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journal or publication title	Life sciences
volume	118
number	2
page range	347-356
year	2014-11
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URL	http://hdl.handle.net/2241/00123593

doi: 10.1016/j.lfs.2014.05.007

Life Sciences 118 (2014) 347–356

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Potential amelioration of upregulated renal HIF-1alpha–endothelin-1 system by landiolol hydrochloride in a rat model of endotoxemia

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ARTICLE INFO

Article history:
Received 4 November 2013
Accepted 9 May 2014
Available online 17 May 2014

Keywords:
Kidney
Landiolol hydrochloride
Endothelin
Endotoxemia
Rat model

ABSTRACT

Aims: Endothelin (ET)-1 is the best known potent vasoconstrictor and has been implicated in pathogenesis of sepsis-associated acute kidney injury (AKI) in human or lipopolysaccharide (LPS)-induced AKI in animal models. We have previously shown that ET-1 is highly up-regulated in renal tissues and in plasma after LPS administration. Here, we investigated whether landiolol hydrochloride, an ultra-short-acting beta-blocker, can play an important role in ameliorating levels of LPS-induced up-regulation of renal HIF-1α–ET-1 system and inflammatory cytokines in a rat model of endotoxemia.

Main methods: Male Wistar rats at 8 weeks of age were either administered with: a) lipopolysaccharide (LPS) only for three hours (3 h) or b) LPS, followed by continuous administration of landiolol for 3 h; c) third group was only treated with vehicle.

Key findings: At 3 h after LPS administration there was: a) minimal injury in kidney tissues; b) circulatory levels of creatinine, blood urea nitrogen and NGAL increased and c) expression of inflammatory cytokines, such as TNF-α, IL-6 and iNOS increased at the level of both circulatory and renal tissues. In addition, LPS significantly induced renal expression of ET-1 and HIF-1α compared to control. Finally, treatment of LPS-administered rats with landiolol for 3 h normalized elevated serum markers of renal injury and up-regulated levels of renal HIF-1α–ET-1 system with normalization of TNF-α.

Significance: Taken together, these data led us to conclude that landiolol ameliorates the up-regulation of HIF-1α–ET-1 system in minimally morphologically-injured kidney and normalizes biomarkers of renal injury in early hours of endotoxemia of a rat model.

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Introduction

Sepsis is a clinical syndrome caused by severe infection and is characterized by a systemic inflammatory reaction with organ dysfunction (Hotchkiss and Karl, 2003; Vandijck et al., 2006). Organ failure is known to occur in 33.6% of septic shock patients (Martin et al., 2003). The systemic inflammatory response may be initiated by entry of bacterial lipopolysaccharide (LPS) or other microbial components into the lymphatic and circulatory systems. Once the sepsis cascade is triggered, a systemic inflammatory response will ensue and, if unregulated, will lead to multiple organ failure. Acute renal failure as well as acute kidney injury (AKI) are common and critical complications of sepsis and septic shock (Palevsky et al., 2008). The combination of acute renal failure and sepsis contributes to the high mortality rate as much as 70% compared to 45% mortality among patients with acute renal failure alone (Schrier and Wang, 2004).

The mechanisms underlying renal dysfunction are still not fully understood, but could involve hypercytokinemia, endothelial and epithelial cell injury and circulating factors (Lopes et al., 2007; Palm et al., 2004). Levels of cytokines, including interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), are significantly higher in sepsis-related acute renal failure patients compared to non-sepsis cases (Murugan et al., 2010). If uncorrected, pathogenesis takes a worse turn following endothelial activation, as it leads to increased capillary permeability, systemic vasodilation, and, ultimately, multi-organ failure (MOF). In addition, a previous study suggests that changes in the vasoconstrictor tone of the afferent and efferent arterioles may be crucial in inducing the loss

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http://dx.doi.org/10.1016/j.lfs.2014.05.007
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of glomerular filtration rate (GFR), contributing to the pathogenesis of sepsis-related acute renal failure (Bellomo et al., 2011; Langenberg et al., 2006).

Endothelin (ET)-1, a potent vasoconstrictor (Yanagisawa et al., 1988), has been implicated in the pathogenesis of sepsis and is induced by hypoxia inducible factor 1-alpha (HIF-1α), which is a transcription factor that responds to hypoxia. Plasma levels of ET-1 have been shown to be significantly higher in septic patients (Battistini et al., 1996) and their levels (ET-1) have a clear correlation with morbidity and mortality in septic patients, suggesting an involvement of ET in the pathogenesis of septic shock in humans (Pittet et al., 1991). Further, it has been suggested that ET contributes to the dysfunction of several vital organ systems in septic shock. In the kidney, ET-1 may affect function of renal microcirculation by inducing constriction in afferent and efferent arterioles mediated by the ET receptor, ET-A (Schildroth et al., 2011). In addition, in rats with diabetic renal injury, selective antagonism of ET-A reduces macrophage infiltration, suggesting that ET-A activation also contributes to renal inflammation (Sasser et al., 2007). In our previous study we have shown that ET-1 is highly upregulated in renal tissues, as well as in plasma after LPS administration and there is a potential imbalance in the expression of vaso-regulatory molecules of renal tissue (Yamaguchi et al., 2006).

On the other hand, landiolol hydrochloride, an ultra-short-acting (a half-life of 4 min) and highly cardio-selective beta-1 blocker, recently has become useful in treating various medical problems, such as management of acute rapid heart rate and prevention of atrial fibrillation after cardiac surgery (Iguchi et al., 1992). The drug has already been approved as an emergency treatment of supraventricular tachyarrhythmia in patients in Japan. Landiolol has also been reported to have a lesser effect on blood pressure compared to esmolol (Mio, 2006). Recent studies have demonstrated that co-treatment with landiolol protects against acute lung injury and cardiac dysfunction in a rat model of LPS-induced systemic inflammation, which is associated with significant reduction in serum levels of the inflammation mediator, HMGB-1 and histological lung damage (Hagiwara et al., 2009). However, these earlier studies have not investigated whether landiolol has renal protective effects in endotoxemia.

In the current study, we investigated whether landiolol hydrochloride can play an important role in ameliorating elevated renal levels of LPS-induced HIF-1α–ET-1 system and inflammatory cytokines and renal injury markers in the early hours of endotoxemia of a rat model.

Materials and methods

Animal preparation

Male Wistar rats (200–250 g, 8 weeks old) were used in all experiments. Endotoxemia was induced by the intra-peritoneal (IP) administration of bacterial LPS from *Escherichia coli* 055: B5 (15 mg/kg), dissolved in sterile saline (Yamaguchi et al., 2006). The rats were ($n = 59$) randomized into group 1 (control, $n = 17$), group 2 (control + landiolol hydrochloride, $n = 8$), group 3 (LPS, $n = 17$) and group 4 (LPS + landiolol hydrochloride, $n = 17$).

Groups 1 and 2 received an equal volume of vehicle (sterile saline; 2 ml/body), without LPS. And, it should be noted that LPS (15 mg/kg, intraperitoneal) was administered at time 0 in groups 3 and 4, and then the rats were killed after 3 h. However, for groups 2 and 4, 15 min before LPS or vehicle administration, landiolol hydrochloride was administered continuously intravenously (100 μg/kg/min). From our pilot studies, we found this to be the minimal dose of landiolol, which can normalize the LPS-induced hyperdynamic state in the early hours of endotoxemia. In addition, we wanted to use the dose of landiolol at a level that exerts minimal effects on blood pressure in endotoxemic rats.

All rats were killed by Nembutal (sodium pentobarbital, IP, 80 mg/kg body weight) at 3 h after LPS or vehicle only. The blood samples were collected from a polyproprene tube catheter inserted into the left carotid artery for blood gas analysis, and renal tissues were harvested gently, snap-frozen in liquid nitrogen, and stored at −80 °C. All animals received care and the experimental procedures were approved by the Animal Care and Use Committee of University of Tsukuba.

Measurements of hemodynamic parameters

Rats were anesthetized with isoflurane inhalation (1.5%, 1 l/min) and a microtip pressure transducer catheter (SPC-320, Millar Instruments, Houston, TX, USA) was inserted into the left carotid artery, as described in our previous study (Yamaguchi et al., 2006) at the end of the 3 h experimental protocol. Next, arterial blood pressure and heart rate were monitored with a pressure transducer (model SCK-590, Gould, Ohio, USA) and recorded with the use of a polygraph system (amplifier, AP-601G, Nihon Kohden, Tokyo, Japan; Tachometer, AT-601G, Nihon Kohden; and thermal-pen recorder, WT-687G, Nihon Kohden). It is important to note here that all the experimental animals went through the same type of anesthetic conditions during hemodynamic measurements.

Echocardiography

Echocardiography was performed using a Vevo 2100 high-frequency ultrasound system (VisualSonics, Inc., Ontario, Canada), which includes an integrated rail system for consistent positioning of the ultrasound probe (Yang et al., 2013) at the end of the 3 h experimental protocol. The fur from the chest was electrically clipped and a gel was applied prior to shaving. The animals were placed on a heating pad and connected to an electrocardiogram (ECG), while rectal temperature was monitored to maintain a body temperature of 38 ± 0.1 °C. A 35 MHz linear transducer (VisualSonics, RMV 707, Inc., Ontario, Canada) was used for imaging. An optimal parasternal long axis (LAX) cine loop (i.e. visualization of both the mitral and aortic valves, and maximum distance between the aortic valve and the cardiac apex) of >1000 frames/s was acquired using the ECG-gated kilohertz visualization technique. The probe was then rotated 90° and positioned 6 mm below the mitral annulus, i.e. at the level of the papillary muscles. Three parasternal short-axis (SAX) M-mode sequences were stored. Fractional shortening (FS) was calculated in the M-mode image as $FS = (EDD - ESD) / EDD$, where EDD and ESD are the end-diastolic and end-systolic diameters, respectively.

Measurement of kidney injury markers

Serum creatinine levels were measured using Creatinine (Serum) Assay Kit from Wako Pure Chemical Industries (LTD, Osaka, Japan). Blood urea nitrogen (BUN) levels were measured using Urea Nitrogen (BUN) Colorimetric Detection Kit (Arbor Assays, Ann Arbor, Michigan). Plasma urea levels were measured using Urea Colorimetric Assay Kit (BioVision, Milpitas, California). Lastly, creatine phosphate kinase (CPK) activities were measured using Creatine Kinase Activity Colorimetric Assay Kit (BioVision Milpitas, California).

Histopathology examination

Histopathological studies were performed to determine the extent of renal micro-morphological injury in LPS-induced endotoxemia and recovery by administration of landiolol hydrochloride. Kidney tissues were fixed in 4% buffered formalin solution, dehydrated, embedded in paraffin, and then sliced into 5-μm-thick sections. After deparaffinization, slides were stained by hematoxylin and eosin (H&E).

Enzyme-linked immunosorbent assay for plasma and renal NGAL, TNF-α, IL-6, iNOS and ET-1

The concentration of each respective protein/peptide of interest in plasma/serum and renal tissue extracts was determined using the following kits: plasma and renal neutrophil gelatinase-associated lopocalin (NGAL) which is a highly predictive biomarker of acute kidney injury (Han et al., 2012) (Abcam, Cambridge, UK) and serum and renal levels of TNF-α, IL-6, inducible nitric oxide synthase (iNOS) and ET-1

(R & D Systems, Minneapolis, MN), according to the manufacturer's protocol.

Western blotting for renal HIF-1α, ECE-1, ET-A receptor, ET-B receptor

Ice-cold kidney tissues were minced with scissors, homogenized, centrifuged and then the concentration of the protein (supernatant) was determined using the bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL). Samples were boiled in reducing SDS sample buffer for 5 min, loaded onto an SDS-PAGE (4–15% polyacrylamide) gel, subjected to electrophoresis, and electrophoretically transferred to polyvinylidine difluoride filter membrane. To reduce non-specific binding, the membrane was blocked for 2 h at room temperature with 5% non-fat milk in PBS (137 mM NaCl, 2.7 mM KCL, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4) containing 0.1% Tween 20, incubated overnight at 4 °C with primary antibodies in PBS-Tween buffer, washed three times with PBS-Tween buffer, and then the membrane was incubated with a suitable secondary antibody coupled to horseradish peroxidase for 60 min at room temperature. The blots were then washed three times in PBS-Tween buffer and subsequently visualized with an enhanced chemiluminescence detection system (Amersham) and exposed to an X-ray film (Fuji Photo Film). The intensity of total protein bands per lane was evaluated by densitometry. Negligible loading/transfer variation was noted between samples. Moreover, beta-actin was used as a loading control. Commercially available and well-characterized antibodies were used in the present study, namely: anti-HIF-1α antibody (Abcam, Cambridge, UK), ET-A receptor antibody (Alomone Labs, Jerusalem, Israel), ET-B receptor antibody (Alomone Labs, Jerusalem, Israel) and endothelin-converting enzyme-1 (ECE-1, Santa Cruz Biotechnology, Inc. Europe).

RNA preparation and real-time quantitative polymerase chain reaction

Total RNA from kidney tissue was isolated using RNeasy (Qiagen, Tokyo, Japan). After isolation, DNase I treatment, and quantification, RNA was reverse transcribed to cDNA by Omniscript RT using a first-strand cDNA synthesis kit (Qiagen). The reaction was performed at 37 °C for 60 min.

The mRNA expression levels of target genes were analyzed by real-time quantitative PCR with TaqMan probe using an ABI Prism 7700 sequence detector (PerkinElmer Applied Biosystems, Foster, CA). The gene-specific primers and TaqMan probes were synthesized from Primer Express version 1.5 software (PerkinElmer) according to the published cDNA sequences for each gene, as previously described (Shimojo et al., 2006, 2007). The expression of GAPDH mRNA was used as an internal control. The PCR mixture (25 μl total volume) consisted of forward and reverse primers for each gene (PerkinElmer) at 450 nM each, FAM-labeled primer probes (PerkinElmer) at 200 nM, and TaqMan Universal PCR Master Mix (PerkinElmer). Each PCR amplification was performed in triplicate as follows: 1 cycle at 95 °C for 10 min and 40 cycles at 94 °C for 15 s and 60 °C for 1 min. The quantitative values of target mRNAs were normalized by GAPDH mRNA, because GAPDH mRNA expressions were more stable among all the samples than other internal controls such as β-actin and 18S ribosomal RNA. Primers and probes are as follows: TNF-α forward: 5′-CCAGGAGAAAGTCAGCCTCCT-3′, TNF-α reverse: 5′-TCATACCAGGGCTTGAGCTCA-3′, and TNF-α probe: 5′-AGAGCCCTTGCCCTAAGGACACCCCT-3′; IL-6 forward primer: 5′-ACAGCCACTGCCTTCCCTAC-3′, IL-6 reverse: 5′-TCTCATTTCCAAGATCTCCC-3′, and IL-6 probe: 5′-CACAGAGGATACCACCCACA-3′; iNOS forward: 5′-GTGGGTGGCCTCGAGTTC-3′, iNOS reverse: 5′-CCAATCTCGGTGCCCATGTAC-3′, and iNOS probe: 5′-CTGCCCCTTCAATGGTT-3′; HIF-1α forward: 5′-CTATGGAGGCCAGAAGAGGGTAT-3′, HIF-1α reverse: 5′-CCCACATCAGGTGGCTCATAA-3′, and HIF-1α probe: 5′-AGATCCCTTGAAGCTAG-3′, ET-1 forward: 5′-TCTACTTCTGCCACCTGGACAT-3′; ET-1 reverse: 5′-GAAGGGCTTCCTAGTCCATACG-3′, and ET-1 probe: 5′-CATCTGGGTCAACACTCC-3′; ET-A forward: 5′-GAATCTCTGCGCTCTCAGTGT-3′, ET-A reverse: 5′-GAGACAATTTCAATGGCGGTAATCA-3′, and ET-A probe: 5′-CAGGAAGCCACTGCTCT-3′; ET-B forward: 5′-GCTGGTGCCCTTCATACAGA-3′, ET-B reverse: 5′-CTTAGAGCACATAGACTCAACACTGT-3′, and ET-B probe: 5′-ATCCCCACAGAAGCCT-3′; ECE-1 forward: 5′-TCAGACAAGTCTCCACACTCATCA-3′, ECE-1 reverse: 5′-CCAGGTTCCACATCATGTAGTTGTT-3′, and ECE-1 probe: 5′-ACAGCACCGACAAATG-3′; GAPDH forward: 5′-GTGCCAAAAGGGTCATCATCTC-3′, reverse: 5′-GGTTCACACCCATCACAAACATG-3′, and probe: 5′-TTCCGCTGATGCCCC-3′.

Statistical analysis

The results were expressed as mean ± SE, and the means were compared by a one-way factorial analysis of variance, followed by Scheffé's test for multiple comparisons. Differences were considered significant at $p < 0.05$.

Results

Blood gas parameters, hemodynamics, and serum inflammatory markers (Table 1)

Blood gas analysis was performed in order to determine whether the rats used in the present study were endotoxemic. Arterial PaO_2 was found to be significantly reduced in LPS-administered rats and landiolol treatment had no significant effect on levels of arterial PaO_2. Blood lactate concentrations were increased dramatically after LPS was given and partly normalized with the treatment of landiolol. We also assessed the hemodynamic parameters in the rats after LPS administration. Both the systolic and diastolic blood pressure levels were significantly lower 3 h after LPS administration and were unaffected by landiolol treatment. Heart rate was significantly increased in LPS administered group compared to control group, and significantly decreased in LPS-administered rats treated with landiolol. % FS measured with echocardiograph was also significantly hyperkinetic in LPS-administered group compared to control group. However, hyperdynamic state induced by LPS administration was significantly normalized in LPS + landiolol group. Plasma urea and CPK levels did not differ among the experimental groups. Serum levels of inflammatory cytokines, namely, TNF-α, IL-6 and iNOS, were significantly increased after LPS administration, as determined by ELIZA. However, landiolol treatment did not change serum levels of these cytokines with statistical significance. Plasma ET-1 levels also significantly increased in LPS and LPS + landiolol groups compared with control and

Table 1
Blood gas analysis, hemodynamic parameters and biochemical parameters.

	Control	LPS	LPS + landiolol
n	8	7	8
pH	7.40 ± 0.07	7.43 ± 0.06	7.44 ± 0.06
PaO_2, torr	98.6 ± 2.7	87.3 ± 5.8*	100.3 ± 10.3
$PaCO_2$, torr	37.4 ± 2.0	36.4 ± 1.8*	30.4 ± 3.2*
BE, mmol/l	−0.9 ± 0.9	−4.0 ± 2.6	−5.4 ± 2.2*
Lac, mmol/l	0.8 ± 0.3	2.9 ± 0.6*	1.8 ± 0.2*,#
HCO_3^-, mmol/l	25.4 ± 0.6	23.4 ± 1.2*	22.1 ± 1.2*
Systolic BP, mm Hg	125 ± 4	108 ± 6*	102 ± 8*
Diastolic BP, mm Hg	84 ± 6	70 ± 4*	67 ± 4*
Heart rate, bpm	461 ± 14	510 ± 21*	441 ± 10#
FS, %	41.1 ± 1.1	45.6 ± 1.3*	41.6 ± 1.3#
Plasma Urea, nmol/μl	1.44 ± 0.02	1.70 ± 0.23	1.47 ± 0.12
Plasma CPK, mU/ml	19.9 ± 5.3	22.9 ± 5.1	21.9 ± 5.7
Serum TNF-α, pg/ml	12.6 ± 2.7	182.3 ± 5.8*	187.3 ± 6.3*
Serum IL-6, pg/ml	16.4 ± 3.0	30.4 ± 5.8*	28.4 ± 4.2*
Serum iNOS, U/ml	7.3 ± 1.3	17.8 ± 2.7*	18.1 ± 2.5*
Plasma ET-1, pg/ml	5.2 ± 0.9	33.0 ± 5.6*	30.4 ± 5.2*

LPS, lipopolysaccharide; BE, base excess; Lac, lactate; BP, blood pressure; FS, fractional shortening; CPK, creatine phosphate kinase; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; ET-1, endothelin 1; Data are mean ± S.E.

* $p < 0.05$ vs. control.

\# $p < 0.05$ vs. LPS groups.

landiolol treatment did not have any significant effect on the elevated plasma ET-1 levels in endotoxemic rat.

Histopathology after LPS and landiolol administration

Evaluation of renal histology using H&E stain did not reveal any evidence of intraglomerular thrombosis and/or disseminated intravascular coagulation in any of the treated animals (Fig. 1). Besides, these sections showed only minimal tubular degenerative changes, i.e., focal tubular dilatation with mild swelling or thinning, partly detachment of the epithelial cells at 3 h after LPS administration, as well as in the group treated with LPS followed by landiolol administration for 3 h (Fig. 1). There was no remarkable infiltration of inflammatory cells in kidney tissue after LPS administration (Fig. 1). Morphologically, no obvious differences were recognized between renal tissues from rats treated with LPS alone and those treated with LPS plus landiolol (Fig. 1).

Biochemical investigations of renal injury parameters

To determine whether LPS caused any elevation of circulatory renal injury parameters, we analyzed circulatory levels of BUN, creatinine and NGAL in endotoxemic rats. Serum BUN and serum creatinine concentrations were significantly increased after LPS administration compared to control rats. Further, landiolol treatment prevented their increase in endotoxemic rats (Fig. 2A and B). NGAL, recently known as an early marker of acute kidney injury (Koyner et al., 2012; Mishra et al., 2005), exhibited a similar pattern as levels of serum BUN and creatinine. NGAL levels in plasma and renal tissue were significantly increased after LPS administration compared to control rats and normalized by landiolol treatment (Fig. 2C and D).

mRNA and protein expression levels of inflammatory molecules (TNF-α, IL-6 and iNOS) in renal tissues

To assess the expression patterns of potential inflammatory cytokines in endotoxemic renal tissues, we determined the mRNA expression and protein levels of TNF-α, IL-6 and iNOS in LPS-administered renal tissues. Both the mRNA and protein expression levels of TNF-α, IL-6 and iNOS were elevated in kidney tissue after LPS administration, compared to control group (Fig. 3) and landiolol treatment only normalized the elevated levels of TNF-α (Fig. 3A and B), but not the other inflammatory molecules, notably IL-6 and iNOS (Fig. 3C, D, 3E and 3F).

mRNA and protein expression levels of HIF-1α in renal tissues

To ascertain whether LPS administration causes any hypoxia in renal tissues, we investigated both the mRNA and protein expression levels of HIF-1α in this tissue. HIF-1α mRNA expression and protein levels were also markedly increased after LPS administration in kidney tissues and landiolol treatment significantly normalized the elevated levels of these molecules (Fig. 3G and H) at both levels, i.e., the mRNA and protein.

Expression of ET system components in renal tissues at mRNA and protein levels

We also sought to ascertain whether ET system components are altered in the early hours of endotoxemic renal tissues, by investigating the expression pattern of various components of ET system at both mRNA and protein levels. Prepro ET-1 mRNA and ET-1 peptide levels were found to be elevated in renal tissue after LPS administration, compared to the control group. Further, landiolol treatment significantly ameliorated the elevated levels of these molecules of interest (Fig. 4A and B). ET-A receptor mRNA and protein expression levels in kidney tissue tended to increase after LPS administration but did not reach statistically significant levels (Fig. 4C and D). ET-B receptor and ECE-1 mRNA and protein expression levels were significantly increased in LPS group in renal tissues and landiolol treatment significantly normalized these expressions in endotoxemic rats (Fig. 4E, F, G and H).

Effects of landiolol on control rats

Finally, to determine whether landiolol treatment affects the hemodynamic and ET expression in control rats, we treated control rats with landiolol for three hours. When control rats were treated with landiolol, systolic blood pressure and heart rate decreased significantly (Fig. 5A and C). On the other hand, regarding diastolic blood pressure and % FS, no statistically significant difference was found between the different groups (Fig. 5B and D), with or without landiolol. Further, landiolol had no effect on plasma as well as renal levels of ET-1 in the control group (Fig. 5E and F).

Discussion

The key findings of the present study are that: 1) treatment of LPS-administered rats with landiolol for three hours potentially normalized the elevated levels of blood lactate and serum levels of renal injury parameters, namely creatinine and BUN, associated with NGAL, without

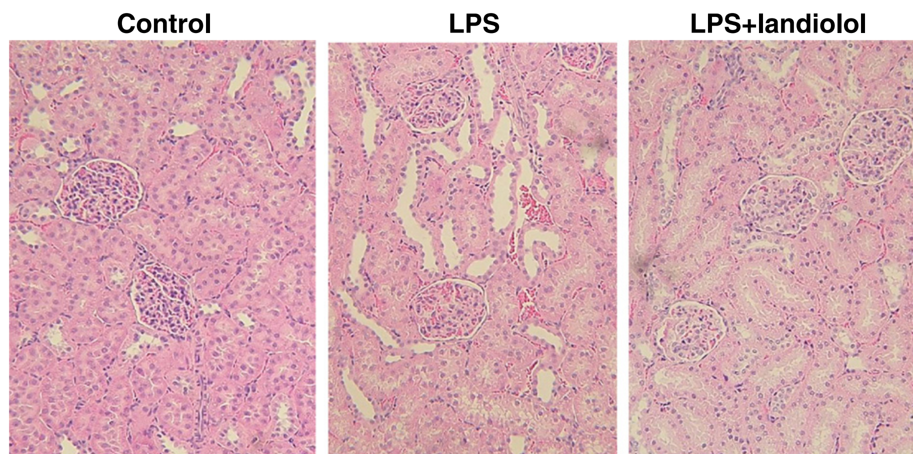

Fig. 1. Morphological findings by hematoxylin and eosin staining in the kidney tissues in control rats, 3 h after lipopolysaccharide (LPS) administration, and 3 h after LPS plus landiolol hydrochloride administration. Magnification, 400×.

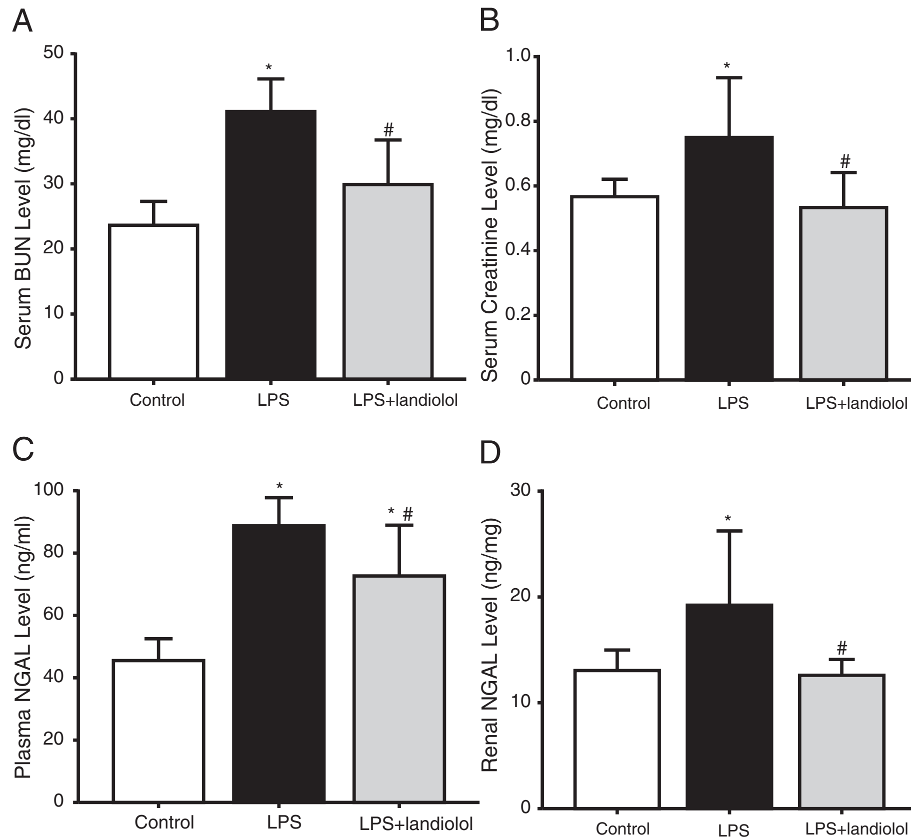

Fig. 2. Serum blood urea nitrogen (BUN) level (A), serum creatinine level (B), plasma neutrophil gelatinase associated lopocalin (NGAL) level (C) and renal NGAL level (D) of the control, 3 h LPS-administered rats, and landiolol treated 3 h LPS-administered rats. Data are shown as mean ± SE (n = 15). *p < 0.05 vs. control, #p < 0.05 vs. 3 h LPS-administered rats.

an effect on circulatory TNF-α, IL-6, iNOS, and ET-1 levels; 2) landiolol treatment only significantly normalized renal levels of TNF-α, without affecting renal levels of other inflammatory cytokines, notably IL-6 and iNOS; 3) treatment of LPS-administered rats with landiolol for three hours potentially normalized the elevated levels of the renal HIF-1α-ET-1-system and 4) there was minimal tissue injury in the kidney after LPS administration, which was unaffected by landiolol treatment.

Our preliminary time course study conducted at various time points (0 h, 1 h, 3 h, 6 h, 10 h, 16 h, 24 h, n = 10 for each group) showed that LPS induced high levels of plasma lactate, elevated heart rate (HR) and increased percent of fractional shortening (% FS), i.e., a hyperdynamic state during the early hours of endotoxemia. These elevated endpoints were greatly normalized by landiolol as early as 3 h compared to rats administered with only LPS. This reversal of the hyperdynamic state in endotoxemia by landiolol resulted in reduced cardiac output, the recovery of systemic peripheral circulation and the improvement of arterial deoxygenation during all the time points of sepsis, and consequently led to an improved survival rate. Based on these earlier observations, the 3 h time point was selected to investigate in depth, the effects of landiolol on various pathogenesis associated with organ dysfunction in endotoxemic rats. Wu et al (2007) have previously shown that renal peritubular capillary flow is dramatically compromised and also the mRNA expression levels of pro-inflammatory cytokines, such as ICAM-1 and VCAM-1, are clearly up-regulated as early as 2 h after LPS administration, leading ultimately to renal injury (Wu et al., 2007). In addition, Gupta et al (2007) demonstrated an obvious reduction in cortical peritubular capillary flow in the early phase of endotoxemia, that is, at 3 h post-LPS administration, using two-photon imaging (Gupta et al., 2007). These data show that an early phase of microvascular dysfunction, following LPS administration, appears to play a key role in initiating and extending tubular injury associated with significant inflammatory activation that contributes to the extension of renal dysfunction (Gupta et al., 2007). Based on these earlier findings, the present study investigated the effects of ultra-short acting drug (landiolol) on the tissue expression of endotoxemia related potential inflammatory cytokines, the HIF-1α and ET system during the early pathogenic phase (3 h) of AKI.

We show here significant increases in levels of serum creatinine, BUN, NGAL and levels of renal NGAL after LPS administration, indicating impairment of kidney function in the endotoxemic models. Recently, increase in urinary and plasma NGAL has been reported to be a reliable predictor of AKI progression (El Housseini et al., 2013). NGAL is expressed in various tissues of the body, and its expression is markedly induced in injured epithelial cells in the kidney. In the present study, minimal renal injury at the histological level was seen at 3 h after LPS administration. In our previous study, we showed that blood pressure continued to decrease even at 10 h after LPS administration, using Wistar rats (Yamaguchi et al., 2006), although the extent of decrease was not abrupt and remarkable. It is possible that it may be the reason why there were no remarkable histological changes in renal tissues after LPS administration. Thus, collectively, the endotoxemic models used in the present study had impairment of key renal function parameters, minimal morphological injury as well as decrease of systemic blood pressure. In fact, the experimental investigation of sepsis-associated AKI induced by LPS is usually of short duration due to the high mortality associated with the doses required to induce AKI (Sharfuddin et al., 2012). This model also tends not to recapitulate the

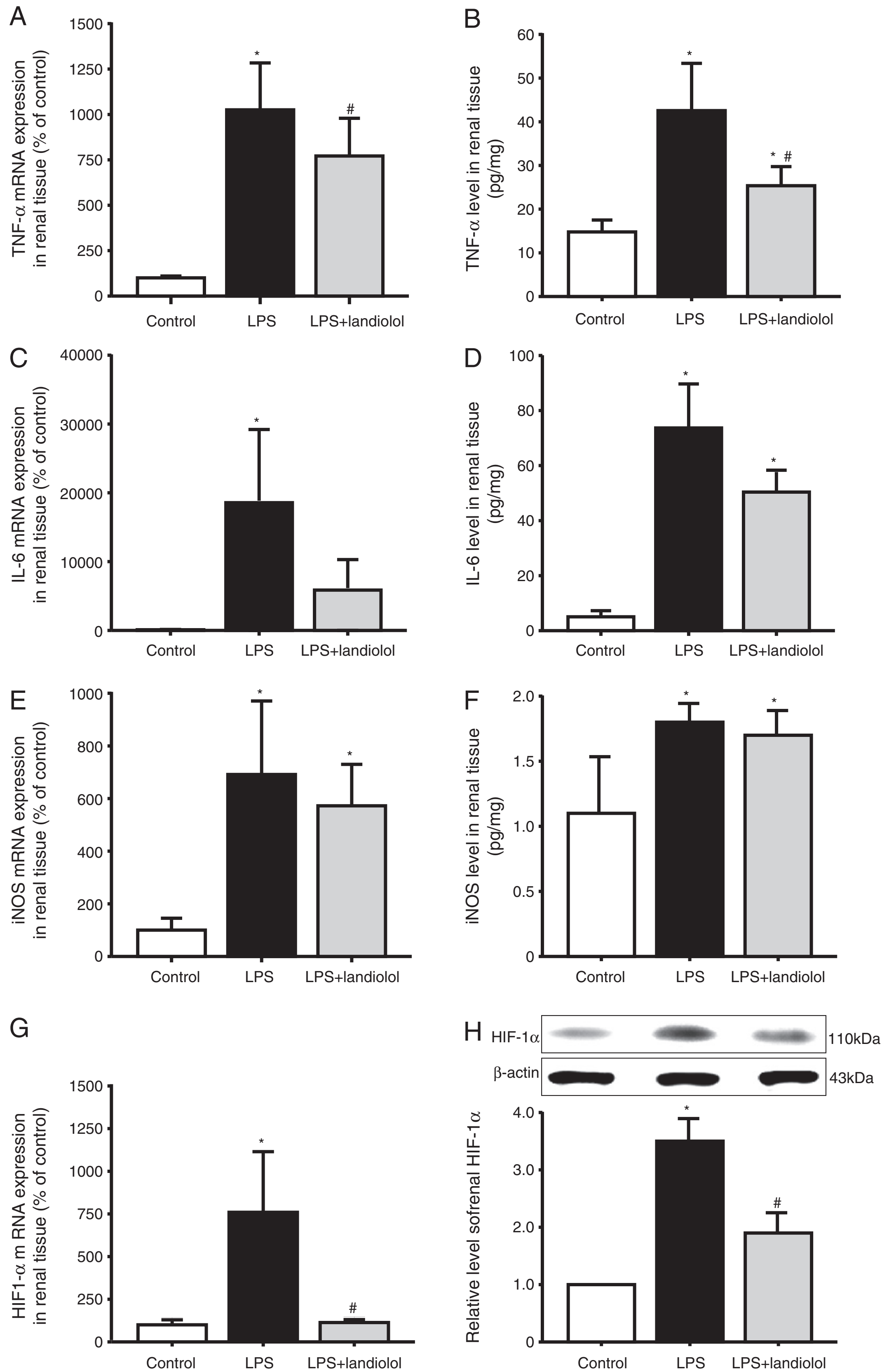

Fig. 3. mRNA and protein expression levels of TNF-α (A and B), IL-6 (C and D), iNOS (E and F), and HIF-1α (G and H) in kidney tissues of the control, 3 h LPS-administered rats, and landiolol treated 3 h LPS-administered rats. mRNA expression levels were analyzed by real-time quantitative PCR. GAPDH was used as internal control. In each of the experiments, the control was normalized as 100%. Data are shown as mean ± SE (n = 15). *p < 0.05 vs. control, #p < 0.05 vs. 3 h LPS-administered rats. Protein expression was analyzed by ELISA and immunoblotting. The panel of bands, just above the histogram, shows representative blots of animal and/or treatment, as described above (immunoblot analysis) (H). β-actin was used as loading control. The intensity of the bands was potted as histograms, as shown below the panel. The band obtained with control is normalized as 1.0. Data are shown as mean ± SE (n = 15). *p < 0.05 vs. control, #p < 0.05 vs. 3 h LPS-administered rats.

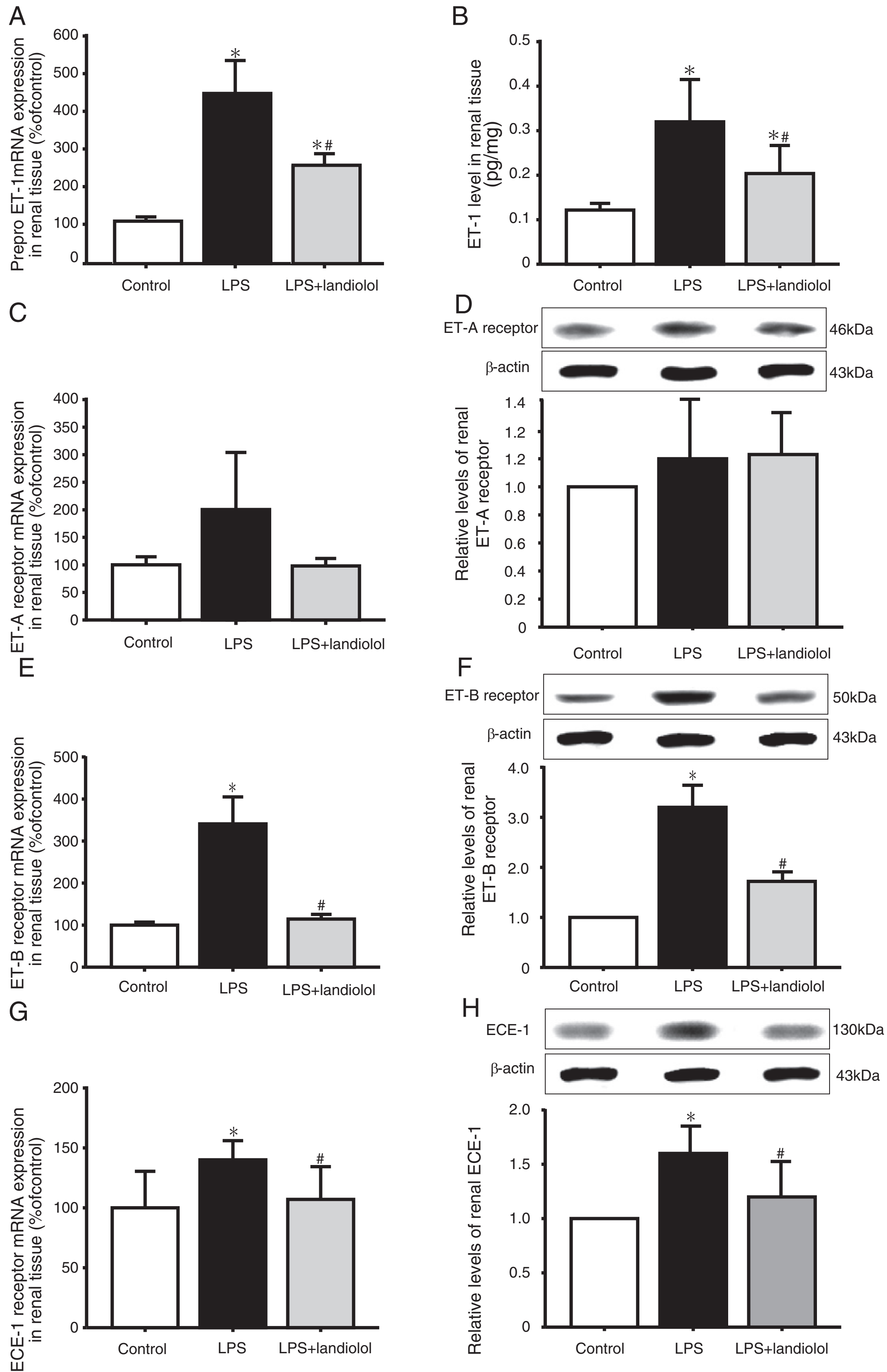

Fig. 4. mRNA and protein expression levels of preproET-1 (A and B), ET-A (C and D), ET-B (E and F) receptors, and ECE-1 (G and H) in kidney tissues of the control, 3 h LPS-administered rats, and landiolol treated 3 h LPS-administered rats. mRNA expression levels were analyzed by real-time quantitative PCR. GAPDH was used as internal control. In each of the experiments, the control was normalized as 100%. Values are mean ± SE (n = 15). *p < 0.05 vs. control, #p < 0.05 vs. 3 h LPS-administered rats. Protein expression was analyzed by ELISA and immunoblotting. The panel of bands, just above the histogram, shows representative blots of the type of animal and/or treatment, as described above (immunoblot analysis). β-actin was used as loading control. The intensity of the bands was potted as histograms, as shown below each panel. In each of the experiments, the band obtained with control is normalized as 1.0. Values are mean ± SE (n = 15). *p < 0.05 vs. control, #p < 0.05 vs. 3 h LPS-administered rats.

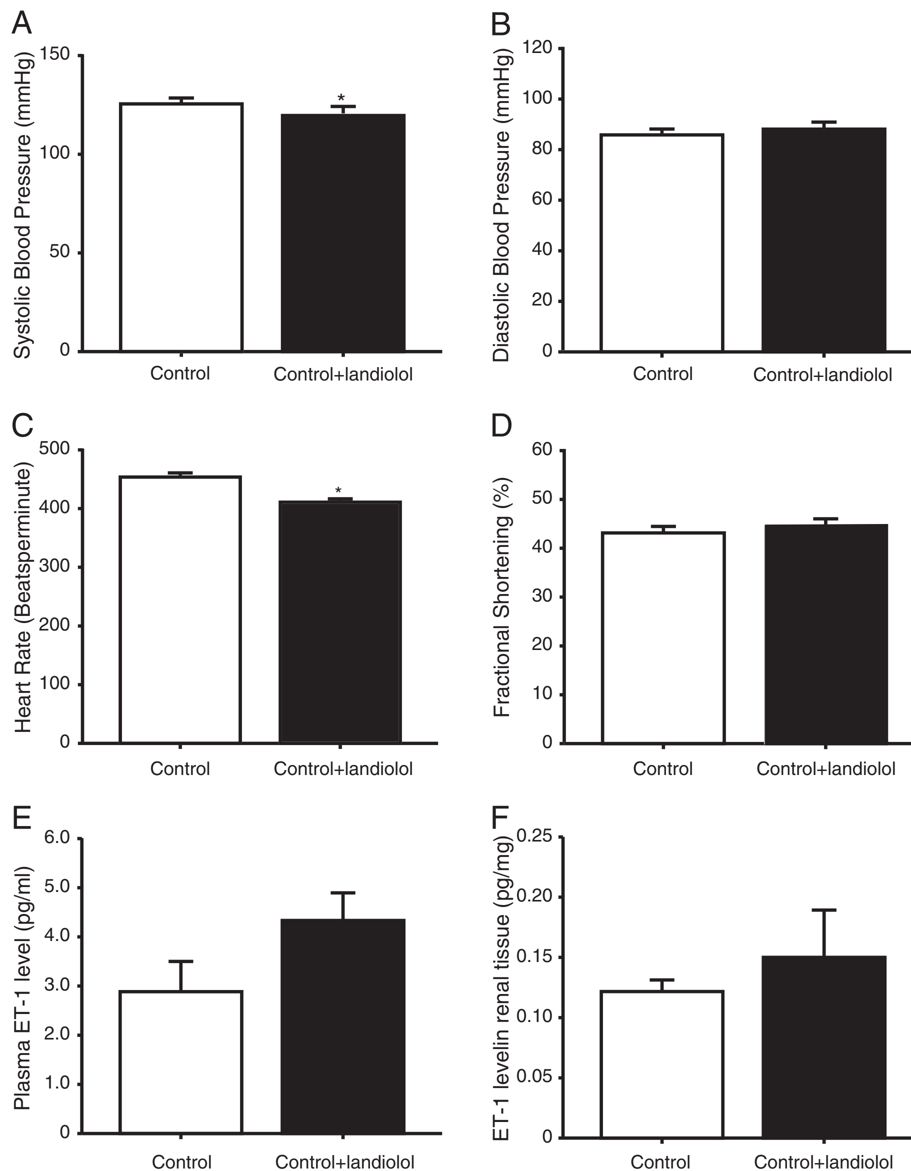

Fig. 5. Systolic blood pressure (A), diastolic blood pressure (B), heart rate (C), % fractional shortening (%FS) (D), plasma and renal tissue ET-1 levels (E and F) of the control and landiolol treated control rats. Data are shown as mean ± SE (n = 8). *p < 0.05 vs. control.

early kidney inflammation or sufficient morphological changes of human sepsis, though it tends to be a vasoconstrictive one (Sharfuddin et al., 2012). Consistent with this earlier report, we did not observe a significant amount of inflammatory cell infiltration in kidney tissues after induction of endotoxemia in the present study. In contrast, the endotoxemic models used in the present study showed increase in levels of inflammatory cytokines, such as TNF-α, IL-6 and iNOS both at circulatory and renal levels. Consistent with our previous studies (Jesmin et al., 2004, 2006, 2007) and earlier reports from other labs (Cohen, 2002; Norman et al., 1995), increase in levels of plasma and renal TNF-α was noted in the earlier hours of endotoxemia. Similarly, consistent with our previous study (Yamaguchi et al., 2006), we also show here elevated levels of serum and renal iNOS after LPS administration. The elevation of IL-6, as seen in current study after LPS administration, has been supported by data from a previous study (Song et al., 2012). Thus, in this context, an adequate inflammatory reaction was observed during the early hours of endotoxemia in the current model, both at renal and circulatory levels through upregulation of inflammatory cytokines. However, morphologically, remarkable inflammatory cell infiltration was absent in the kidney tissue after LPS administration.

Consistent with previous observations, the LPS-induced endotoxemia in the present study resulted in a rapid and profound elevation of plasma ET-1 at circulatory levels (Yamaguchi et al., 2006). The main mechanism responsible for this rise is believed to be the upregulation of ET-1 synthesis in vascular endothelia of various organs, a consequence of endothelial injury (Yamaguchi et al., 2006). Importantly, plasma ET-1 levels correlate positively with the severity of endotoxemia in patients (Pittet et al., 1991) and are lower in survivors than in non-survivors of septic shock (Takakuwa et al., 1994). The data showing the significant alterations in mRNA expression of ET-1 system [ET-1, ET receptors, ECE] of kidney tissue in endotoxemia from the present study were consistent with our previous article, where we clearly demonstrated the time-dependent alteration of various components of ET system in kidney tissues in endotoxemia (Yamaguchi et al., 2006). Over-expression of ET-1 is sufficient to induce structural and functional changes, including glomerular and tubule-interstitial fibrosis, because ET-1 causes hypoxic injury due to the constriction of peritubular capillaries and stimulates mesangial cell proliferation (Richter, 2006). Chronic ischemia and hypoxia that exist in the presence of renal interstitial fibrosis (RIF) increase the expression of hypoxia-inducible

factor-1alpha (HIF-1α). In this study, we also detected the increased expression level of HIF-1α in kidney tissue after LPS administration, suggesting that hypoxia remained in the kidney tissues after LPS administration.

Although both ET receptors appear to be upregulated in kidney tissues after LPS administration, as revealed here, significant elevation was observed in the case of ET-B receptor. In fact, upregulation of the ET signaling pathway and NF-kappaB has been shown to play an important role in the ARF of septic shock and a potential amelioration of renal lesions was achieved by suppressing the ET(A) and ET(B) receptors in the renal cortex following CPU0213 medication (He et al., 2006). Thus therapeutics focusing on the ET receptors may be preferable for the prevention of kidney injury in sepsis.

The most important finding of the present study is data showing amelioration of the elevated levels of the HIF-1α–ET-1 axis by landiolol in endotoxemic renal tissues. The ultra-short-acting beta-blocker, landiolol, which has a potent efficacy and dose adjustability, was developed in Japan for the emergency treatment of tachyarrhythmias in human and animals (Iguchi et al., 1992; Motomura et al., 1998). Recent studies have demonstrated that co-treatment with landiolol protects against acute lung injury and cardiac dysfunction in a rat model of LPS-induced systemic inflammation (Hagiwara et al., 2009). In that study, landiolol treatment caused a significant reduction in serum levels of the inflammation mediator, HMGB-1, as well as histological lung damage (Hagiwara et al., 2009). However, whether landiolol has any effect on ET system in endotoxemia is yet to be demonstrated. The current investigation is the first to show the effectiveness of landiolol on reversing the elevated levels of the HIF-1α–ET-1 system in renal tissues of endotoxemic rats. For now, we have no obvious explanation to describe the underlying mechanism of landiolol in reversing the elevated levels of ET system in endotoxemic rats. It is important to note that landiolol had no effect on the elevated plasma levels of inflammatory cytokines, namely TNF-α, IL-6 and iNOS. Surprisingly, landiolol normalized the renal augmented expression of TNF-α, but failed to normalize the elevated renal levels of IL-6 and iNOS after LPS administration. The underlying mechanisms for the differential effects of landiolol on circulatory and renal levels of inflammatory cytokines (TNF-α, IL-6 and iNOS), as observed in the present study, for now cannot be accounted for. More specifically, the exact process regulating reversal of elevated renal expression of TNF-α by landiolol for now is unclear, and thus warrants in depth exploration in future studies. Also, we show that landiolol normalizes the elevated levels of biochemical markers of renal injury, such as creatinine, BUN and NGAL. The decrease in levels of renal injury parameters by landiolol indicates that it (landiolol) may be effective in regulating the progression of renal dysfunction in sepsis. In addition, in the present study, introduction of landiolol improved PaO_2 and lactate levels in LPS-administered rats, the crucial features of endotoxemia. Thus, we believe that landiolol has reno-protective effects on endotoxemic kidney through the reversal of augmented levels of renal injury parameters, up-regulated HIF-1α–ET-1 axis, and increased expression of inflammatory cytokine, such as TNF-α.

The use of adrenergic antagonist with beta1-selectivity has a therapeutic advantage over non-selective beta-adrenergic antagonists in the treatment of patients with peripheral vascular disease, diabetes mellitus and obstructive pulmonary diseases (Weir et al., 2012). Recently, a lot of clinical studies have shown the efficacy and safety of *landiolol*, an ultra-short acting beta-1-selective blocker, on intraoperative and postoperative tachyarrhythmias in critical conditions (Taenaka and Kikawa, 2013), such as coronary artery bypass grafting, cardiac valve surgery, and other thoracic surgeries (Plosker, 2013). These critical conditions are vulnerable to acute tubular necrosis/acute kidney injury, which is also associated with increased mortality. In general, the short-term administration of a beta-blocker usually results in a reduction of GFR and effective renal plasma flow (Weir et al., 2012). In contrast, nebivolol, a beta-1-selective blocker, has vasodilator properties and has been shown to increase GFR and renal plasma flow through mechanism of increased synthesis of vasodilator nitric oxide (Greven and Gabriëls, 2000). The degree of specificity of beta-adrenergic blockers for beta-1- and beta-2-receptors might be expected to influence renal function, to the same extent as intrinsic partial agonist activity (Weir et al., 2012). So far, no clinical report shows that landiolol causes obvious renal dysfunction. However, equally, we did not find a clinical report that shows any safe or efficacious use of landiolol for kidney conditions in patients that have endotoxemia. The present finding is the first evidence that landiolol treatment in the early hours of endotoxemia is effective in reversing the circulatory levels of renal injury parameters and levels of inflammatory cytokines, as well as elevated HIF-ET. Future studies are needed to clarify these issues.

Another important finding of the present study is that landiolol only significantly affects heart rate in both control and endotoxemic rats. In contrast, both the circulatory and renal ET-1 levels in control rats were not affected by landiolol. Thus, the potential reversal of renal ET-1 levels by landiolol in endotoxemic rats, as reported here, may be specifically attributed to landiolol in sepsis. It is important to note that no significant change in blood pressure of LPS plus landiolol administered animals, implying that the normalization of up-regulated renal ET-1 levels in endotoxemia by landiolol is independent of blood pressure changes. In depth investigations are warranted in the future regarding this issue.

The present study was conducted during the early hours of endotoxemia, when the circulatory inflammatory cytokines were highly up-regulated and renal injury markers began to increase. However, signs of renal injury at the morphological level were still absent or insignificant. On the other hand, under these conditions, levels of renal HIF-1α and ET-1 were significantly up-regulated in the endotoxemic rats. Based on these data, we speculate that expression of the renal HIF-1α–ET-1 system begins to be altered prior to the onset of obvious renal injury in experimental endotoxemia and landiolol significantly ameliorates molecular changes that arrests progression of renal damage during sepsis.

Conclusion

Our present study shows that landiolol hydrochloride normalizes serum levels of potential renal injury markers, renal levels of inflammatory cytokine, such as TNF-α and up-regulated expression of HIF-1α and ET-1 system in kidney tissues in the early hours of endotoxemia in a rat model with a minimal/absent level of morphological renal injury.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research B and C from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (22390334, 23592025, 23406037, 23406016, 23406029, 24406026, 25462812 and 25305034). We greatly thank Ono Pharmaceutical Company (Tsukuba, Japan) for the kind gift of landiolol hydrochloride. There is nothing to disclose on the financial assistance from Ono Pharmaceutical Company for the present study.

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