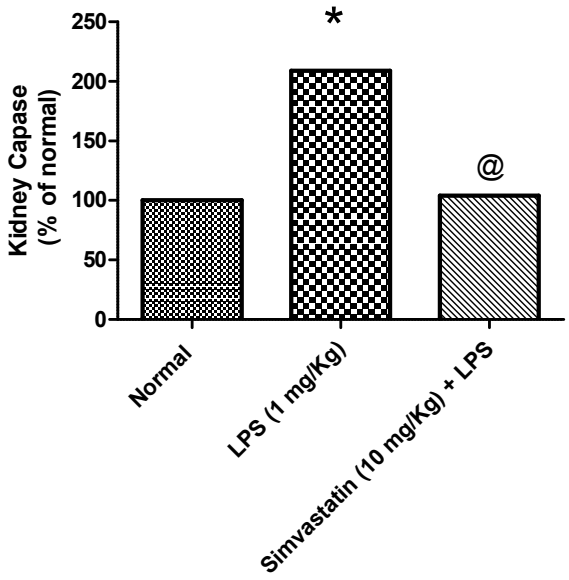
Data are expressed as mean percentage (n = 6-8).
* Significantly different from normal group at $p < 0.05$.
@ Significantly different from LPS group at $p < 0.05$.

Figure 3. Effect of simvastatin pretreatment on kidney contents of (a) MDA, (b) GSH (c) NO_x and activities of (d) SOD and (e) CAT of LPS-treated mice



Data are expressed as mean percentage (n = 6-8).
* Significantly different from normal group at $p < 0.05$.
@ Significantly different from LPS group at $p < 0.05$.

Figure 4. Effect of simvastatin pretreatment on kidney contents of (a) IL-10, (b) IL-1β as well as gene expression of (c) HMGB1 and (d) IκB-α of LPS-treated mice



Data are expressed as mean percentage (n = 6-8).
* Significantly different from normal group at $p < 0.05$.
@ Significantly different from LPS group at $p < 0.05$.

Figure 5. Effect of simvastatin pretreatment on kidney caspase-3 activity of LPS-treated mice

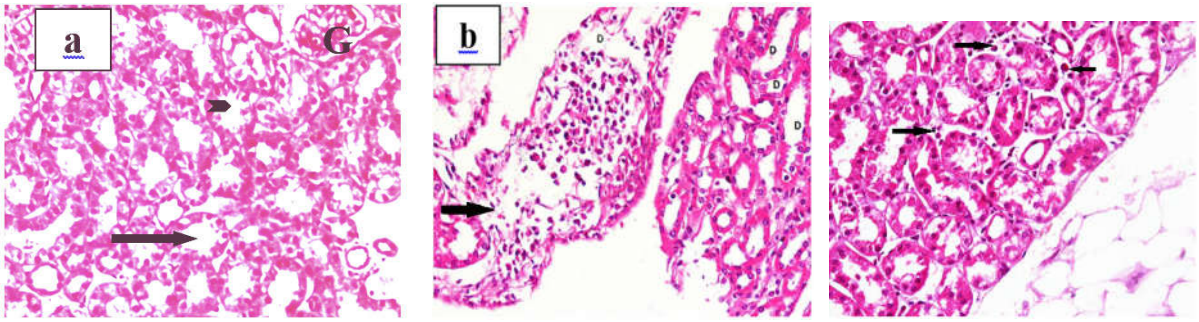


Figure 6. Photomicrographs of mice kidneys sections stained with H&E (400x): (a) control group showing the normal architecture of renal tissue composed of a number of glomeruli (G) embedded among a great number of different tubules most prominently the proximal convoluted tubules (arrow head) and the distal convoluted tubules (arrow). (b) LPS-3h group showing tubular dilatation (D) and degeneration of lining epithelium (arrow) (c) simvastatin-pretreated mice for LPS-3h group showing minimal tubular vacuolization (black arrow)

Pretreatment with simvastatin decreased kidney *IκB-α* gene expression by 75% as compared to LPS-control group (Figure 4c). LPS significantly decreased kidney *HMGB1* gene expression in the LPS-3h group by 37% as compared to the normal group. Pretreatment with simvastatin decreased kidney *HMGB1* gene expression by 56% as compared to LPS-control group (Figure 4d).

Apoptosis biomarkers

LPS significantly elevated caspase-3 activity in the LPS-3h group by 2 fold as compared to normal group. Simvastatin pretreatment normalized the caspase-3 activity (Figure 5).

Histopathological examination of kidney

Kidneys from normal mice showed healthy architecture composed of glomeruli embedded among numerous tubules: the proximal convoluted tubules lined with pyramidal cells and the distal convoluted tubules lined with cuboidal cells (Figure 6a). Kidneys from LPS-3h group showed degeneration of tubular lining epithelium (score 1) and tubular dilatation (score 2) (Figure 6b). Simvastatin pretreatment prevented LPS-induced degeneration in the tubular epithelium in LPS-3h group as well as tubular cells vacuolization (score 0), dilatation or casts (score 0) (Figure 6c).

DISCUSSION

In the present study, mice treated with LPS exhibited AKI as illustrated by increased plasma creatinine and urea levels parallel to elevated kidney LDH activity, *NGAL* expression and total cumulative histopathological score (Cunningham *et al*., 2002). The observed LPS-induced functional impairment and tissue damage may be attributed to augmented oxidative stress and apoptosis (Okoko and Ndoni, 2009).

NGAL gene is a well-established early marker of AKI (Supavekin *et al*., 2003) whose expression was previously shown to be enhanced in cortical tubular epithelia following LPS administration (Han *et al*., 2012). In the current investigation, LPS triggered oxidative stress as reflected in increased kidney MDA and NO levels coupled with decreased antioxidants: GSH content, SOD and CAT activities. It has been known that oxidative stress plays an important role in the development of LPS-induced AKI (Cunningham *et al*., 2004). Recognition of LPS by TLR4 initiates signaling pathways that induce production of proinflammatory (e.g. IL-1β) and anti-inflammatory (e.g. IL-10) cytokines. The balance of pro-inflammatory and anti-inflammatory cytokines plays a pivotal role in keeping the host homeostasis (Schetter and Harris, 2011). Stress-exposed organs activate their defense systems to cope with stress which is known as preconditioning (Heemann *et al*., 2000). LPS triggers the release of anti-inflammatory cytokines to balance and control the inflammatory response (Jaffer *et al*., 2010), which may explain the observed increase in IL-10 and suppressed IL-1β production. *In vivo*, IL-10 has been reported to attenuate macrophage-induced glomerular injury (Hashimoto *et al*., 2001) and inhibit production of a variety of proinflammatory cytokines by monocytes and neutrophils. *NF-κB* is a DNA binding protein that takes part in the regulation of multiple inflammatory responses by adjusting gene expression (Homaidan *et al*., 2003). Its activity is regulated through interaction with IκB that sequesters it in an inactive form in the cytoplasm. Multiple stimuli can activate *NF-κB* signaling by degradation of IκB and release of the *NF-κB*, which translocates to the nucleus and regulates transcriptional activation of the target genes (Wong and Tergaonkar, 2009). Therefore, the observed increase in *IκB-α* RNA expression level suggested enhanced inhibition of *NF-κB* activation with subsequent suppression of inflammatory mediators production. Such inhibition of *NF-κB* may partly explain the observed preconditioning. *HMGB1*, a non-histone nuclear protein, belongs to the group of molecules known as alarmins (Andersson and Tracey, 2011). *HMGB1*can be passively released from necrotic cells or actively secreted by activated immune cells in response to inflammatory signals (Scaffidi *et al*., 2002; DeMarco *et al*., 2005). Excessive *HMGB1* release was found to play a crucial role in the pathogenesis of acute and chronic inflammation. Released *HMGB1* can bind to several cell surface receptors, including TLR4 (Park *et al*., 2004), which results in activation of *NF-κB* and increased cytokines production (Yang *et al*., 2010). Therefore, the observed decrease in kidney *HMGB1* RNA expression level in both the LPS-3h group may also contribute to diminished *NF-κB* activation that underscores the observed preconditioning.

Enhanced apoptosis is associated with the activation of caspase cascade. Caspase-3 is a downstream effector in this cascade that directly mediates apoptosis (Linkermann *et al*., 2012) and is regarded as a pivotal indicator of apoptosis during AKI (Yang *et al*., 2013). LPS can induce caspase activation in the kidney via several possible mechanisms such as the generation of ROS (Stehlik *et al*., 1998) or stimulation of iNOS expression (Speyer *et al*., 2003). NO can activate caspases through its effects on mitochondria (Brown and Borutaite, 2002) or through generation of reactive nitrogen species (Brune, 2002). In the present study, pretreatment with simvastatin (HMG-CoA reductase inhibitor) improved renal function, as measured by plasma urea and creatinine as well as LDH activity. Simvastatin also decreased the observed tubular vacuolar degeneration as indicated by kidney total cumulative histopathological score. Statins have been shown to have beneficial effects in many kidney diseases including ischemia-reperfusion injury, transplantation and chronic kidney disease (Mason, 2005). It was found that HMG-CoA reductase was expressed in glomerular and peritubular vascular networks as well as tubular epithelial cells of native kidneys. Therefore, simvastatin treatment prevented microvascular permeability as well as creatinine and NGAL levels. Interestingly, the mevalonate pathway or cholesterol synthesis pathways are activated in cortical tubules after sepsis induced by LPS. Simvastasin improved acute kidney injury through effects on systemic circulation, direct effects on the renal vasculature and subsequent reversal of tubular hypoxia as well as a systemic anti-inflammatory action. Moreover, simvastatin has direct HMG-CoA reductase-independent effects on leukocytes (Weitz-Schmidt *et al*. 2001) as well as activation of innate and adaptive immune responses (Tuuminen *et al*., 2013). In the current study, the LPS-induced increase in TBARS and NOx contents was abolished by simvastatin pretreatment and enhanced SOD and CAT activities in mouse kidney. Statins possess antioxidant properties by reducing lipid peroxidation (Wilson *et al*., 2001) and ROS production (Wassmann *et al*., 2001). Statins promote systemic antioxidant effects through the suppression of distinct oxidation pathways included myeloperoxidase-derived and nitric oxide-derived oxidants (Cordle and Landreth, 2005).

In the current investigation, the LPS-induced increase in IL-10 and decrease in IL-1β contents was augmented by simvastatin pretreatment. Numerous studies suggest inhibitory effects of statins on proinflammatory cytokine production, such as IL-1β, in several cells including microglia and mononuclear cells. Several in vitro and in vivo models suggest a statin induction of IL-10 (Zeiser *et al*., 2007). Upregulation of IκBα gene expression was observed in the present study with simvastatin pretreatment. The suppression of the immune response by statins is mainly attributed to impaired cell activation and adhesion as well as *via* the down-regulation of NF-κB encoding the transcription of many immune genes. Statins are powerful inhibitors of the inflammatory process (Greenwood and Mason, 2007). Some of these anti-inflammatory properties of statins are related to the inhibition of HMG-CoA reductase (Kwak *et al*., 2000), whereas others are independent of blocking HMG-CoA reductase activity (Weitz-Schmidt *et al*., 2001). In a similar study, atorvastatin was shown to markedly suppress HMGB1-induced TLR4 expression, NF-κB nuclear translocation and DNA binding activity in endothelial cells. These findings indicate that atorvastatin attenuates HMGB1-induced vascular endothelial activation. The underlying mechanism involves, at least in part, inhibition of TLR4/NF-κB-dependent signaling pathway (Yang *et al*. 2010). In the current study, the LPS-induced increase in caspase-3 activity was abolished by simvastatin pretreatment. Slijper *et al*. (2010) found that treatment with simvastatin resulted in a significant decrease in cell apoptosis rate following intestinal ischemic-reperfusion. Dibazar *et al*. (2008) published similar conclusions regarding this positive effect of simvastatin to inhibition of inflammation and apoptotic pathway. In

summary, simvastatin pretreatment can significantly ameliorate LPS-induced AKI through reduction of oxidative stress, proinflammatory cytokines and apoptosis via inhibiting HMG-CoA reductase pathway. Thus, interventions involving HMG-CoA reductase can protect against LPS-induced kidney injury, which indicates that simvastatin may represent an alternative treatment for preventing kidney injury in septic diseases.

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