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Original Article

Urotensin-II receptor contributes to the pro-inflammatory TLR4/MyD88 NF-κB/iNOS/NO pathway-mediated cardiovascular response to systemic lipopolysaccharide challenge in a septic shock model in rats

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Abstract

Urotensin (U)-II through the U-II receptor (UT) (the orphan G protein-coupled receptor; GPR14) plays an important role in the pathogenesis of many cardiovascular and renal diseases characterized by increased production of vasodilatory and pro-inflammatory mediators. This study tested the hypothesis of whether UT contributes to the pro-inflammatory TLR4/MyD88/NF-κB/iNOS/NO pathway-mediated changes in the cardiovascular response to systemic lipopolysaccharide (LPS) challenge in a rat model of septic shock. SB-710411, a UT antagonist, was used to test this hypothesis. Rats were injected with SB-710411 1 hour following an injection of saline or LPS. A tail-cuff device was used to record the mean arterial pressure and heart rate values of rats. Serum U-II and nitrite levels and U-II, GPR14, TLR4, MyD88, NF-κB, IL-1β, and iNOS mRNA expression in the cardiovascular and renal tissues were measured. Mean arterial pressure was reduced and heart rate was increased at 4 hours following LPS injection. In addition to the levels of U-II and nitrite in the sera of rats injected with LPS, the expression of U-II, GPR14, TLR4, MyD88, NF-κB, IL-1β, and iNOS was increased in the cardiovascular and renal tissues. SB-710411 at 0.01 mg/kg dose ameliorated the changes induced by LPS, excepting the increased serum nitrite level. These findings suggest that UT contributes to hypotension and tachycardia mediated by the TLR4/MyD88/NF-κB/iNOS/NO pathway, accompanied by an increase in pro-inflammatory cytokine expression in tissues related to the cardiovascular and renal systems, in response to systemic LPS challenge in rats.

Keywords: Septic shock, Urotensin-II receptor, Cardiovascular response, TLR4/MyD88/NF-κB/iNOS/NO pathway.

1. Introduction

Septic shock and multiple organ failure syndrome are life-threatening medical emergencies that can be caused by endotoxins, such as lipopolysaccharides (LPS), released from Gram-negative bacteria like Escherichia coli (E. coli), contributing significantly to global morbidity and mortality [1-3]. Mortality increases particularly in patients with various dysfunctions in vital organs such as the heart, kidney, brain, lung, and liver [1,3]. Additionally, advances in the management of septic shock are progressing very slowly. The reasons for this are (1) the physiopathology of septic shock is complex due to the contribution of various mediators, (2) the interaction of mediators, and (3) the variability of symptoms and prognosis among individuals [1,3-5]. Studies in recent years have focused on specific therapeutic approaches aimed at the underlying cause and molecular level. Most of these approaches focus on the signaling mechanisms triggered in the host by the lipid A moiety of lipopolysaccharide (LPS), also called "endotoxin", which is a component of the cell wall of Gram-negative bacteria such as *E. coli* [2,4,5]. Lipid A is responsible for most of the detrimental effects observed in Gram-negative septic shock. The primary effect of lipid A is to activate macrophages and trigger inflammatory events [2,3].

Cluster of differentiation 14 and toll-like receptor (TLR) 4, found on the surface of monocytes, macrophages, neutrophils, and endothelial cells that play a significant role in the response to infectious agents, are receptors that recognize and bind to whole bacteria or bacterial products such as LPS. In particular, in the TLR4/myeloid differentiation primary response protein (MyD)-dependent pathway, nuclear factor (NF)-κB, which is activated and passes into the nucleus, causes an increase in the transcriptional expression of enzymes responsible for the formation of various molecules that cause inflammation. These genes include enzymes such as inducible nitric oxide (NO) synthase (iNOS) and pro-inflammatory cytokines such as interleukin (IL)-1β which are known to play an important role in the physiopathology of septic shock [2,4,5]. Increased formation of vasodilator products such as NO

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[6-10] and decreased synthesis of vasoconstrictor eicosanoids such as 20-hydroxyeicosatrienoic acid [6,9-13] also contribute to suppression of the cardiovascular and renal systems. Even though these findings seem contradictory, vasodilator/vasoconstrictor and pro-inflammatory/anti-inflammatory balances are disrupted during septic shock, mostly in the direction of increased formation of vasodilator and pro-inflammatory products. To re-establish this disrupted balance, interactions are observed at the level of products and/or enzymes that prevent each other's formation and/or effects [4,5]. All of these events explain irreversible vascular hyporeactivity despite appropriate vasoconstrictor therapy in patients with septic shock.

The urotensinergic system plays a substantial role in the physiopathology of numerous diseases, particularly cardiovascular and renal diseases, which are characterized by inflammation and tissue damage caused by the increased production and release of pro-inflammatory cytokines [14-16]. This system in humans consists of two endogenous peptides, urotensin (U)-II and U-II-related peptide, and a G protein-coupled receptor (GPCR), U-II receptor (UT) [17,18]. UT is also known as "G protein receptor (GPR) 14", "UII-R1", "UTR2", and "sensory epithelial neuropeptide-like receptor" [17]. UT, shown to be expressed in cell and nuclear membranes, is a group A GPCR from the rhodopsin family [17,18]. As an endogenous ligand, human U-II has been reported to selectively bind to a rat orphan receptor, GPR14, currently referred to as "UT" [17,19,20]. UT/GPR14 is found in varying amounts in vascular smooth muscle and endothelial cells in addition to cardiac myocytes and renal tissue [19,20]. Activation of UT in various cells by U-II activates complex and multiple signaling pathways, leading to different effects [15,16].

U-II is one of the endogenous vasoconstrictor peptides known to date in mammals, even more potent than endothelin-1 [19]. On the other hand, Hillier et al. [21] reported that U-II had no vasoconstrictor effect in arteries with intact endothelial layers isolated from patients. Moreover, U-II has a potent vasodilator activity in the human small pulmonary artery (PA), abdominal arteries, and small resistance vessels, a major determinant of vascular resistance [22]. Based on the results of these studies, it has been suggested that the effects of U-II are dependent on arterial diameter [23]. According to the results of various studies, the release of NO from the endothelial cells of small vessels which cause vasodilation plays a critical role in balancing the vasoconstrictor effect of U-II on smooth muscle cells [24,25]. The results of all these studies suggest that the complex effects of U-II may be due to the type of artery, its diameter, and the integrity of endothelial cells, as well as the amount of UT in the tissue or the activation of a secondary UT signaling pathway [23].

In recent years, U-II has been implicated in the regulation of cardiovascular and renal homeostasis with promising therapeutic applications based on UT antagonism. Therefore, the U-II/UT signaling system has been considered a drug target for the treatment of various diseases related to hemodynamic disturbances associated with systemic inflammation, including septic shock [14-16]. There are numerous preclinical and clinical studies using UT agonists and peptide and non-peptide competitive antagonists targeting the urotensinergic system for the management of diseases in which it plays a role in the pathogenesis [14-16]. For UT in rats, *in vivo* studies were performed in rodents using the peptide antagonist urantide [26,27], the non-peptide antagonist palosuran [28], and the peptide antagonist Cpa-D-Cys-Pal-D-Trp-Lys-Val-Cys-Cpa-NH2 (SB-710411) [29-31], which is reported to be a rat-selective peptide antagonist. On the contrary, an important issue to emphasize is that although urantide, palosuran, and SB-710411 are known as UT/GPR14 receptor antagonists, these substances can act as full or partial agonists for UT in a species-specific manner [14,15]. It been also reported that SB-710411 is an antagonist for UT in rat aorta [29] and a full agonist in monkey TA, common carotid artery, renal artery (RA), and superior mesenteric artery (SMA) [30]. In addition, urantide and SB-710411 were described as "low efficacy partial agonists" for UT based on the results of a study conducted by Behm et al. [26] on human embryonic kidney cells and isolated rat, cat and monkey aorta, femoral artery, RA, and mesenteric artery. There are also *in vivo* studies in the literature with urantide, palosuran, and SB-710411 in various sepsis models [32-37]. In a study conducted by Nitescu et al. [36] in an LPS-induced endotoxemia model in rats, it was reported that urantide did not prevent the increase in mean arterial pressure (MAP), heart rate (HR), and renovascular resistance, suggesting that the renal abnormalities observed in non-hypotensive endotoxemic rats were not mediated by UT. Although neither was performed in a septic shock model, there are only two *in vivo* studies with SB-710411 in the literature. In a study conducted by Liu et al. [38] in a carbon tetrachloride-induced cirrhosis model in rats, SB-710411 was shown to prevent the increase in systemic U-II levels in addition to U-II and UT expression in the liver. Luo et al. [31] also reported that SB-710411 could correct changes in cardiovascular hemodynamic parameters such as a decrease in MAP and HR in an ischemia/reperfusion (I/R) model established in rats. Collectively, the findings of studies performed on rodent models of septic shock have shown beneficial effects of UT antagonism on systemic inflammation, tissue injury, cardiac dysfunction, and survival [32-35,37]. Hence, these data led us to hypothesize that UT may contribute to the hemodynamic changes that occurred in the LPS-induced septic shock model in rats. However, the contribution of UT to the hemodynamic changes associated with vasodilatory and pro-inflammatory mediator production via the TLR4/MyD88/NF-κB/iNOS pathway in the arterial vasculature and vital organs of rats with septic shock has not been explored. Therefore, we tested the hypothesis of whether UT contributes to the pro-inflammatory TLR4/MyD88/NF-κB/iNOS/NO pathway-mediated changes in the cardiovascular response to systemic challenge to LPS in the rat model of septic shock by using selective UT antagonist, SB-710411. It is expected that this study may contribute to the development of selective UT antagonists in ongoing efforts to improve the management of septic shock due to bacterial infections.

2. Materials and methods

2.1. Animals

Experiments were carried out on male Wistar rats aged 8 to 16 weeks and weighing 200-360 g (n=36) (Research Center of Experimental Animals, Mersin University, Mersin, Turkey). The animals were kept under a controlled temperature of 24°C with 50% humidity, and 12-hours light/dark cycle with free access to water and standard rat chow. All of the experimental protocols were approved by

the Mersin University Experimental Animals Local Ethics Committee (Protocol number: 2023/34; Approval date: July 24, 2023). The experiments were performed following the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Induction of septic shock model

The rat model of septic shock model induced by LPS (*E. coli* LPS, O111:B4; L4130; Sigma Chemical Co., St. Louis, MO, USA) was used in this study, as previously described [6-13,39]. The rats were injected intraperitoneally (i.p.) with saline (n=6), LPS (10 mg/kg) (n=6), saline+SB-710411 (0.01 mg/kg) (n=6), LPS+SB-710411 (0.0001 mg/kg (n=6), LPS+SB-710411 (0.001 mg/kg) (n=6), and LPS+SB-710411 (0.01 mg/kg) (n=6). SB-710411 (4 ml/kg) (071-15; Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) was administered 1 hour following injection of saline (4 mL/kg) or LPS (4 ml/kg). Saline- and LPS-injected rats were used as control and septic shock groups, respectively. LPS and SB-710411 were dissolved in saline. Although there were studies performed with UT antagonists urantide and palosuran in LPS-, LPS/D-galactosamine-, and cecal ligation and puncture (CLP)-induced endotoxic shock, acute liver failure, polymicrobial sepsis, and endotoxemia models in mice and rats [32-37], there was no study performed with SB-710411 in these models. To determine the lowest effective dose of SB-710411 that can significantly prevent the decrease in I/R-induced MAP and increase in HR without causing mortality, the dose range of SB-710411 (0.0001, 0.001, and 0.01 mg/kg; i.p.) was selected considering its effects on MAP, HR, and cardiac UT protein expression when injected at 1 and 2 µg/kg (intravenous; i.v.) doses in rats in the I/R model [31]. A tail-cuff device (MAY 9610 Indirect Blood Pressure Recorder System, Commat Ltd., Ankara, Turkey) was used to record MAP and HR values of the animals at times 0 and 1, 2, 3, and 4 hours. Under xylazine (7 mg/kg; 0.35 ml/kg; i.p.)+ketamine (90 mg/kg; 0.9 ml/kg; i.p.) anesthesia 4 hours following the saline or LPS injection, and blood sample, TA, RA, heart, and kidney were taken from these animals. Euthanasia was carried out by the exsanguination of rats. Serum samples prepared from the blood taken were stored at -80°C to determine systemic U-II and nitrite levels. The tissues to be used in messenger ribonucleic acid (mRNA) expression studies were rapidly frozen in liquid nitrogen and stored at -80°C. There were no potential confounding factors during the experiments.

2.3. Determination of serum levels of U-II and nitrite

U-II levels in serum as an indicator of systemic U-II formation were measured using a Rat Urotensin II (UII) enzyme-linked immunosorbent assay (ELISA) Kit (MBS264715; MyBiosource, Inc.; San Diego, CA, USA) according to the manufacturer's instructions. The diazotization method, based on the Griess reaction, which is sensitive to nitrite ions, was used to measure the levels of serum nitrite, one of the stable metabolites of NO, as an indicator of systemic NO formation [8].

2.4. mRNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

PolyATtract® System 1000 with Magnetic Separation Stand (Z5420; Promega, Madison, WI, USA) was used to isolate mRNAs from the frozen tissue powders and complementary deoxyribonucleic acids (cDNAs) were synthesized by Reverse Transcription System (A3500; Promega) as suggested by the manufacturer. cDNAs for U-II, GPR14, TLR4, MyD88, NF-κB, iNOS, IL-1β, α-smooth muscle actin, α-skeletal muscle actin, and β-actin were synthesized from 1 µL of eluted mRNA by the RT-PCR method with the primer pairs listed in Table 1 [39-42]. The polymerase chain reaction (PCR) cycling parameters for cDNAs for U-II, GPR14, TLR4, MyD88, NF-κB, iNOS, IL-1β, α-smooth muscle actin, α-skeletal muscle actin, and β-actin are shown in Table 1. The RT-PCR products (20 µL) along with diluted DNA marker (5 µL) (G6951; Promega) were submitted to electrophoresis in 1% agarose gels prestained with 1 µL diluted ethidium bromide dye (10 mg/mL) in distilled water (final concentration of 0.08 µg/ml gel) and visualized under 302 nm ultraviolet light using a gel-imaging system (EC3-CHEMI HR Imaging System; Ultra-Violet Products, UVP, Cambridge, UK). The intensity of each band for U-II, GPR14, TLR4, MyD88, NF-κB, IL-1β, and iNOS mRNA were normalized to the α-smooth muscle actin for TA and RA, α-skeletal muscle actin for heart, and β-actin for kidney and expressed as relative densitometric units. To quantify the relative densities of bands, Image J densitometry analysis software (Image J 1.54g, Wayne Rasband, National Institute of Health, Bethesda, MD, USA) was used.

2.5. Statistical analysis

Data are expressed as means ± standard error of means (SEM). The suitability of the data for a normal distribution was evaluated using Bartlett's test. For data normally or not normally distributed, parametric or nonparametric statistical analysis was conducted using a one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons or Kruskal-Wallis test followed by Dunn's test, respectively, to determine differences from the saline- or LPS-injected groups. GraphPad Prism 10 version 10.0.3 (275) (GraphPad Software, San Diego California USA) was used to perform statistical analysis and generate the figures. A P *v*alue < 0.05 was considered to be statistically significant. No notable outliers or inconsistencies were observed in the data.

3. Results

3.1. Contribution of UT to the cardiovascular response to systemic LPS challenge in rats

To investigate the contribution of UT to the cardiovascular response to systemic LPS challenge, MAP and HR values of saline-, LPS-, and/or SB-710411-injected rats were measured. LPS-injected rats presented a gradual decrease in MAP and an increase in HR during the experimental period compared to the control group (Table 2). MAP was reduced by 44 mmHg and HR increased by 130 beats per minute (bpm) 4 hours following LPS injection into the rats (P<0.05). When SB-710411 was injected at a dose of 0.0001 mg/kg, it prevented the decrease in MAP values observed at the 3rd hour induced by LPS (P<0.05), but 5 of the rats died at the 4th hour, and MAP and HR could not be recorded from the remaining 1 rat. When the LPS+SB-710411 group was compared with the LPS-injected group, it was found that the decrease in MAP (but not the increase in HR) 2, 3, and 4 hours following LPS injection was prevented by SB-710411 (0.0001 mg/kg) (P<0.05). SB-710411, at a dose of 0.01 mg/kg, prevented

Table 1. Primer sequences for RT-PCR and PCR cycling parameters used for cDNAs for U-II, GPR14, TLR4, MyD88, NF-κB, iNOS, and IL-1β.

Primers	PCR cycling parameters
U-II - TA, RA, heart, and kidney ***Forward:*** *5′-TGC CTG CTC TTC GTA GGA CT-3′* ***Reverse:*** *5′-AGA GCC TTC CTC AAG CTT-3′*	Preheating for 5 min at 94°C; denaturation for 30 seconds at 94°C, annealing for 30 seconds at 53°C, and elongation for 45 seconds at 72°C (25 cycles); final elongation 10 min at 72°C
GPR14 - TA, RA, heart, and kidney ***Forward:*** *5′-TCT GAG CCT GGA GTC TAC AAC AAG CT-3′* ***Reverse:*** *5′-CCA AAG TGC CAG TCC TTA GTG ACG T-3′*	Preheating for 5 min at 94°C; denaturation for 30 seconds at 94°C, annealing for 30 seconds at 53°C, and elongation for 45 seconds at 72°C (25 cycles); final elongation 10 min at 72°C
TLR4 - TA, RA, heart, and kidney ***Forward:*** *5′-GTG GAA GTT GAA CGA ATG GA-3′* ***Reverse:*** *5′-TGG ATG ATG TTG GCA GCA-3′*	Preheating for 5 min at 94°C; denaturation for 30 seconds at 94°C, annealing for 30 seconds at 53°C, and elongation for 45 seconds at 72°C (30 cycles); final elongation 10 min at 72°C
MyD88 - TA, RA, heart, and kidney ***Forward:*** *5′-TCG CGC ATC GGA CAA ACG-3′* ***Reverse:*** *5′-GCA ATG GAC CAG ACA CAG GT-3′*	Preheating for 5 min at 94°C; denaturation for 30 seconds at 94°C, annealing for 30 seconds at 53°C, and elongation for 45 seconds at 72°C (30 cycles); final elongation 10 min at 72°C
NF-κB - TA, RA, heart, and kidney ***Forward:*** *5′-AGT TGA GGG GAC TTT CCC AGG C-3′* ***Reverse:*** *5′-GAT TCG AGT ATT AGT TCA TGG A-3′*	Preheating for 5 min at 94°C; denaturation for 30 seconds at 94°C, annealing for 30 seconds at 53°C, and elongation for 45 seconds at 72°C (30 cycles); final elongation 10 min at 72°C
IL-1β - TA, RA, heart, and kidney ***Forward:*** *5′-GGC AAC TGT CCC TGA ACT CAA C-3′* ***Reverse:*** *5′-AAG CTC CAC GGG CAA GAC ATA-3′*	Preheating for 5 min at 94°C; denaturation for 30 seconds at 94°C, annealing for 30 seconds at 53°C, and elongation for 45 seconds at 72°C (30 cycles); final elongation 10 min at 72°C
iNOS - TA, RA, heart, and kidney ***Forward:*** *5′-ACC TAC TTC CTG GAC ATC AC-3′* ***Reverse:*** *5′-ACC CAA ACA CCA AGG TCA TG-3′*	Preheating for 5 min at 94°C; denaturation for 30 seconds at 94°C, annealing for 30 seconds at 53°C, and elongation for 45 seconds at 72°C (30 cycles); final elongation 10 min at 72°C
α-Smooth muscle actin - TA and RA ***Forward:*** *5′-GAC ACC AGG GAG TGA TGG TT-3′* ***Reverse:*** *5′-GTT AGC AAG GTC GGA TGC TC-3′*	Preheating for 5 min at 94°C; denaturation for 20 seconds at 95°C, annealing for 30 seconds at 60°C, and elongation for 30 seconds at 72°C (25 cycles); final elongation 10 min at 72°C
α-Skeletal muscle actin - Heart ***Forward:*** *5′-CTC TCT CTC CTC AGG ACG ACA ATC-3′* ***Reverse:*** *5′-CAG AAT GGC TGG CTT TAA TGC TTC-3′*	Preheating for 5 min at 94°C; denaturation for 15 seconds at 95°C, annealing for 15 seconds at 56°C, and elongation for 45 seconds at 72°C (40 cycles); final elongation 10 min at 72°C
β-Actin - Kidney ***Forward:*** *5′-TCA TGA AGT GTG ACG TTG ACA TCC GT-3′* ***Reverse:*** *5′-CCT AGA AGC ATT TGC GGT GCA CGA TG-3′*	Preheating for 5 min at 94°C; denaturation for 15 seconds at 94°C, annealing for 30 seconds at 55°C, and elongation for 60 seconds at 72°C (35 cycles); final elongation 10 min at 72°C

Table 2. Effects of SB-710411 on the changes in MAP and HR values induced by LPS in rats.

	Time after saline or LPS injection (hours)				
	0	**1**	**2**	**3**	**4**
Saline	125.90 ± 0.90 (n=6)	127.00 ± 0.86 (n=6)	126.90 ± 1.05 (n=6)	128.60 ± 1.04 (n=6)	129.00 ± 0.60 (n=6)
MAP (mm/Hg)	349.20 ± 8.42 (n=6)	342.50 ± 5.89 (n=6)	338.10 ± 5.56 (n=6)	338.50 ± 4.56 (n=6)	339.30 ± 5.73 (n=6)
HR (bpm)					
LPS					
MAP (mm/Hg)	127.90 ± 1.18 (n=6)	88.50 ± 0.94 (n=6)$^{*\cdot}$	88.06 ± 0.59 (n=6)$^{*\cdot}$	83.56 ± 1.51 (n=6)$^{*\cdot}$	84.00 ± 1.60 (n=6)$^{*\cdot}$
HR (bpm)	330.90 ± 9.46 (n=6)	407.90 ± 6.57 (n=6)$^{*\cdot}$	430.50 ± 11.55 (n=6)$^{*\cdot}$	453.50 ± 15.05 (n=6)$^{*\cdot}$	460.50 ± 12.82 (n=6)$^{*\cdot}$
SB-710411 (0.01 mg/kg)					
MAP (mm/Hg)	128.30 ± 0.35 (n=6)	128.10± 0.45 (n=6)	125.6 ± 1.72 (n=6)	128.40 ± 0.36 (n=6)	127.70 ± 0.32 (n=6)
HR (bpm)	346.40 ± 6.25 (n=6)	337.70 ± 8.14 (n=6)	339.60 ± 7.77 (n=6)	335.90 ± 9.17 (n=6)	340.10 ± 7.19 (n=6)
LPS+SB-710411 (0.0001 mg/kg)					
MAP (mm/Hg)	127.80 ± 0.57 (n=6)	88.72 ± 0.51 (n=6)$^{*\cdot}$	100.00 ± 7.68 (n= 5)$^{*\cdot}$	109.00 ± 4.07 (n= 5)$^{*\#\cdot\blacklozenge}$	---
HR (bpm)	346.90 ± 6.22 (n=6)	414.50 ± 4.33 (n=6)$^{*\cdot}$	411.00 ± 18.05 (n= 5)$^{*\cdot}$	506.80 ± 14.97 (n= 5)$^{*\cdot\blacklozenge\S}$	---
LPS+SB-710411 (0.001 mg/kg)					
MAP (mm/Hg)	128.20 ± 0.64 (n=6)	89.72 ± 0.58 (n=6)$^{*\cdot}$	116.30 ± 7.17 (n=6)$^{\#\blacklozenge}$	119.90 ± 3.43 (n=6)$^{\#\blacklozenge}$	117.60 ± 4.11 (n=6)$^{\#\blacklozenge}$
HR (bpm)	363.60 ± 6.16 (n=6)	425.20 ± 9.88 (n=6)$^{*\cdot}$	455.20 ± 5.37 (n=6)$^{*\cdot\blacklozenge}$	467.50 ± 4.97 (n=6)$^{*\cdot\blacklozenge}$	492.80 ± 4.07 (n=6)$^{*\cdot\blacklozenge\S}$
LPS+SB-710411 (0.01 mg/kg)					
MAP (mm/Hg)	128.30 ± 0.37 (n=6)	88.44 ± 0.58 (n=6)$^{*\cdot}$	114.30 ± 1.91 (n=6)$^{\cdot\#\blacklozenge}$	126.90 ± 1.05 (n=6)$^{\#\blacklozenge\S}$	127.40 ± 0.71 (n=6)$^{\#\blacklozenge\S}$
HR (bpm)	330.70 ± 3.63 (n=6)	419.90 ± 1.29 (n=6)$^{*\cdot}$	432.00 ± 9.96 (n=6)*	408.60 ± 9.75 (n=6)$^{*\#\cdot\S}$	356.10 ± 9.10 (n=6)$^{\#\blacklozenge\S\ddagger}$

Data are expressed as means ± SEM. *P<0.05 versus corresponding value seen in the saline-injected group. $^{\#}$P<0.05 vs. value corresponding to that measured in the LPS-injected group. $^{\cdot}$P<0.05 vs. time 0 h value to that measured within a group. $^{\blacklozenge}$P<0.05 vs. time 1 h value to that measured within a group. §P<0.05 vs. time 2 h value to that measured within a group. ‡P<0.05 vs. time 3 h value to that measured within a group.

the LPS-induced fall in MAP and the rise in HR at 2 and 3 h following its injection (P<0.05). MAP and HR values in rats injected with SB-710411 (0.01 mg/kg) were also not different from the control group (P>0.05). Consequently, sera and tissues of rats treated with 0.01 mg/kg dose of SB-710411 were used for further experiments.

3.2. SB-710411 prevented the rise in serum U-II level associated with enhanced tissue expression of U-II and its receptor, GPR14, induced by LPS

To explore whether UT antagonism also affects systemic U-II formation as well as expression of U-II and its receptor in the vascular, cardiac, and renal tissues during septic shock, serum U-II levels and U-II/GPR14 mRNA expression in the TA, RA, heart, and kidney of saline-, LPS-, and/or SB-710411-injected rats were measured. LPS injection into rats resulted in a rise in serum U-II level (Figure 1A) associated with U-II and GPR14 expression in the TA (Figure 1B, 2A), RA (Figure 1C, 2B), heart (Figure 1D, 2C), and kidney (Figure 1E, 2D) when compared to the control group (P<0.05). SB-710411 treatment prevented the LPS-induced rise in serum U-II level and

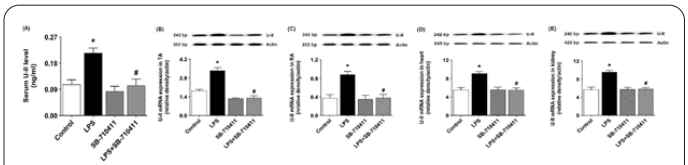

Fig. 1. SB-710411 prevented the LPS-induced rise in (A) serum U-II levels and enhancement in the expression of U-II mRNA in the (B) TA, (C) RA, (D) heart, and (E) kidney of rats. Data represent mean ± SEM (n=4-6). *P<0.05 vs. saline-injected group; $^{\#}$P<0.05 vs. LPS-injected group.

enhancement in the expression of U-II and GPR14 mRNA (Figures 1 and 2) (P<0.05). Serum U-II level and tissue U-II/GPR14 expression in rats injected with SB-710411 were not different from the control group (Figures 1 and 2) (P>0.05).

3.3. SB-710411 prevented the enhanced tissue expression of pro-inflammatory TLR4/MyD88/NF-κB/IL-1β pathway induced by LPS

To further explore whether UT antagonism can influence the expression of pro-inflammatory TLR4/MyD88/

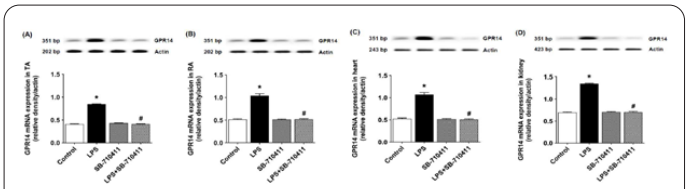

Fig. 2. SB-710411 prevented the LPS-induced enhancement in the expression of GPR14 mRNA in the (A) TA, (B) RA, (C) heart, and (D) kidney of rats. Data represent mean ± SEM (n=4). *P<0.05 vs. saline-injected group; #P<0.05 vs. LPS-injected group.

NF-κB/IL-1β pathway in the vascular, cardiac, and renal tissues during septic shock, TLR4, MyD88, NF-κB, and IL-1β mRNA expression in the TA, RA, heart, and kidney of saline-, LPS-, and/or SB-710411-injected rats were measured. In the LPS-injected rats, expression of TLR4, MyD88, NF-κB, and IL-1β was found to be enhanced in the TA (Figure 3A), RA (Figure 3B), heart (Figure 3C), and kidney (Figure 3D) compared to the control group (P<0.05). The LPS-induced enhancement in the expression of TLR4, MyD88, NF-κB, and IL-1β was prevented by SB-710411 treatment (Figure 3) (P<0.05). Tissue expression of TLR4, MyD88, NF-κB, and IL-1β in rats injected with SB-710411 was not different from the control group (Figure 3) (P>0.05).

3.4. SB-710411 prevented the enhanced tissue expression of iNOS, but not rise in serum nitrite level, induced by LPS

In order to explore the effect of UT antagonism on the systemic NO formation and expression of iNOS through the TLR4/MyD88/NF-κB pathway in the vascular, cardiac, and renal tissues during septic shock, serum nitrite levels and iNOS mRNA expression in the TA, RA, heart, and kidney of saline-, LPS-, and/or SB-710411-injected rats were measured. LPS injection into rats resulted in a rise in serum nitrite level (Figure 4A) associated with iNOS expression in the TA (Figure 4B), RA (Figure 4C), heart (Figure 4D), and kidney (Figure 4E) when compared to the control group (P<0.05). SB-710411 prevented the enhanced tissue iNOS expression, without affecting the rise in serum nitrite level induced by LPS (Figure 4) (P<0.05). Serum nitrite level and tissue iNOS expression in rats injected with SB-710411 were not different from the control group (Figure 4) (P>0.05).

4. Discussion

The results of the study demonstrate that a rat-selective peptide UT antagonist, SB-710411, prevents the LPS-induced (1) fall in MAP and rise in HR, (2) increase in serum levels of U-II, but not nitrite, and (3) increase in the mRNA expression of U-II, GPR14, TLR4, MyD88, NF-κB, IL-1β, and iNOS in the cardiovascular and renal tissues of rats in the septic shock model. The findings of this study may also ensure the first evidence that UT contributes to the hypotension and tachycardia mediated by the TLR4/MyD88/NF-κB/iNOS/NO signaling pathway accompanied by an increase in pro-inflammatory cytokine expression (Figure 5).

In the literature, there are *in vivo* studies in rodents using urantide, which is an antagonist for UT in rats [26,27], palosuran [28], and SB-710411, which is reported to be a selective antagonist, especially in rats [26,29-31]. In a study conducted in an LPS-induced endotoxic shock

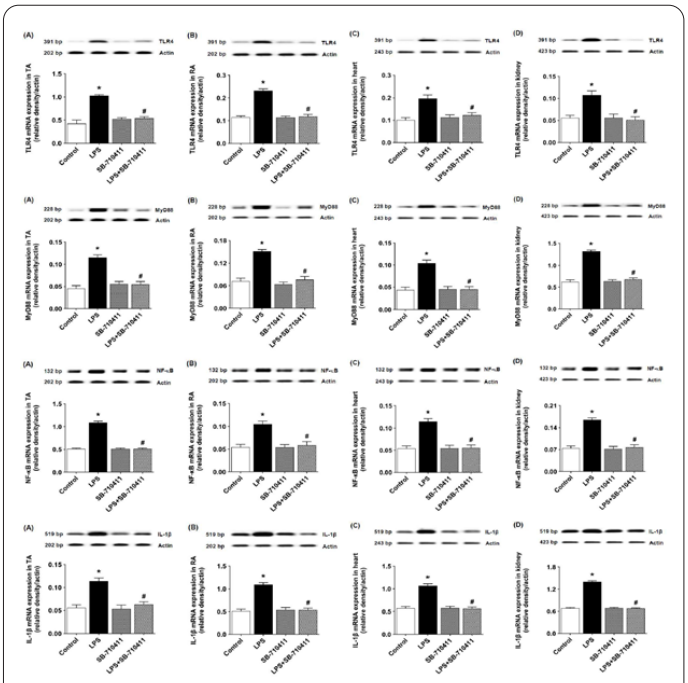

Fig. 3. SB-710411 prevented the LPS-induced enhancement in the expression of TLR4, MyD88, NF-κB, and IL-1β mRNA in the (A) TA, (B) RA, (C) heart, and (D) kidney of rats. Data represent mean ± SEM (n=4). *P<0.05 vs. saline-injected group; #P<0.05 vs. LPS-injected group.

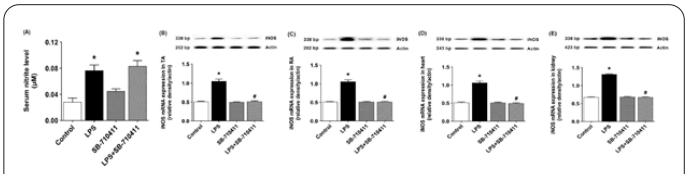

Fig. 4. Effects of SB-710411 on the LPS-induced rise in (A) serum nitrite levels and enhanced iNOS mRNA expression in the (B) TA, (C) RA, (D) heart, and (E) kidney of rats. Data represent mean ± SEM (n=4-6). *P<0.05 vs. saline-injected group; #P<0.05 vs. LPS-injected group.

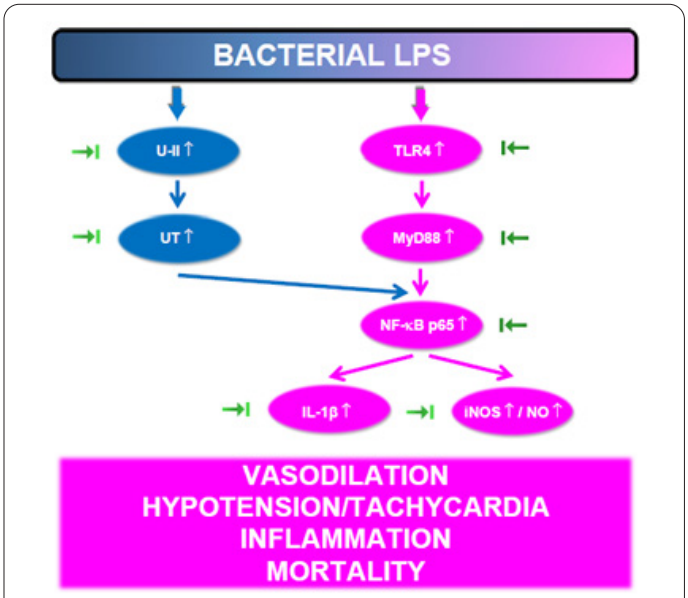

Fig. 5. The proposed mechanism for the effect of a rat-selective UT antagonist SB-710411 on vasodilation, hypotension, tachycardia, inflammation, and mortality in the LPS-induced septic shock model in rats. , Increase; ↓, decrease; , sites of the counter-effect of SB-710411.

model in mice, urantide injected at (i.v.) doses of 43 and 430 µg/kg was shown to prevent cardiac dysfunction by increasing plasma IL-1β levels and NF-κB expression in the kidney and liver, as well as prolonging lifespan [33]. In the LPS/D-galactosamine-induced acute liver failure model in mice, urantide injected at an i.p. dose of 0.6 mg/kg was reported to prevent the increase in serum and liver U-II and IL-1β levels, U-II, UT, and IL-1β mRNA expression in the liver, IκBα phosphorylation, and subsequently nuclear translocation and binding to DNA of NF-B, as well as prolonging life span [34,35]. According to the results of a study conducted on a polymicrobial sepsis model induced by the CLP method in mice, palosuran injected at i.p. doses of 30 and 100 mg/kg was shown to prevent the increase in mRNA expression of GPR14, IL-1β, and NF-κB in the lung, as well as the increase in vascular congestion, edema, and inflammatory cell infiltration [37]. In another study conducted on the same model, urantide and palosuran, when injected at i.p. doses of 1.2 mg/kg and 100 mg/kg, respectively, prevented the increase in mRNA expression of not only U-II and GPR14 but also NF-κB, and IL-1β in the lung, as well as edema, alveolar wall thickness, and inflammatory cell infiltration [32]. In the rat endotoxemia model induced by LPS, it was reported that urantide was injected at i.v. doses of 0.2 mg/kg and then 1.2 mg/kg/h for 2 hours did not prevent the increase in MAP, HR, and renovascular resistance [36]. These authors suggested that UT did not mediate the renal abnormalities observed in non-hypotensive endotoxemic rats. Although neither was performed in a septic shock model, there are only two *in vivo* studies with SB-710411 in the literature. In a study conducted by Liu et al. [38] on a carbon tetrachloride-induced cirrhosis model in rats, it was shown that SB-710411 injected at a hypodermal dose of 1 µg/kg prevented the increase in plasma U-II levels and U-II and UT expression in the liver. In a study conducted in a myocardial I/R model in rats, Luo et al. [31] reported that SB-710411 injected at doses of 1 and 2 µg/kg (i.v.) prevented the changes in hemodynamic parameters such as a decrease in MAP and HR, ST-segment elevation in the electrocardiogram, serum lactate dehydrogenase and creatine phosphokinase-MB activities, and cardiac troponin I levels in addition to an increase in UT protein expression, as well as reducing myocardial infarct size, necrosis, interstitial edema, and inflammatory cell infiltration. Although studies have been conducted with UT antagonists urantide and palosuran in LPS-, LPS/D-galactosamine-, and CLP-induced endotoxic shock, acute liver failure, polymicrobial sepsis, and endotoxemia models in mice and rats [32-37], there is no study with using SB-710411 in these models.

The results of our previous studies indicated that systemic LPS challenge in rats results in (1) hypotension and tachycardia [6-13,39], (2) vascular hyporeactivity in the TA and SMA [7,9], (3) enhanced systemic and/or tissue levels of iNOS/NO [7-10], and (4) increased expression and/or activity of MyD88 [11], NF-κB p65 [11,39], IL-1β [12], and iNOS [6,7,10] in the TA, RA, SMA, PA, heart, kidney, brain, lung, and/or liver. In this study, the reduction in MAP and the rise in HR in response to LPS injection into rats over the 4-hour course of the experiment was also observed. Moreover, these changes were associated with (1) elevated serum levels of U-II and nitrite and (2) increased mRNA expression of TLR4, MyD88, NF-κB p65, IL-1β, and iNOS in the TA, RA, heart, and kidney tissues of rats with septic shock at 4 hours following LPS injection. According to the results of the studies conducted within the scope of this study, in the group injected with SB-710411 at a dose of 0.0001 mg/kg together with LPS, the decrease in MAP, but not HR, values 3 hours following LPS injection could be prevented. In addition, 5 of the rats in this group died at 4 hours following LPS injection, and MAP and HR recordings could not be obtained from the remaining 1 rat. In the group injected with SB-710411 at a dose of 0.001 mg/kg, it was found that the decrease in MAP, but not HR, values 2, 3, and 4 hours following LPS injection was prevented. When SB-710411 was injected into rats at a dose of 0.01 mg/kg, it was able to prevent LPS-induced hypotension and subsequent tachycardia without causing mortality. The observed dose-dependent effects on LPS-induced changes in MAP and thus HR are consistent with the results of studies reporting that SB-710411 can be a full agonist, antagonist, or partial agonist for UT in arteries isolated from various species and primarily responsible for the regulation of blood pressure [26,29,30]. In addition, although the increase in serum U-II levels was prevented in rats injected with SB-710411 at a dose of 0.01 mg/kg with LPS, no change was observed in the increased serum nitrite levels. The findings of this study are align with previous research on UT in cardiovascular responses [31,36] and also consistent with the results of studies on the effects of SB-710411 on the decrease in MAP [31], and the increase in plasma U-II levels [38] although it was not used in the rat model of septic shock induced by LPS. In addition, our findings are consistent with the preventive effect of urantide, another UT antagonist, on the increase in serum and liver U-II levels in the LPS/D-galactosamine-induced acute liver failure model in mice [34,35]. On the other hand, the inability of SB-710411 to diminish the increase in systemic NO formation caused by LPS is consistent with the results of studies showing that the formation and release of various vasodilator mediators, especially NO, from the endothelial cells of small vessels play a critical role in balancing the vasoconstrictor effect of U-II on smooth muscle cells [24,25]. Consequently, this study has shown for the first time that SB-710411, when injected into rats at the lowest effective dose of 0.01 mg/kg, can prevent LPS-induced hypotension and subsequent tachycardia, as well as increased systemic U-II formation and expression of U-II, GPR14, TLR4, MyD88, NF-κB, IL-1β, and iNOS in the cardiovascular and renal tissues, without causing mortality or decrease in systemic NO formation.

This study provides evidence for the first time that SB-710411, presumably owing to not only its antagonistic effect for UT but also by suppressing both U-II/UT- and TLR4/MyD88/NF-κB/iNOS/NO-related signaling pathways accompanied by an increase in pro-inflammatory cytokine expression, prevents the hypotension and tachycardia in response to systemic LPS challenge in the rat model of septic shock. The results of the current study suggest that the use of systemically applicable selective UT antagonists, such as SB-710411, as a potential antihypotensive, antiarrhythmic, and antiinflammatory drug candidate could be a promising approach for the treatment of septic shock due to bacterial infections. Our data also suggest that inhibition of additional nonspecific targets other than UT also contributes to the preventive effect of SB-710411 on the hemodynamic changes induced by LPS. Therefore,

the effects of SB-710411 on the nonspecific targets and/or pathways should be investigated further. While UT antagonism can prevent LPS-induced hypotension and tachycardia, exploring the long-term effects of treatment with SB-710411 using single or repeated doses during septic shock would also provide important information regarding its sustained therapeutic benefits. Further detailed in vitro, in vivo, and ex vivo studies regarding the possible side and toxic effects of SB-710411 in vital organs such as the heart and kidney, as well as the brain, lung, and liver, will provide scientific support for the clinical use of selective UT antagonists in the treatment of septic shock for various reasons. In addition, since the number of preclinical and clinical studies on the treatment approaches using UT antagonists like SB-710411, especially for the treatment of systemic inflammatory diseases associated with hemodynamic disturbances, has been increasing in recent years, it can be suggested that the translational potential of these antagonists in clinical settings for the management of various diseases including septic shock.

A limitation of this study is that whether SB-710411 exerts its beneficial effects directly by inhibiting the expression of U-II/UT and/or indirectly by suppressing increased expression and/or activity of the TLR4, MyD88, NF-κB, IL-1β, and iNOS in the cardiovascular and renal tissues during LPS-induced septic shock. Therefore, additional experiments need to be conducted in UT knockout septic rats to elucidate the molecular mechanisms of the beneficial effects of SB-710411 in the septic shock model. Since the data primarily focused on mRNA expression rather than levels of unphosphorylated and/or phosphorylated U-II, UT, TLR4, MyD88, NF-κB, IL-1β, and iNOS proteins, further detailed experiments at the molecular level are also required to explore the effects of SB-710411 on the interactions between these signaling pathways in the specific cells of cardiovascular and renal tissues expressing UT such as myocardial, endothelial, vascular, and renal tubular cells isolated from septic rats. Since animal models provide basic information about the pharmacodynamic and pharmacokinetic properties of drugs as well as their toxicities that other methods cannot replace, animal models are essential in developing new therapeutic approaches for septic shock despite several limitations. Hence, considering the critical role of U-II/UT pathway during endotoxemia, re-evaluation of the effects of SB-710411 in more clinically relevant hyperdynamic and hypodynamic animal models may provide a novel therapeutic target for the treatment of patients with septic shock.

Informed consent
The authors declare that no humans were used in this study.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Interest of conflict
The authors declare no conflict of interest/competing interests.

Author' contribution
BT conceptualized and conceived the research design, analyzed the data, and drafted the manuscript. BT, MA, SPS, and EI carried out the experiments. All authors read and approved the final manuscript.

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