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1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU), a soluble epoxide hydrolase inhibitor, lowers L-NAME-induced hypertension through suppression of angiotensin-converting enzyme in rats

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Abstract. – OBJECTIVE: This study evaluated the efficacy of the soluble epoxide hydrolase (sEH) inhibitor, TPPU on chronic NG-Nitro L-arginine methyl ester (L-NAME)-induced hypertension in rats and its effects on plasma Angiotensin II (Ang II), cardiac Angiotensin-converting enzyme (ACE) and Angiotensin II receptor type 1 (AT1R) expressions.

MATERIALS AND METHODS: Forty Sprague Dawley rats were divided into 5 groups. Two groups served as control and received orally either vehicle or TPPU (3 mg/kg) for five weeks. The other three groups were given L-NAME (50 mg/kg/day) in drinking water for five weeks. Two weeks after the L-NAME treatment, animals received orally either saline or TPPU (3 mg/kg/day) or lisinopril (10 mg/kg/day) daily for 3 weeks. Blood pressure (BP) was measured weekly. At the end of the experiment, plasma Ang II, cardiac ACE and AT1R protein and gene expressions were determined.

RESULTS: L-NAME caused a significant increase in BP of the animals. TPPU and lisinopril resulted in normalization of L-NAME-induced hypertension. They also caused a significant reduction in Ang II and ACE protein and gene expressions compared to L-NAME and vehicle-treated animals.

CONCLUSIONS: This study demonstrates that TPPU effectively lowers L-NAME-induced hypertension in rats. The mechanism of its antihypertensive effect is likely mediated by the suppression of ACE gene and protein expression, leading to a lower Ang II level.

Key Words:
Soluble epoxide hydrolase inhibitors, TPPU, L-NAME induced hypertension, Angiotensin converting enzyme, Angiotensin type 1 receptors.

Introduction

The administration of soluble epoxide hydrolase inhibitors increases the tissue levels of epoxyeicosatrienoic acids (EETs) and lowers blood pressure in several animal models of hypertension[1-3]. Blood vessel dilatation in response to EETs was not inhibited by L-NAME[4,5]. Scholars[6,7] have shown that sEH inhibitors failed to lower blood pressure in L-NAME-induced hypertension and L-NAME abolished the antihypertensive effect of the sEH inhibitors in angiotensin-dependent hypertension. Chronic administration of L-NAME leads to activation of the renin angiotensin system (RAS)[8]. Since sEH inhibitors are more effective in angiotensin-mediated hypertension, they are likely to be effective in chronic L-NAME induced hypertension. Inhibition of sEH resulted in significant reduction of plasma and renal angiotensin II levels[9-11]. Therefore, suppression of Ang II levels is the main factor mediating the antihypertensive effects of sEH inhibitors. In this study,

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we evaluated the efficacy of the sEH inhibitor, 1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea (TPPU), in L-NAME-induced hypertension and its effects on Ang II, ACE and AT1R.

Materials and Methods

Animals

Male Sprague Dawley rats weighing 190-220 g were obtained from the animal house facility at college of pharmacy, King Saud University, Riyadh Saudi Arabia. Rats were acclimatized for one week under a temperature of 22-25ºC and 12-hour light-dark cycles. They were given standard chow and allowed free access to water. All studies followed the guidelines of the National Committee for Ethics and Care of Experimental Animals, King Saud University, Riyadh. The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies[12].

Chemicals

TPPU was purchased from Cynthia labs (Davis, CA, USA), and L-NAME from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The reagents and kits for Western Blot were purchased from Abcam (Burlingame, CA, USA). Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and membranes were purchased from Bio-Rad (Des Plaines, IL, USA). PCR kits and reagents were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Experimental Design

A total of 40 Sprague Dawley rats were divided randomly into 5 groups (n=8). One group (Control group) received orally Tween 80 as vehicle for 3 weeks. Another group (TPPU group) received TPPU (3 mg/kg daily by oral gavage) for five weeks. The other three groups were given L-NAME (50 mg/kg daily in drinking water) for 5 weeks to induce hypertension. Two weeks after the L-NAME treatment, animals received Tween 80; control vehicle group) or TPPU (3 mg/kg; L-NAME-TPPU group) or Lisinopril (10 mg/kg; L-NAME-LISINO) for 3 weeks.

Blood pressure (BP) was measured by non-invasive tail-cuff technique (Model MK-2000, Murom chi Kikai Co., Ltd., Tokyo, Japan). Average basal blood pressure measurements were done before and weekly throughout the experiment. At the end of the experimental periods, the animals were anesthetized, and 5 ml of blood was collected from the heart in EDTA-containing tubes. Blood was immediately centrifuged, and plasma was stored at -80°C until the time of analysis. The animals were sacrificed and the hearts were excised. The heart weight was measured, and its heart weight index was determined (heart weight in mg/body weight in g). Half of the heart was rapidly put in liquid nitrogen and kept frozen at -80°C until the time of analysis.

Measurement of Plasma Angiotensin II

Plasma Ang II was measured by Enzyme-Linked Immunosorbent Assay (ELISA) using angiotensin II EIA kit from Sigma-Aldrich (St. Louis, MO, USA). Assay procedure was done according to manufacturer's protocol.

Analysis of ACE and AT1R Protein Expressions by Western Blotting

After cardiac tissue homogenization, Radio Immunoprecipitation Assay (RIPA) lysis buffer was used to extract and isolate proteins according to the manufacturer's instructions. Forty micrograms of extracted protein were electrophoresed by 10% Sodium Dodecyl Sulfate-Polyacrylamide Gel (SDS-PAGE) electrophoresis. The separated proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% bovine serum albumin and then incubated overnight with polyclonal anti-ACE, anti-AT1R antibodies (Abcam, Cambridge, MA, USA) at 4°C. The membranes were then washed and incubated with a horseradish peroxidase-conjugated secondary antibody at 37°C for 1 hour. Stained bands were visualized by enhanced chemiluminescence. Protein bands were quantified using image processing software.

Quantitation of ACE and AT1R Gene Expression

RNA extraction was performed using RNA extraction kit (Analytik Jena AG, Germany) according to manufacturer instructions. First strand Complementary DNA (cDNA) was prepared from total RNA extracted from each sample by using cDNA reverse transcription kit (Thermo Fisher Scientific, Grand Island, NY, USA), following the manufacturer protocol. Quantitative analysis of mRNA expression was performed by real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR; Applied Biosystems, Foster City, CA,

USA) using the cDNA synthesized from the heart tissue specimens. The sequence of primers was as follows: ACE: (forward)5′-CGAACCCACTTT-GATGCTG-3', (reverse) 5′-GAGAAGTGGAG-GTGGATGGTCT-3'; AT1R: (forward) 5'-TG-GGAATA1TTGGGAACAGC-3', (reverse) 3'-GTGAATATTTGGTGGGGAAC-5'. GAPDH: (forward) 5'-TGGCATGGACTGTGGTCATG -3', (reverse) 5'-TGGGTGTGAACCACGAGAAA -3'.

The PCR protocol involved initial heating to 95°C for 300 s for denaturation. This is followed by 40 thermal cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 20 s. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as a reference gene. The Cycle threshold (CT) for the target sequence among the various treatments was compared for relative quantification. Relative gene expression was calculated as described by Livak[13]. First ΔCT value was obtained by subtracting CT of the reference gene from samples CT (CT experimental – CT reference gene). ΔΔCT was determined using the equation:

$$\Delta\Delta CT - target = (\Delta CT\ treatment - \Delta CT\ control)$$

Fold change of gene expression was calculated to be equal to 2^(-ΔΔCT).

Statistical Analysis

Values are expressed as means +/- SEM. Statistical analysis of the differences between groups was done using one-way analysis of variance (ANOVA) followed by Tukey Kramer test. A probability value of less than 0.05 was considered significant.

Results

Effect of TPPU on L-NAME-Induced Hypertension

The administration of L-NAME (50 mg/kg daily orally; p.o) resulted in a progressive rise in BP starting after one week of treatment (Figure 1). BP increased from 89.6 ± 2.5 mmHg before treatment to 157.3 ± 2.7 mmHg after 5 weeks of treatment. This increase was significantly different compared to control vehicle treated animals (p<0.0001). TPPU (3 mg/kg, p.o) and lisinopril (10 mg/kg, p.o) were started after two weeks of L-NAME administration. TPPU caused significant reduction in the systolic BP within one week of treatment. BP was restored to bassline value (88.6 ± 5.0 mmHg) in the third week of TPPU treatment (p<0.0001). Lisinopril had a similar effect. No appreciable change in blood pressure

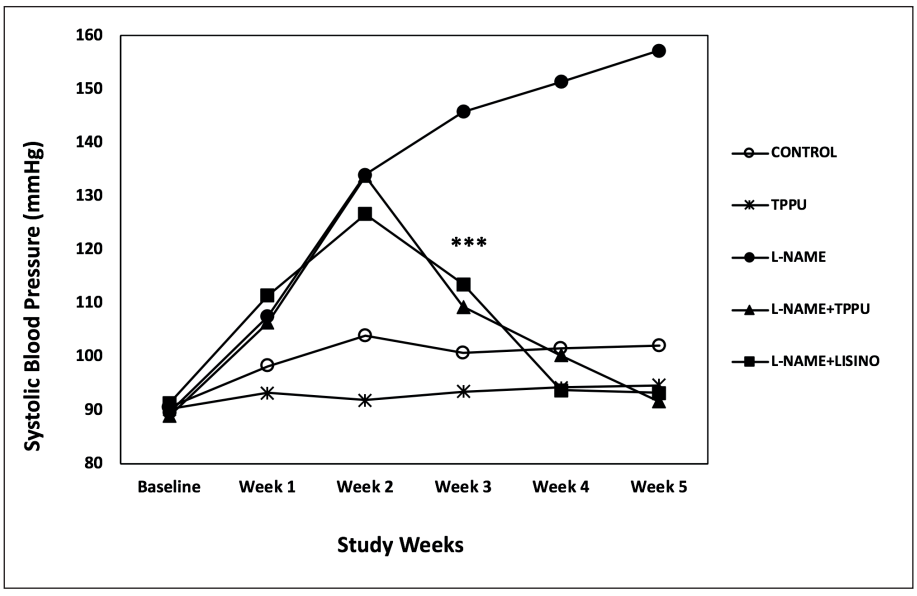

Figure 1. The effects of TPPU and lisinopril on systolic blood pressure in L-NAME-induced hypertension in rats. Animals were given L-NAME (50 mg/kg daily in drinking water) for 5 weeks to induce hypertension. Two weeks after the L-NAME treatment, animals received Tween 80; control vehicle group) or TPPU (3 mg/kg; L-NAME-TPPU group) or Lisinopril (10 mg/kg; L-NAME-LISINO) for 3 weeks. In parallel experiments animals were also given TPPU alone (TPPU group). Blood pressure was monitored weekly. Data is presented as means ± SEM of systolic blood pressure; mmHg (n=8). ***Indicates a significance level at p<0.0001 compared to the L-NAME group.

was observed in the control vehicle treated animals (data not shown). Animals treated with TPPU alone had low blood pressure compared to baseline value for control vehicle treated animals (Figure 1). There were no significant differences in heart weights among animal groups.

Effects of L-NAME, TPPU, and Lisinopril on plasma Ang II level

As shown in Figure 2, the mean of plasma level of Ang II in the control (vehicle treated) group was 1.44± 0.33 ng/ml. Animals treated with L-NAME and vehicle exhibited significant reduction in Ang II level to a mean of 0.79±0.07 ng /ml (p<0.05). TPPU and vehicle treated animals showed similar significant reduction in the level of Ang II (p<0.05). When TPPU was given with L-NAME, Ang II level decreased to 0.42±0.11 ng/ml, compared to the L-NAME treated g (p<0.05). In the lisinopril-treated animals, plasma Ang II level was significantly decreased to 0.28±0.06 ng/ml compared to the L-NAME treated animals' group (p<0.01).

ACE Gene and Protein Expression

Figure 3 shows that ACE mRNA expression increased in the L-NAME group by 1.34-fold (non-significant). TPPU alone did not cause any significant change in ACE gene expression. When TPPU was given with L-NAME, it suppressed ACE mRNA expression to 59% compared to control group (p<0.01). Lisinopril decreased ACE gene expression to 41% compared to control group (p<0.01).

ACE protein expression was increased by 1.38-fold in the L-NAME group compared to the control group (Figure 4). TPPU (alone) increased ACE protein expression by 1.31-fold compared to control group. These changes in ACE protein expression were not significant. When TPPU was given with L-NAME, it decreased ACE protein expression to 67% of the level in the control group (p<0.05). Lisinopril also caused a significant decrease (62%) in ACE protein expression (p<0.01).

AT1R Gene and Protein Expressions

L-NAME caused mild suppression of AT1R mRNA expression. TPPU (alone) did not cause a significant change in AT1R gene or protein expression. When TPPU was given with L-NAME, it led to a mild non-significant increase in AT1R gene and protein expression. Lisinopril caused a similar non-significant effect.

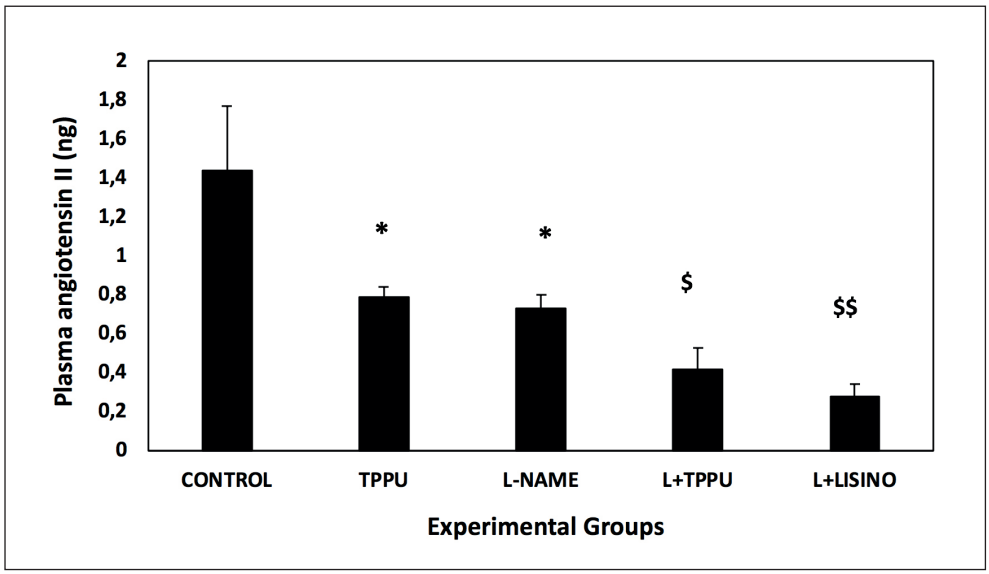

Figure 2. Effects of L-NAME, TPPU and Lisinopril on plasma angiotensin II levels. Animals were given L-NAME (50 mg/kg daily in drinking water) for 5 weeks to induce hypertension. Two weeks after the L-NAME treatment, animals received Tween 80; control vehicle group) or TPPU (3 mg/kg; L-NAME-TPPU group) or Lisinopril (10 mg/kg; L-NAME-LISINO) for 3 weeks. In parallel experiments animals were also given TPPU alone (TPPU group). Data are expressed as mean + SEM (no=8). Blood was withdrawn from animals, plasma was prepared and stored at – 80 °C until the time of analysis. *Indicates a significance level at p < 0.05 compared to control group. $ Indicates a significance level at p<0.05 compared to L-NAME group. $$ Indicates a significance level at p < 0.01 compared to L-NAME group.

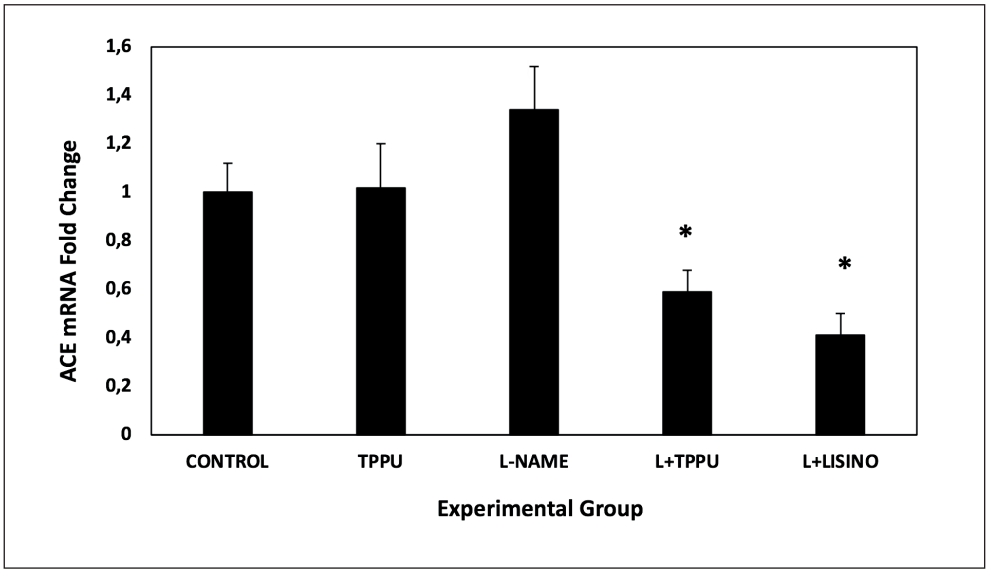

Figure 3. Effects of L-NAME, TPPU and lisinopril on ACE mRNA expression in rats. Gene expression is shown as a fold change relative to Control group. Animals were given L-NAME (50 mg/kg daily in drinking water) for 5 weeks to induce hypertension. Two weeks after the L-NAME treatment, animals received Tween 80; control vehicle group) or TPPU (3 mg/kg; L-NAME-TPPU group) or Lisinopril (10 mg/kg; L-NAME-LISINO) for 3 weeks. In parallel experiments animals were also given TPPU alone (TPPU group). At the end of treatment, animals were killed and cardiac tissue specimens were taken for measurement of ACE protein expression by Western Blot analysis. Data are expressed as mean + SEM (n=8). *Indicates a significance level at $p < 0.01$ compared to the control group.

Discussion

This study showed that TPPU caused significant BP lowering effect in L-NAME-induced hypertension. The reduction in BP was observed in the first week of treatment returning to normal levels after two weeks of TPPU therapy. It was previously reported that the sEH inhibitor N-adamantyl-N-dodecylurea (ADU) failed to lower BP in the L-NAME-induced experimental model of hypertension[6]. Another study[7] showed that the antihypertensive effect of sEH inhibitor, cis-4-(4-(3-adamantan-1-ylureido) cyclohexyloxy benzoic acid (c-AUCB) in the angiotensin-dependent hypertension in Ren-2 transgenic rats was completely prevented by the simultaneous administration of L-NAME. These investigators[6,7] suggested that the failure of sEH inhibitors to lower BP might indicate that the antihypertensive action of these compounds is dependent on nitric oxide (NO) pathway. In both studies, L-NAME was used for 3-7 days and sEH inhibitors were given for 3 days only. Therefore, their findings probably reflect the effects of short-term sEH inhibition in acute L-NAME hypertension model. Acute L-NAME administration was reported to cause profound vasoconstriction of renal vessels together with marked reduction of renal blood flow and glomerular filtration rate[14]. This was associated with significant decrease in sodium excretion. Although short-term sEH inhibition lowered BP in angiotensin-dependent hypertension but no appreciable change in the Ang II level in kidney was observed[9,15]. This indicates that the BP lowering effect of short-term sEH inhibition is not mediated via suppression of Ang II. The early antihypertensive effect of sEH inhibitors occurs through diuretic and natriuretic actions[16]. sEH inhibitors produce protective physiological effects *via* increasing endogenous level of Epoxyeicosatrienoic acids (EETs). EETs have been reported[16,17] to decrease sodium reabsorption in the renal proximal tubules by blocking the sodium–hydrogen exchanger. Previous scholars[18] have revealed that EETs reduce sodium reabsorption in the renal cortical collecting ducts by blocking epithelial sodium channels. These findings suggest that BP lowering effect of sEH inhibitors is most likely occur *via* increased sodium excretion and diuresis. L-NAME may likely antagonize these properties of EETs on the kidney preventing BP lowering effect of sEH inhibitors. This hypothesis is supported by another study showing that L-NAME reduced glomerular filtration rate and urinary sodium excretion in angiotensin-dependent hypertension which was not improved with

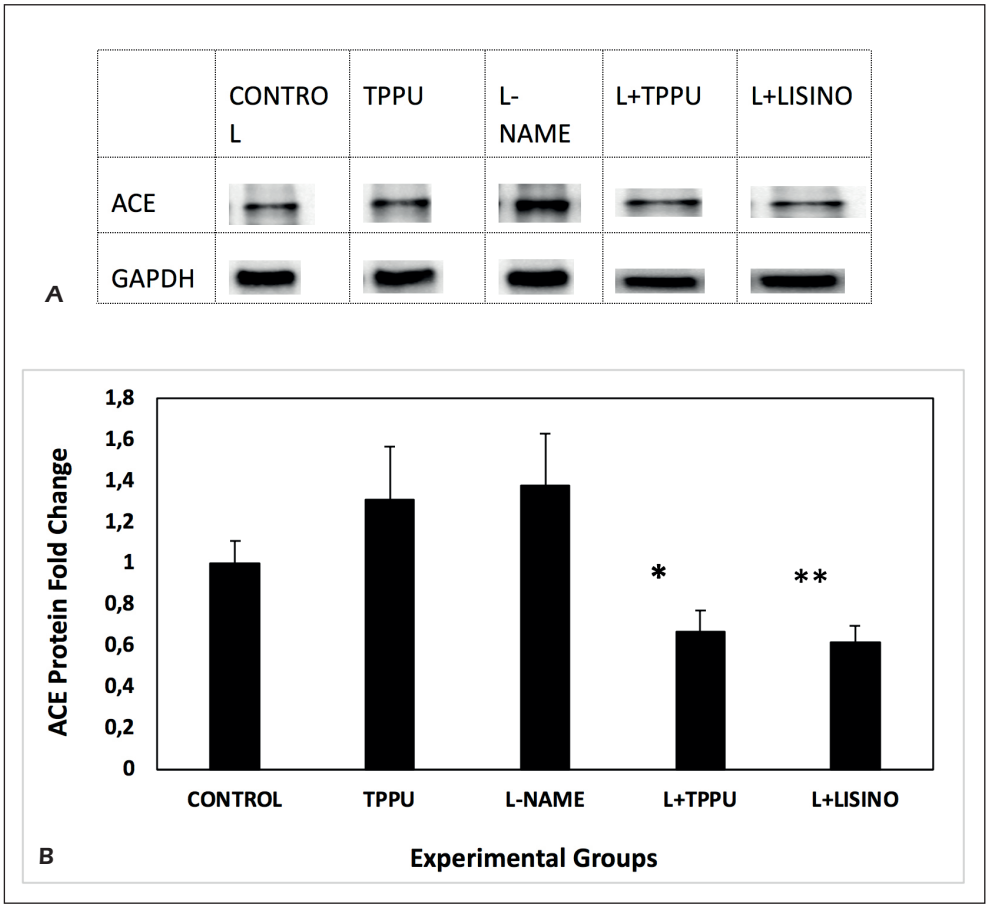

Figure 4. Effects of L-NAME, TPPU and lisinopril on ACE protein expression. **A**, Representative Western blots of ACE protein expression from the three groups. Protein expression is quantified relative to GAPDH protein signals. **B**, ACE protein expression fold change relative to control group. Animals were given L-NAME (50 mg/kg daily in drinking water) for 5 weeks to induce hypertension. Two weeks after the L-NAME treatment, animals received Tween 80; control vehicle group) or TPPU (3 mg/kg; L-NAME-TPPU group) or Lisinopril (10 mg/kg; L-NAME-LISINO) for 3 weeks. Data are expressed as mean ± SEM (n=6). * Indicates a significance level at $p < 0.05$ compared to the control group. ** Indicates a significance level at $p < 0.01$ compared to the control group.

the administration of cis-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyl-oxy]-benzoic acid (c-AUCB)[7].

Chronic administration of L-NAME has been shown to produce structural changes in the kidneys together with activation of the renin-angiotensin system[14,19,20]. ACE inhibitors or Ang II receptor blockers reversed the L-NAME-induced hypertension[21,22]. In the current study the administration of TPPU for three weeks was highly effective in lowering BP in chronic L-NAME induced hypertension. Failure of sEH inhibition to lower BP in the presence of L-NAME, reported previously, is probably due to the acute effects of L-NAME on renal blood flow and glomerular filtration[7].

In the present study L-NAME caused significant suppression of plasma Ang II level. Concurrent administration of TPPU with L-NMAE caused further significant suppression of plasma level of Ang II. This suggests that the BP lowering effect of TPPU in chronic L-NAME-induced hypertension is at least partly due to suppression of plasma and possibly tissue Ang II levels. c-AUCB, the sEH inhibitor, has been reported[9,10] to produce significant suppression of elevated plasma and intrarenal Ang II levels in angiotensin-dependent hypertension model in rats. The suppression of Ang II level with TPPU treatment in the current study occurred despite significantly suppressed levels by L-NAME. This emphasizes the idea that sEH inhibitors lower BP in various animal models through suppression of Ang II production. However, in view of the complexity of pathophysiological pathways involved in the L-NAME induced model of hypertension, other

mechanisms, such as anti-inflammatory and suppression of oxidative stress by sEH inhibitors may be involved in the observed anti-hypertensive effect of TPPU in the current investigation.

Chronic L-NAME administration caused a slight non-significant increase in cardiac ACE mRNA and ACE protein expressions. When TPPU was given together with L-NAME, it resulted in significant reductions in ACE mRNA and ACE protein expressions when compared to L-NAME alone. To our knowledge, this is the first study that evaluates ACE gene and protein expression in relation to treatment with sEH inhibitors in L-NAME induced hypertension. The findings of this study indicate that suppression of Ang II level is likely to be due to suppression of ACE. Chronic treatment of angiotensin-dependent hypertension with the sEH inhibitor c-AUCB resulted in reduction of plasma and kidney Ang II/Ang I ratio, reflecting suppression of ACE activity[9]. Plasma renin activity was unchanged or increased following treatment with EETs or sEH inhibitors[9,23]. This further supports the idea that sEH inhibitors lower plasma Ang II by suppressing ACE. TPPU caused non-significant increase in AT1 receptor gene and protein expressions. This may be a response to decreased plasma and tissue Ang II levels[24].

Conclusions

The results of this study demonstrate that the sEH inhibitor TPPU effectively lowers L-NAME-induced hypertension indicating that its effect is independent of NO-pathway. The mechanism of its antihypertensive effect is likely mediated by suppression of ACE gene and protein expression, leading to lower Ang II level. TPPU had minimal effect on AT1 receptor. Since L-NAME induced hypertension model involves other biochemical and renal function derangements, future studies will be carried out to explore the role of sEH inhibitors in the L-NAME induced biochemical and renal function derangements.

Acknowledgments

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Conflict of Interests

The authors declare that they have no conflict of interest.

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