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The effects of novokinin, an AT2 agonist, on blood pressure, vascular responses, and levels of ADMA, NADPH oxidase, and Rho kinase in hypertension induced by NOS inhibition and salt

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Background/aim: The effects of AT_2 receptor agonist novokinin on blood pressure, eNOS, NADPH oxidase, protein arginine methyltransferases (PRMTs), and Rho kinase in hypertension were investigated. Furthermore, in isolated thoracic aorta rings, contractile and dilator responses were studied.

Materials and methods: To develop hypertension, L-NAME was administered intraperitoneally and salt was given with tap water (1%) for 4 weeks. Novokinin was administered intraperitoneally for the last 2 weeks. Blood pressures were measured using the tail-cuff method and enzyme levels by real-time polymerase chain reaction in aortic tissues.

Results: Blood pressure increased significantly in hypertensive rats. Novokinin reduced the blood pressure in the hypertensive group. While the contractile responses to increasing doses of angiotensin II were increased, vascular reactivity (Emax) and sensitivity (EC50) to acetylcholine were decreased in hypertensive rats. In novokinin-treated hypertensive groups, the EC50 value decreased and the Emax value for acetylcholine significantly increased. The levels of Rho kinase and PRMT expression increased and the level of eNOS expression decreased in the hypertensive group. In novokinin-treated rats, ADMA, NADPH oxidase, and Rho kinase tended to decreased, but these changes did not reach statistical significance.

Conclusion: Although further studies are needed to determine its effectiveness, the AT2 agonist novokinin may be a novel agent that is promising in terms of protective effects for the treatment of hypertension.

Key words: ADMA, AT2 receptor, hypertension, nitric oxide, novokinin, Rho kinase

1. Introduction

Hypertension remains a challenging clinical problem. Although recent advances have been made in the treatment of hypertension, the majority of patients treated with antihypertensive agents not optimally controlled. Additional pharmacologic treatments could ameliorate this situation (1). There are many responsible mechanisms that cause essential hypertension. Endothelial dysfunction characterized by a reduction in the bioavailability of nitric oxide (NO) and increased plasma volume induced by excessive salt intake may play important roles in the development and persistence of hypertension (2).

The renin–angiotensin–aldosterone system (RAAS) is an important pharmacologic target because it plays a substantial role in the development of hypertension-related organ damage. Although the RAAS can be blocked by angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs), these pharmacologic manipulations do not completely suppress the RAAS and the development of target organ damage because ACE inhibitors and ARBs cause the reduction of negative feedback and lead to a compensatory increase in renin synthesis (3). Therefore, additional novel methods for RAAS blockade may provide better control of hypertension and prevention of organ damage.

Angiotensin II (Ang-II) induces vasoconstriction, aldosterone secretion, sympathetic activation, renal sodium reabsorption, and cell growth via the activation of AT1 receptors. Increasing experimental evidence demonstrates that Ang-II type-2 receptor (AT2-R) activation elicits vasodilatation, sodium excretion, blood pressure reduction, and antigrowth, proapoptotic, and

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antiinflammatory effects and thereby counteracts the effects of AT1 receptors (4,5). It has also been shown that AT2-R regulates RAAS activity by inhibiting renin biosynthesis (6). AT2-R activation leads to stimulation of bradykinin, nitric oxide, and prostaglandin production (7). Due to this effect, AT2 may enhance renal perfusion. These findings imply that AT2-R may be a potential novel therapeutic approach in hypertensive conditions.

The RAAS is associated with many enzymes and molecules that contribute to the development and maintenance of hypertension, such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, Rho kinase, asymmetric dimethylarginine (ADMA), and endothelial nitric oxide synthase (eNOS). Elevated plasma concentrations of ADMA, an endogenous nitric oxide synthase inhibitor, were reported in cardiovascular diseases, such as hypertension, hypercholesterolemia, coronary artery disease, and acute coronary syndrome, as well as diabetes mellitus, all of which are associated with endothelial dysfunction (8). ADMA is generated by protein methyltransferase (PRMT) (9). NADPH oxidase enzymes are the major sources of reactive oxygen species in the vessel wall. Ang-II activates NADPH oxidase via AT_1 receptor and leads to endothelial dysfunction. Rho kinase induces the enhancement of vascular tone and decreases the blood flow indirectly through negative effects on eNOS activity (10). Oxidative stress-related parameters such as NADPH oxidase, Rho kinase, and ADMA are important pharmacological targets for the treatment of cardiovascular diseases.

Novokinin is a new potent antihypertensive peptide acting through the AT_2 receptor. Its effect is not known on blood pressure in N-[omega]-nitro-L-arginine methyl ester (L-NAME)- and salt-induced hypertension. According to our knowledge, the effects of novokinin on eNOS, NADPH oxidase, ADMA, and Rho kinase in hypertension are unclear, as are the ways in which it affects vascular functions (Ang-II and α_1-adrenergic-induced contractions, acetylcholine (ACh)-mediated dilator responses).

Many studies have revealed that the interaction between NO and the autonomic nervous system plays an important role in the regulation of the cardiovascular system (11). The cholinergic system is active in balancing arterial hypertension. The cholinergic neurotransmitter ACh may induce eNOS enzyme.

In this study, to determine the contributions of NADPH oxidase, Rho kinase, ADMA, PRMT, and eNOS and to investigate the effect of an AT2-R agonist (novokinin) on these parameters and systolic blood pressure in hypertension, Sprague Dawley male rats were given L-NAME and salt. Additionally, α1-adrenergic contractions, ACh-induced relaxation, and the roles of AT2-R and Rho kinase in Ang-II-induced contractions in vessels in all groups were studied.

2. Materials and methods

2.1. Chemicals

Phenylephrine (Phe), ACh, L-NAME, novokinin, and Ang-II were purchased from Sigma Aldrich (St. Louis, MO, USA). All drugs were dissolved in saline.

2.2. Animals and treatments

Twenty-eight Sprague Dawley male rats, weighing 240–280 g, were housed in quiet rooms with a 12-h light/dark cycle (0700–1900 hours) and allowed a commercial standard rat diet and water ad libitum. The experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (DHEW Publication (NIH) 8523, 1985). This study was approved by the relevant institutional animal ethical committee (211-5-79).

Rats were divided into four groups. There were 7 rats in each group. L-NAME and salt were given to rats for development of hypertension. L-NAME was injected intraperitoneally (40 mg/kg per day, once a day) and salt (1%) was administered with drinking water. Novokinin (0.1 mg/kg per day) was administered intraperitoneally for 2 weeks. 1) Control group: Saline was administered at 0.5 mL intraperitoneally per day for 4 weeks. 2) Novokinin: After physiological serum (0.5 mL per day), novokinin was given during the last 2 weeks intraperitoneally. 3) Hypertension (HT) group: L-NAME and sodium chloride were administered for 4 weeks. 4) Hypertension (HT) + novokinin group: L-NAME and sodium chloride were administered for 4 weeks, and also novokinin was given in last 2 weeks intraperitoneally (Figure 1).

2.3. In vivo studies

Systolic blood pressure (SBP) was measured by tail-cuff method (MAY BPHR 9610-PC, Commat Ltd., Ankara, Turkey) at the beginning of the protocol and the 14th and 28th days of experiments, as described previously in restrained animals (12). Five measurements were taken for each rat and then the average thereof was calculated.

2.4. In vitro studies

The freshly harvested thoracic aortas of rats decapitated under urethane anesthesia were cleaned from fat and connective tissues. Rings, each approximately 0.4 cm long, were prepared from a segment of thoracic aorta and mounted on FDT 05 (MAY Force–Displacement Transducer) MP 36 (BİOPAC) in temperature-controlled baths (37 °C) of 20 mL containing Krebs Henseleit buffer (mmol/L: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11; pH 7.4) and oxygenated with a 95% O_2 + 5% CO_2 mixture. Vessels were equilibrated for 60 min at 2.0 g resting tension, with changes of bathing fluid every 15 min.

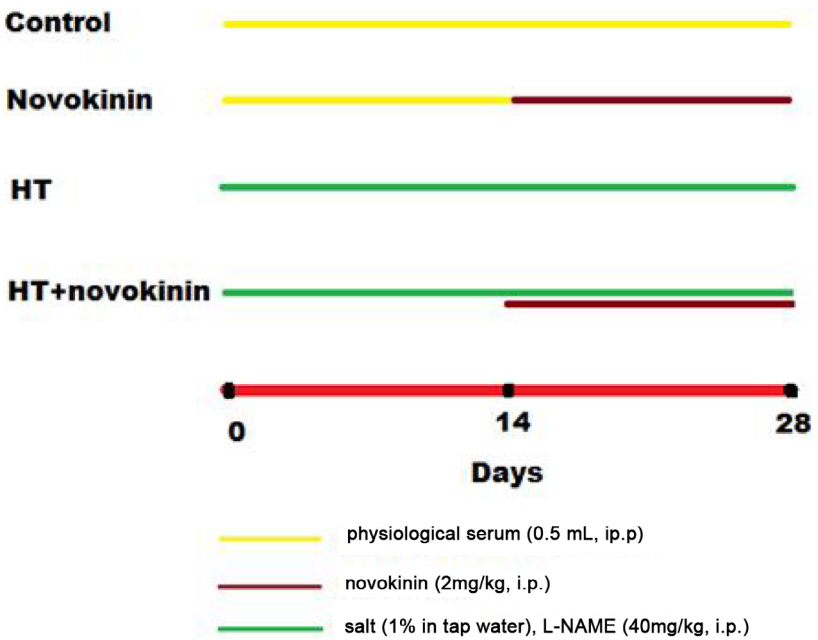

Figure 1. Experimental protocol.

The presence of the endothelium was tested by application of ACh. The dose–response curve of Phe was determined by the application of increasing doses of Phe (from 10^{-9} to 10^{-4} M) and a submaximal dose of Phe was obtained. After the contraction of the submaximal dose of Phe, at the plateau of the contraction response to Phe, doses of Ach were applied (from 10^{-9} to 10^{-5} M) and the dose-response curve of Ach was obtained. The 50% maximal effective concentration (EC50) and Emax (maximal contraction for Phe and maximal relaxation for Ach) values of Phe and ACh were calculated. To investigate the roles of AT1 and AT2 receptors and the Rho kinase pathway, Ang-II (10^{-11} to 10^{-6} M/L) dose–response curves were calculated for Phe (10^{-6}) precontracted vessels.

2.5. Real-time polymerase chain reaction

Gene expression was analyzed using real-time polymerase chain reaction (RT-PCR). Sections of the thoracic aorta weighting 20 mg were prepared for each sample, and the tissue was disintegrated by MAGNA laser. A genomic RNA purification kit was used for isolation of RNA (High Pure PCR Tissue RNA Kit, Cat. No. 12033674 0019). Using total RNA isolated from real-time PCR, cDNA synthesis was performed. Related parameters and mRNA sequences were amplified by PCR using specific primers. Related to the amplification kit (eNOS Assay ID 500 228, 502 043 ID Protein Arginine Methyltransferases (PRMT) Assay, Enzymatic Assay ID 502 973, 500 277 ID Rho Kinase Assay, Roche), the LightCycler and TaqMan Master Kit (Roche Cat. No. 04535286001 or 04735536001) were used. Amplifications (LightCycler, Roche) were performed in a RT-PCR device (LightCycler, Roche). Messenger RNA expression was normalized by beta-actin and expressed as the ratio of target to control value.

2.6. Data analysis

Data were analyzed using SPSS 21.0. One-way ANOVA was performed and post hoc multiple comparisons were made using the Tukey HSD test. Results are presented as mean ± SEM; $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. Blood pressure

In the HT and HT + novokinin groups, SBP increased at the 14th day significantly (Table 1). While there was no significant difference between 14th and 28th day blood pressure in the HT group, SBP decreased significantly in the HT + novokinin group at the 28th day ($P < 0.05$) (Table 1). There was no significant reduction in the novokinin group at the 28th day (Table 1).

3.2. Vasoconstrictor responses to phenylephrine

There was no significant difference between groups in vasoconstrictor responses (data not shown).

3.3. Vasodilator responses to acetylcholine

In the HT group, the ACh EC50 value was significantly higher than in the control group ($P < 0.05$; Table 2). The ACh Emax value was significantly lower in the HT group compared to the control group ($P < 0.05$). Novokinin application alone significantly reduced the EC50 value compared to the control. The ACh Emax value was significantly higher in the novokinin group than the control ($P < 0.05$). In the HT + novokinin group, Emax values increased significantly compared to the HT group ($P < 0.05$) (Table 2).

Table 1. Systolic arterial blood pressure at baseline and 14th and 28th days in all experimental groups.

Groups	Baseline	Day 14	Day 28
Control	107.09 ± 2.46	108.54 ± 3.58	109.07 ± 3.70
Novokinin	115.84 ± 5.05	110.43 ± 4.81	111.28 ± 4.16
HT	114.89 ± 2.59	152.07 ± 5.02 ^{ab*}	154.16 ± 8.72 ^{ab*}
HT + novokinin	116.69 ± 2.01	161.29 ± 7.01 ^{ab*}	134.00 ± 3.84 $^{abc\#}$

Results are represented as mean ± SE.
a, vs. control,
b, vs. novokinin
c, vs. HT
*, vs. baseline
#, vs. day 14, $P < 0.05$.

3.4. Concentration–response curves for Ang-II in Phe-precontracted vessels

In the HT group, the contractile responses to Ang-II at increasing doses (10^{-6} and 10^{-7}) were raised significantly compared with the control group. In the HT + novokinin group, contractile responses to Ang-II were decreased significantly compared with the HT and other treatment groups (Figure 2).

3.5. The expression levels of PRMT, NADPH oxidase, eNOS, and Rho kinase

In the HT group, while the expressions of PRMT, NADPH oxidase, and Rho kinase tended to increase, the expression of eNOS tended to decrease, but not significantly. The expressions of PRMT, NADPH oxidase, and Rho kinase in the HT + novokinin group tended to be lower than in the HT group, but these changes did not reach statistical significance (Figures 3–6, respectively).

Table 2. Relaxant responses to acetylcholine after phenylephrine (10^{-6} M) application at the end of 4 weeks in all groups.

	Relaxant responses in thoracic aorta	
Groups	Emax (%)	EC50 (–log10)
Control	53.17 ± 0.87	6.74 ± 8.22
Novokinin	80.50 ± 0.89^{a}	7.00 ± 8.30^{a}
HT	47.83 ± 0.54^{ab}	6.24 ± 8.15^{ab}
HT + novokinin	52.33 ± 0.98^{bc}	6.58 ± 8.22^{abc}

Results are represented as mean ± SE.
a, vs. control,
b, vs. novokinin
c, vs. HT, $P < 0.05$.

4. Discussion

In the present study, it was found that novokinin lowered the blood pressure, improved ACh dilator responses, reduced Ang-II-induced contraction in NOS inhibition and salt-coadministered hypertensive rats. In the novokinin treatment group, the expressions of Rho kinase, PRMT, and NADPH oxidase tended to be decreased and the expression of eNOS was increased in hypertensive rats.

Endothelial dysfunction, oxidative stress, increased sympathetic activation, and salt retention may play primary roles in hypertension. Salt-loading augments the degree of hypertension and lowers its starting time (13). It has been shown that chronic treatment of rats with L-NAME decreases NO production, causes impairment of endothelial vasodilator function, and results in a progressive increase of blood pressure (14). The current study was done by combining both methods: salt loading and NOS inhibition-induced hypertension.

The cholinergic neurotransmitter ACh may induce eNOS enzyme. eNOS is the enzyme most responsible for the production of vascular NO. Ang-II can increase oxidative stress, and oxidative stress may cause inactivity of NO and this result may provoke cardiovascular diseases (15,16). The negative effect of oxidative stress on NO may disturb the dilator (17) effect of cholinergic system, and thus the sympathetic system may become more dominant in the vascular system. ADMA, an endogenous and competitive NOS inhibitor, is an important molecule that decreases in endothelial NO biosynthesis; accordingly, ADMA may change the cholinergic activity in vessels in the opposite direction (18). Rho kinase is able to phosphorylate eNOS by inactivating the enzymes and may impair NO synthesis (19). The negative correlation between eNOS and Rho kinase may convert the balance between the cholinergic system and sympathetic system in blood pressure in the favor of the sympathetic system.

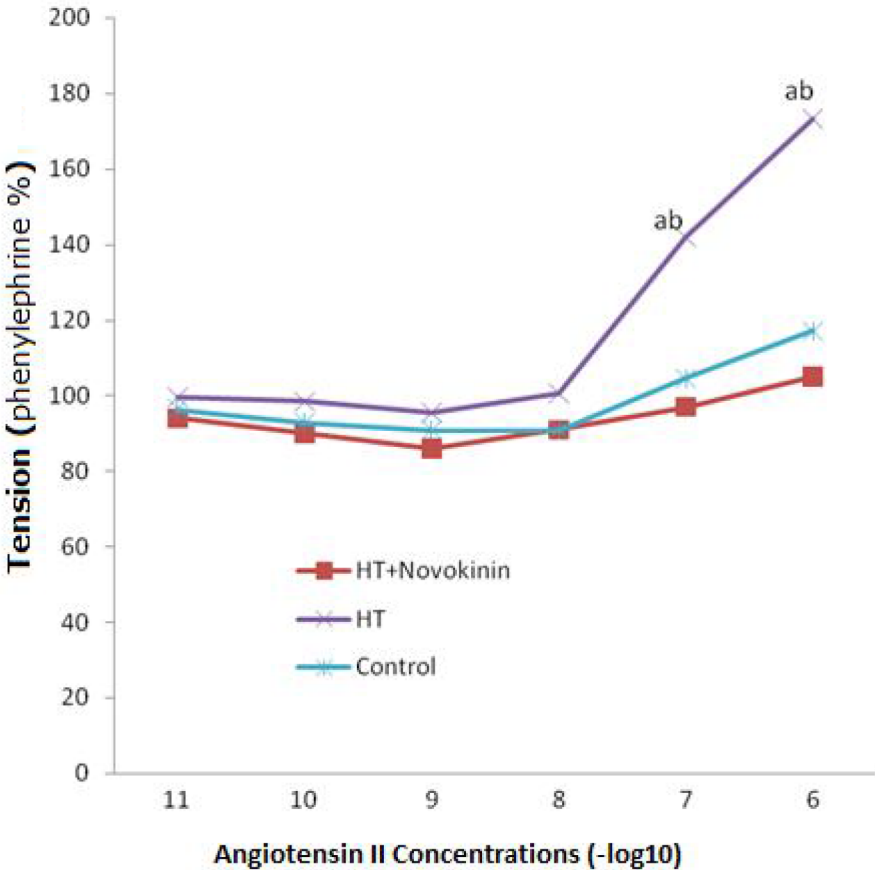

Figure 2. Cumulative dose–response curves produced by angiotensin II in phenylephrine-precontracted aortic ring preparations obtained from rats given HT and HT + novokinin and in control rats. a- vs. control. b- vs. HT + novokinin.

In the present study, the systolic blood pressure increased in the HT and HT + novokinin groups at the 14th day. Blood pressure was numerically higher on the 28th day. Novokinin significantly reduced the SBP compared with the hypertension group. Our findings are similar to previous studies demonstrating that novokinin significantly reduced SBP after oral administration at a dose of 0.1 mg/kg in spontaneously hypertensive rats (20,21). Its hypotensive activity was inhibited by PD123319, an AT2 antagonist, in hypertensive rats. Novokinin did not exhibit hypotensive activity in AT2 receptor-deficient mice (20,21). Yamada et al. (20) showed that novokinin caused vasodilatation in isolated mesenteric arteries. The vasodilator activity of novokinin was blunted by PD123319, indomethacin, and CAY10441 which is a prostaglandin I2 receptor antagonist. Furthermore, it was found that the vasorelaxing activity of novokinin could be inhibited by L-NAME (20,21). In this regard, prostaglandin I2 and NO contribute to the vasorelaxing activity of novokinin.

In our study, Phe-induced contractions were increased in the hypertension group, but not significantly so compared with the control. There was no difference between other groups in Phe contractile responses. In a study by Kalliovalkama et al. (22), L-NAME administration for 4 weeks did not change noradrenaline contractile responses compared with the control. Likewise, results of the addition of losartan to the L-NAME-administered group was not different from the control and L-NAME-administered groups (22). These results are in agreement with our study.

ACh generated significantly lower vasorelaxing response in the hypertension group than the control group in the current study. In a previous study, relaxations with ACh were significantly impaired in spontaneous hypertensive rats (SHRs) compared with the wild type (17). Administration of 10 mg and 100 mg of L-NAME significantly suppressed ACh-induced relaxation in aortic rings compared with the control (23). However, there was no difference between L-NAME-administered (10, 100 mg) groups.

We observed that contractile response to Ang-II increased at high doses of Ang-II (10^{-7} and 10^{-6}). Our

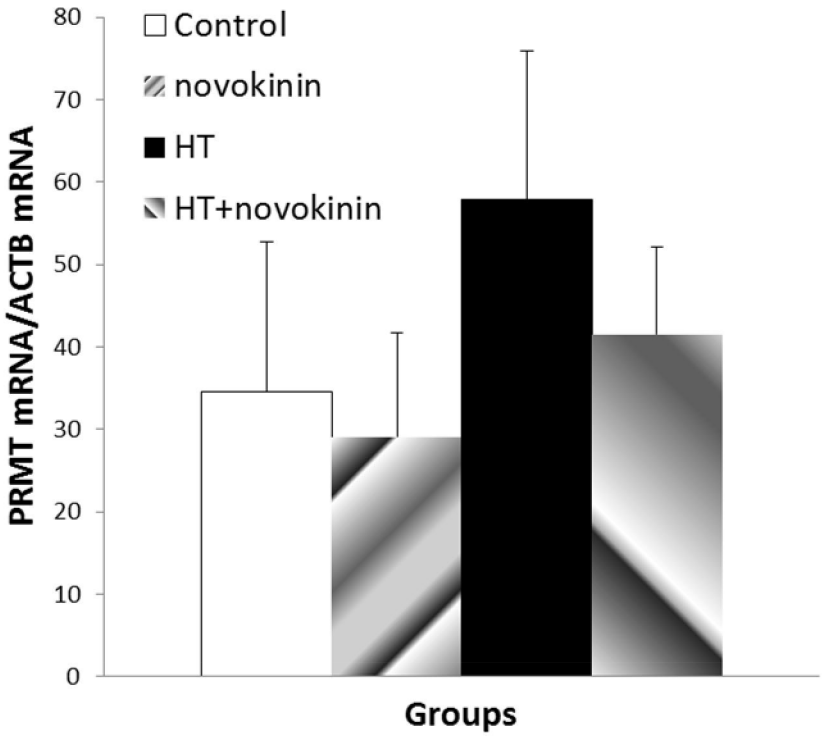

Figure 3. The expression level of PRMT in aortic tissue from all experimental groups.

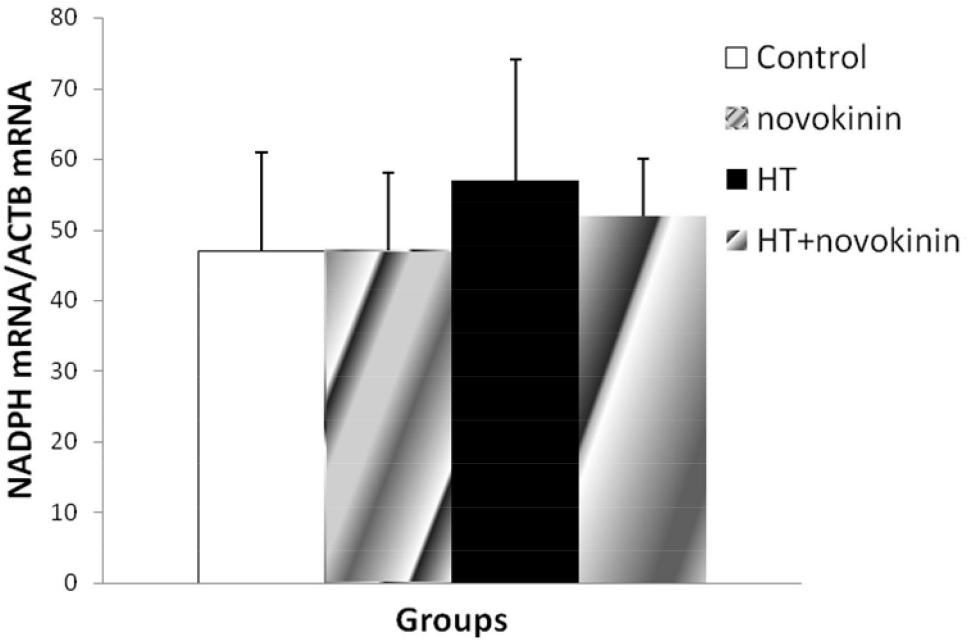

Figure 4. The expression level of NADPH oxidase in aortic tissue from all experimental groups.

findings suggest that the administration of L-NAME and salt may enhance the sensitivity to Ang-II. However, this observation was not shown in the novokinin-treated HT group. It might be argued that novokinin treatment enhanced the antagonism of the AT2 receptor and so AT1-induced vasoconstriction might have fallen. In aortic rings from mice with the 2-kidney, 1-clip Goldbatt hypertension model, the contractile responses to Ang-II

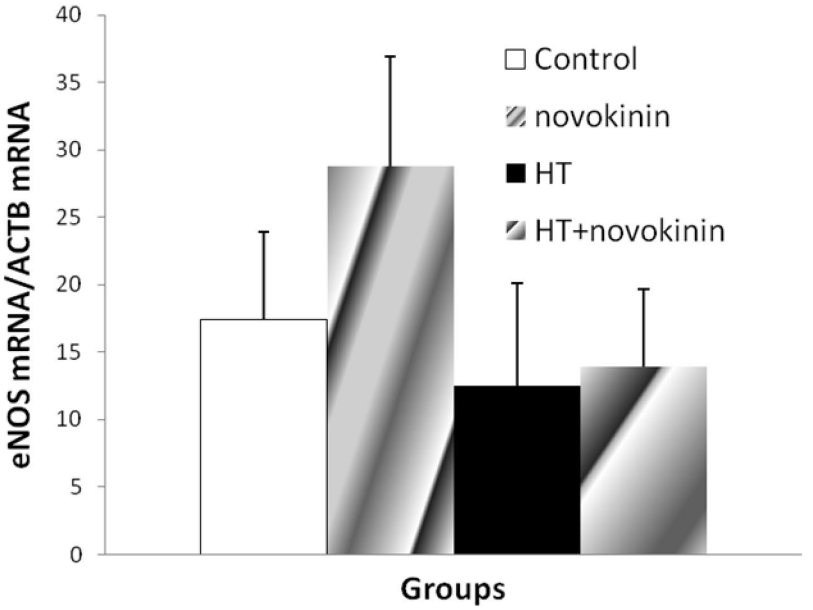

Figure 5. The expression level of eNOS in aortic tissue from all experimental groups.

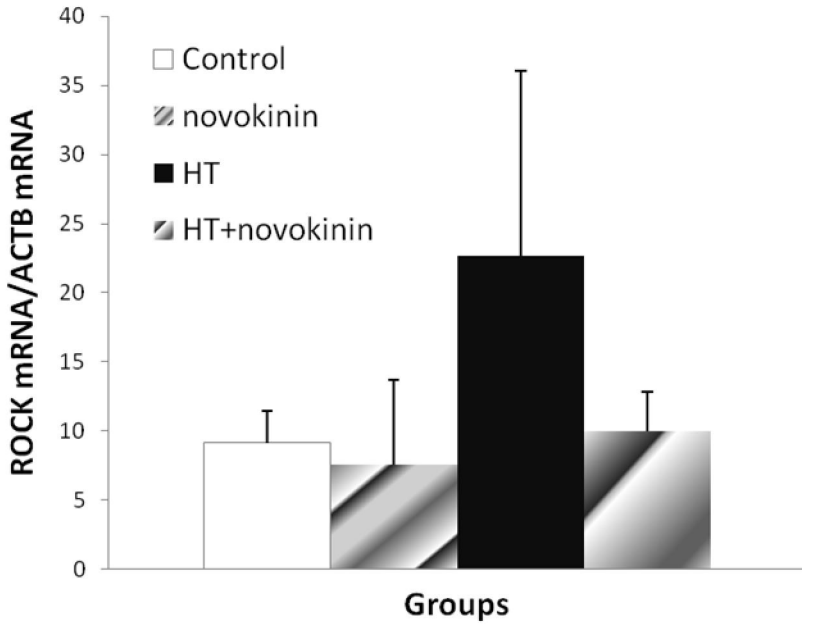

Figure 6. The expression level of Rho kinase in aortic tissue from all experimental groups.

were attenuated compared with sham-operated animals (24). It was estimated that the attenuation of AT1-mediated contraction probably depended on the increased number of AT2 receptors (16).

We explored the expression of Rho kinase, PRMT, NADPH oxidase, and eNOS in the aortic tissues from all experimental groups. It has been suggested that there is a causal link between hypertension and these enzymes. In the hypertension group, eNOS expression was decreased. On the other hand, eNOS expression increased in treatment groups. In a previous study, male Sprague Dawley rats given L-NAME in drinking water for 8 weeks had a decrease of eNOS expression in aortic tissue (25). In L-NAME-treated rats, administration in the last 4 weeks

of either enalapril or losartan completely restored eNOS mRNA levels in aortic tissue (25).

In our study, the expression level of NADPH oxidase increased in the L-NAME- and salt-given group, but NADPH oxidase expression in the novokinin-treated group tended to decrease. In a previous study NADPH oxidase expression in both the telmisartan-treated and the losartan-treated SHRs was significantly lower than in the untreated SHRs (26). In another study it was demonstrated that NADPH oxidase activity in aldosterone-salt-induced hypertension was increased in aortic tissue (27).

In the present study, the expression of PRMT, an enzyme responsible for generating ADMA, was increased in the HT group and decreased in novokinin-treated groups. Several studies have demonstrated that ACE inhibitors or ARBs reduced plasma ADMA levels in patients with HT, diabetes, or cardiovascular diseases (8). In a previous study, it was suggested that ADMA activates the local renin-angiotensin system, and Ang-II augmentation activates NAD(P)H oxidase via the AT1 receptor; the production of superoxide impairs the bioavailability of NO. This mechanism may participate in the development of endothelial dysfunction, which is associated with elevated ADMA levels (16).

Increased activity of the Rho/Rho kinase pathway has been proposed to play an important role in the development and maintenance of hypertension (28). We have observed that Rho kinase expression increased in hypertension, but its expression level was decreased in novokinin-treated groups. In rats treated with L-NAME, although oral administration of Y27632 lowered blood pressure, the level of RhoA was markedly increased in vessels from L-NAME-treated rats (29).

As results of earlier studies, it was indicated that AT2-R shows beneficial effects for preventing cardiovascular diseases, including antiinflammatory, antiapoptotic, and blood pressure-lowering actions (30). Recent preclinical models showed that AT2 agonists are effective molecules and so they may play important therapeutic roles in the treatment of hypertension and enhance the actions of currently available RAS inhibitors (30).

The results of our study show that novokinin might positively affect blood pressure, endothelial function, and oxidative stress-related parameters. The above effects of the AT_2 agonists were shown for the first time in our study. Although further studies are needed to determine the effectiveness of novokinin on cardiovascular diseases, it is argued that novokinin might support or be an alternative to the current treatments.

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