Research Article

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Comparative Effectiveness of Novokinin, Perindopril and Losartan on Blood Pressure, Adma, NADPH Oxidase and Rho Kinase at Renal Tissue in L-Name and Salt Induced Hypertension

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Abstract

There is no sufficiently investigation about the effects of novokinin, AT2 receptor agonist, on target molecules associated with organ pathology including ADMA, NADPH oxidase and Rho kinase. In this study we investigated the effects of novokinin, perindopril and losartan on Rho kinase, ADMA, NADPH oxidase at renal tissue blood pressure, in L-NAME and salt induced hypertension. Additionally, α 1-adrenergic-induced contraction, ach-induced dilator responses in vessels obtained from hypertensive and pharmacological therapy groups were studied. In this study we investigated the effects of novokinin, perindopril and losartan on blood pressure, Rho kinase, ADMA, NADPH oxidase at renal tissue in L-NAME and salt induced hypertension. Additionally, α 1-adrenergic-induced contraction, ach-induced dilator responses in vessels obtained from hypertensive and pharmacological therapy groups were studied.

To develop hypertension, L-NAME was administrated intraperitoneally and drinking water with salt (1%) for 4 weeks. Perindopril, losartan, novokinin were administrated intraperitoneally for 2 weeks. Blood pressure was measured by using tail-cuff method; Rho kinase, ADMA and NADPH oxidase were measured by ELISA at renal tissues. Values are presented as means $\pm$ S.E.M.; compared by one way anova.

Novokinin, perindopril and losartan diminished the level of NADPH oxidase and ADMA at renal tissue compared to hypertension group. Novokinin, perindopril and losartan decreased blood pressure. The greatest reduction of blood pressure was determined in perindopril treatment group. In the hypertensive group, the acetylcholine EC50 value was significantly higher than in the control group and Emax value was significantly lower in hypertensive group compared to control. The application of novokinin, perindopril and losartan were improved the ach induced dilator responses in L-NAME and salt induced hypertension model.

AT2 receptor agonist novokinin may offer protection of target organs such as the kidney. In this regard, further experimental studies are needed to exhibit potential benefits of novokinin in hypertension and end organ damage treatment for advanced clinical research.

Keywords: Novokinin; Blood pressure; Renin angiotensin aldosterone system; ADMA; Vascular responses

Introduction

Hypertension correlates with renal function and is associated with renal pathology. The renin-angiotensin-aldosterone system (RAAS) plays crucial roles in the pathogenesis of progression of arterial hypertension, cardiovascular disease and chronic kidney disease [1]. In addition the RAAS is effective in the control of renal function. The kidney RAAS is included in the adjustment of multiple renal functions, such as the renal blood flow, glomerular filtration, tubular sodium and water reabsorption. Long lasting stimulation of circulatory and tissue RAAS can cause the development and progression of renal pathophysiology. In this regard, making RAAS blockage is a logical therapeutic way in the prevention of renal diseases in hypertensive patients. Angiotensin-converting enzyme inhibitors (ACEis) and angiotensin II AT-1 receptor blockers (ARBs) are major approach to decelerate and treat hypertension, cardiovascular and renal diseases [2]. Despite this therapy, the progression of renal disease is not completely inhibited and residual proportion of hospitalisation and death in patients with cardiovascular pathology remain high. Novel alternative RAAS related strategies are currently being attempted to improve patient outcomes.

Endothelial dysfunction, oxidative stress, increased sympathetic activation, and salt retention play a primary role in hypertension. Salt-loading augments the degree of the hypertension and lowers its starting time [3]. It has been shown that chronic treatment of rats with L-NAME

decreases NO production, impairment of endothelial vasodilator function and causes a progressive increase of blood pressure [4].

Novokinin is a potent hypotensive peptide, designed based on ovokinin, a vasorelaxing and hypotensive peptide derived from ovalbumin [5,6]. It was found that novokinin exhibited an affinity for the AT2 receptor [6]. It was demonstrated that novokinin significantly lowered systolic blood pressure in spontaneously hypertensive rats (SHRs) [7].

Several studies have found that the plasma level of asymmetric dimethylarginine [8] is associated with cardiovascular risk factors such as hypertension, diabetes, hyperlipidemia, and renal disease, and is a prognostic marker for cardiovascular and the renal diseases [9]. Hypertension can lead to ADMA accumulation. In animal models

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positive correlations have been determined between ADMA and proteinuria [10]. Both hypertension and proteinuria are identified major risk factors for the progression of renal disease [11]. Elevation of ADMA is associated with increased renal oxidative stress, can cause induction of glomerular fibrosis via enhancement of synthesis collagen type I and II and fibronectin deposition [12]. ADMA may be a crucial link between cardiovascular and renal disease.

The Rho/Rho kinase pathway have revealed important role in the structure and function of various kidney cells such as tubular epithelial cells, mesangial cells and podocytes [13]. The Rho/Rho kinase pathway also arranges glomerular blood flow and glomerular filtration rate via modulating renal arteriolar contractility [14].

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is the predominant enzyme source for reactive oxygen species (ROS) generation and is established as a pivotal mediator of cell proliferation and matrix accumulation in renal disease [15]. Ang II induces the activity or expression of the NADPH oxidases within the blood vessels, kidney and brain, leading to increased production of ROS. ROS contribute organ damage by altering signalling pathways that regulate cell and organ functions, and leading to the generation of advanced oxidation protein products. Targeting NADPH oxidase may be a novel strategy for the therapeutic intervention of renal injury.

There is no sufficient investigation about the effects of novokinin on target molecules associated with organ pathology including ADMA, NADPH oxidase and Rho kinase. In this study we investigated the effects of novokinin, perindopril and losartan on Rho kinase, ADMA, NADPH oxidase at renal tissue blood pressure in L-NAME and salt induced hypertension. Additionally, α 1-adrenergic-induced contraction, acetylcholine (ach) induced dilator responses in vessels obtained from hypertensive and pharmacological therapy groups were studied. We compared the effects of AT1R antagonist losartan, ACE inhibitor perindopril and AT2R agonist novokinin on these parameters. We attempted to clarify how novokinin affects the paracrine pathways which lead to cardiovascular and renal damage, and we also evaluated whether novokinin is more effective than the other RAAS blockers.

Methods

Animals and groups design

A total of 35 adult male Sprague-Dawley rats (6–8 weeks old) were housed under standard laboratory conditions (24 ± 3°C, 40–60% humidity, a 12-h light and dark cycle). The study has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments. This study was approved by the Animal Ethical Committee of Firat University (Elazığ, Turkey). A commercial pellet diet (Elazığ Food Co., Elazığ, Turkey) was given ad libitum. The animals were divided into five groups: control, Hypertension (HT), HT+perindopril, HT+losartan and HT+novokinin. Each group had seven rats and the experimental period was 4 weeks.

L-NAME and salt were given to rats for development of hypertension. L-NAME was treated intraperitoneally (40mg/kg per day) and salt (8%) was administered with drinking water during 4 weeks. Perindopril (2 mg/kg per day), losartan (2 mg/kg per day), novokinin (0.1 mg/kg per day) were administered intraperitoneally for last two weeks.

Chemicals

Perindopril, losartan, novokinin, L-NAME, acetylcholine and

phenylephrine were purchased from Sigma Aldrich (Inc.St. Louis, MO, U.S.A). All drugs were dissolved in saline.

In-vivo studies: Systolic blood pressure (SBP) was measured using the tail-cuff method (MAY BPHR 9610-PC, Commat Ltd., Ankara, Turkey) at the beginning of the protocol, 14th and 28th days of experiments. Five measurements were obtained for each rat and then the average of these measurements were calculated.

In-vitro studies: Freshly harvested thoracic aortas of decapitated rats were cleaned from fat and connective tissues. The rings each approximately 0.4 mm long were prepared from a segment of thoracic aorta and mounted on FDT 05 (MAY Force-Displacement Transducer) MP 36 (BIO-PAC) in 20 mL temperature controlled baths (37°C) containing Krebs Henseleit buffer mmol /L: NaCl 118, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, and glucose 11, pH 7.4 and oxygenated with 95 % O₂, 5% CO₂ mixture. Vessels were equilibrated for 60 min at 2.0 g resting tension, with changes of bathing fluid every 15 min.

The dose-response curve of phenylephrine was determined by the application of increasing doses of phenylephrine (from 10⁻⁹ to 10⁻⁴ M) and submaximal dose of phenylephrine was obtained. After the contraction of submaximal dose of phenylephrine, increasing doses of acetylcholine (from 10⁻⁹ to 10⁻⁵ M) was performed at the plateau of contraction response to phenylephrine and the dose-response curve of acetylcholine was assessed. The half maximal effective concentration (EC₅₀) and E_{max} (maximal contraction for phenylephrine and maximal relaxation for acetylcholine) values of phenylephrine and acetylcholine were calculated. Ach relaxation responses were included in the vascular endothelium intact rings.

Sample collection: After 24 h of last drug application, rats were decapitated and the kidney tissues were removed for biochemical analysis.

Biochemical analysis

The tissue ADMA, Rho kinase and NADPH oxidase content was determined by ELISA. Rat kidney tissue was cut into small pieces and washed with phosphate-buffered saline and then grinded in a homogenization buffer consisting of 0.05 M Tris-HCl, pH 7.9, 25% glycerol, 0.1 mM EDTA, and 0.32 M (NH₄)₂SO₄ and containing a protease inhibitor tablet (Roche, Germany). All steps were achieved at 4°C.

The lysates were homogenized on ice using a IKA Labortechnik homogenizer. The solution was sonicated in an ice bath to prevent overheating for 15 seconds followed by 3-minute centrifugation at 15000 rpm and 4°C. The supernatant was aliquoted and stored at -80°C. The homogenate was used for determination of ADMA, Rho kinase and NADPH oxidase levels. (ADMA: cusabio Cat No: CSB-E13039r, NADPH oxidase: cusabio Cat No: CSB-EL015959RA, Rho kinase: Eastbiopharm Cat No: CK-E90094 OK)

Data analysis

Data were analyzed by using a commercially available statistical software package. The one-way ANOVA test was performed and post hoc multiple comparisons were made using Tukey HSD test. Results are presented as mean ± SEM; P < 0.05 was regarded as statistically significant.

Results

Blood pressure

In L-NAME and salt treatment groups, SBP increased at 14th day significantly ($p < 0.05$, Table 1). There was no significantly difference between at 14th and 28th day blood pressure in L-NAME and salt coadministrated group. SBP decreased significantly in perindopril, losartan and novokin treatment groups at 28th day ($p < 0.05$). The greatest reduction of blood pressure was determined in perindopril treatment group.

Vasoconstrictor responses to phenylephrine

The phenylephrine concentration-response curve for each group was obtained. There was no significant difference between groups in vasoconstrictor responses.

Vasodilator responses to acetylcholine

Emax and EC50 values are displayed in Table 2. In the hypertensive group, the acetylcholine EC50 value was significantly higher than control group ($p < 0.05$). The Emax value was significantly lower in hypertensive group compared to control ($p < 0.05$).

In the hypertension group, EC50 value was significantly higher than perindopril treated hypertensive group ($p < 0.05$). The Emax value was significantly lower in hypertension group compared to perindopril treated hypertension group ($p < 0.05$). In losartan treated hypertension group EC50 value was significantly lower than hypertension group ($p < 0.05$), but significantly higher than control. In novokin treated hypertension group the Emax increased significantly when compared to hypertension group ($p < 0.05$). EC50 value was significantly lower in novokin treated hypertension group versus hypertension.

The comparison of antihypertensive drug effects on acetylcholine dilator responses

In perindopril treated hypertension group, EC50 was significantly

lower than losartan ($p < 0.05$) and novokin treated hypertension group ($p < 0.05$). EC50 value of novokin treated hypertension group was lower than losartan treated hypertension group ($p < 0.05$).

Biochemical findings

L-NAME and salt administration was increased ADMA level in hypertension group significantly; novokin, perindopril, losartan treatment was decreased ADMA significantly compared with hypertension group ($p < 0.001$) (Figure 1). L-NAME and salt administration enhanced NADPH oxidase level significantly; novokin, losartan and novokin treatment was decreased NADPH oxidase compared with hypertension significantly ($p < 0.001$) (Figure 2).

Rho kinase did not differ between experimental groups (data not shown).

Discussion

In this investigation, two types of RAAS blockade were compared with AT2 receptor agonist novokin. For the first time the present study showed that treatment of novokin diminished the level of NADPH oxidase and ADMA at renal tissue, decreased blood pressure and improved ach induced dilator responses in L-NAME and salt

Figure 1: The comparison of systolic blood pressure (SBP) changes in perindopril, losartan and novokin treatment groups.

Figure 2: Levels of NADPH oxidase at renal tissue in all experimental groups.

Results are represented as mean $\pm$ SE.

a, vs control,
b, vs HT
x, vs baseline
y, vs day 14, $p < 0.05$

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

Groups	Relaxant responses in thoracic aorta	
	Emax	EC50 (μ M)
Control	52.36 $\pm$ 0.76	0.19 $\pm$ 0.007
HT	47.42 $\pm$ 0.61 ^a	0.61 $\pm$ 0.008 ^a
HT+perindopril	52.33 $\pm$ 0.99 ^b	0.14 $\pm$ 0.008 ^{ab}
HT+losartan	51.00 $\pm$ 3.18	0.41 $\pm$ 0.006 ^{abc}
HT+novokin	50.9 $\pm$ 1.37 ^b	0.28 $\pm$ 0.009 ^{abcd}

Results are represented as mean $\pm$ SE.

a, vs control,
b, vs HT
c, vs HT+Perindopril
d, vs HT+Losartan, $p < 0.05$

Table 2: Relaxant responses to acetylcholine at the end of 4 weeks in all groups

induced hypertension model. Moreover it has been demonstrated that the application of perindopril and losartan was decreased ADMA and NADPH oxidase at renal tissue in the current experimental hypertension model.

While a significant reduction in blood pressure was observed with the ACE inhibitor perindopril and the AT1 antagonist, the perindopril treatment provided more higher percentage change compared with losartan. These results are similar to the previous results reported studies reported in the literature. Thoracic aorta Ach Ec50 (mol/L) values were lower in the group treated with perindopril compared to the group treated with losartan. As a result it suggests that, the vascular relaxation capacity was better protected in the perindopril group. The reduction of serum ADMA concentration increase with hypertensive patients by using ACE inhibitors and AT1 receptor antagonist drugs suggest that, these drugs via their protective effects, may contribute in the reduction of ADMA levels which is effective in the development of endothelial dysfunction. In our study both losartan and perindopril reduced the ADMA and NADPH oxidase levels in kidney tissues similarly compared to the HT group.

The current study method was conducted by combining both the following methods: salt loading and NOS inhibition-induced hypertension. In the rats which were administered L-NAME for 8 weeks and losartan last 4 weeks, the blood pressure was decreased significantly compared with alone L-NAME administered group [4,16]. In the same study, the blood pressure showed a similar reduction in enalapril treated hypertension group. We have demonstrated that the blood pressure increased in L-NAME and salt administered groups by the 14th day. The blood pressure was numerically higher by the 28th day, but there was no significant difference between the 14th and 28th days. These results were similar with previous study [17-19]. Treatments of the losartan, perindopril or novokinin to L-NAME and salt administration groups lowered the blood pressure significantly. The percentage reduction in perindopril group in terms of this period and dose was higher than the losartan ($p < 0.05$) and novokinin ($p > 0.05$).

In previous studies, it has been demonstrated that novokinin reduces significantly systolic blood pressure after oral administration at a dose of 0,1mg/kg in spontaneously hypertensive rats [7]. Novokinin did not exhibit hypotensive activity in AT2 receptor deficient mice [7]. In this examination, novokinin significantly reduced blood pressure compared with hypertension group and there was no significant difference between novokinin and other treatment groups. Therefore, novokinin has been reported to have a vasorelaxing effects blocking by PD 123319 in the mesenteric artery from SHR. In the present study, as a novel finding, novokinin raised the sensitivity to ach compared with the losartan treatment group in current model.

In a study by Kalliovalkama et al. demonstrated that [20], L-NAME administration for 4 weeks did not change noradrenaline contractile responses compared with control. Likewise, addition of losartan to L-NAME administered group was not different from control and L-NAME administered groups [20]. Phenylephrine-induced contractions increased in hypertension group but it was not significantly compared with control. There was no difference between other groups in phenylephrine contractile responses. These results are in agreement with our study.

In a previous study, the relaxations to ach were significantly impaired in SHR group compared with Wild-type. Administration of 10 mg and 100 mg L-NAME suppressed significantly acetylcholine-

induced relaxation in aortic ring compared with control [21]. However, there was no difference between L-NAME administered (10, 100 mg) groups. Ach generated significantly lower vasorelaxing response in hypertension group than the control group in current study.

In another study, the dose-dependent relaxation response to acetylcholine was greater in SHR treated either with losartan or with captopril than non treated SHR group [22-23]. In the present study, exposure to perindopril raised significant sensitivity to ach (Ec50) compared with novokinin and losartan treatment groups.

Serum ADMA levels have been reported to be markers of endothelial dysfunction and/or atherosclerosis and to be associated with renal function and also proteinuria in renal diseases [24-25]. Oxidative stress causing upregulation of protein L-arginine methyltransferase (PRMT) expression, predominant enzyme responsible for ADMA formation [26]. The activity of dimethylarginine dimethylaminohydrolase (DDAH), the key enzyme in ADMA degradation, is downregulated by oxidative stress [27]. In a previous study, it has been established that administration of L-NAME increased renal ADMA level and decreased DDAH in SHR [28]. Recent investigations have shown that NOS inhibition caused oxidative stress and furthermore addition of salt may aggravate [19]. In our study L-NAME and salt administration may provoke enhancement of oxidative stress and this rise may induce production of ADMA.

Several studies have proposed that there was a potential interaction between ADMA and the RAAS [29]. Ang II acts AT1 receptors, increases oxidative stress, in part by activating NADPH oxidase and ADMA, which in turn activates the local renin-angiotensin system [30]. Also ACEi and ARB have been shown to decrease plasma ADMA in many studies [31]. RAAS blockers can lower oxidative stress, which has been suggested to be associated with a reduction in serum ADMA levels independently of lowering the BP. In the present examination ADMA levels were tend to decrease at novokinin and losartan treatment groups, but the greatest reduction of ADMA level was observed in the perindopril treatment group. Furthermore this study is the first time exhibit the relationship between ADMA and AT2 agonist, novokinin.

NADPH oxidase is the primary source of ROS in vascular smooth cells in both kidney cortex and medulla [32-33]. Stimulation by angiotensin II and aldosterone, cytosolic subunits of NADPH oxidase can translocate into the mitochondrial membrane and enhance ROS production [15]. In the kidney, NADPH oxidase-derived ROS can influence regulation of renal blood flow, alteration of cell fate and regulation of gene expression. It has been reported that ROS regulated renal blood flow via the reaction of superoxide anion O_2^- with NO, which limited its relaxing effect in afferent arterioles [34]. ROS induce a pro-inflammatory and pro-fibrotic state via both cytokines and the transforming growth factor β (TGF- β). Moreover it has been demonstrated that ROS provoked epithelial-mesenchymal transition and was thought to be one of the major causes of renal fibrosis [35]. NADPH oxidase mediated activation of transcription factors promotes renal interstitial fibrosis, inflammation and renal injury [36].

It has been observed that the application of L-NAME and salt induced the increasing of NADPH oxidase level at renal tissue in the present study. L-NAME and salt may cause enhanced oxidative stress by raise of NADPH oxidase. In another study administration of L-NAME for five weeks was upregulated NADPH oxidase subunits at aortic tissue [37]. In a similar examination, NADPH oxidase level was increased in four weeks L-NAME applied group at aortic tissue [38]. It has been known that L-NAME model of hypertension was associated

with increased systemic oxidative stress [39]. It has been implied that L-NAME and salt might increase oxidative stress by upregulation of NADPH oxidase at renal tissue.

RAAS is critical in the regulation of NADPH oxidase function [40]. Activation of these pathways can be disturbed by inhibiting ACE or by blocking AT1 receptors. In SHR, treatment of losartan and ACE inhibitor fosinopril reduced the elevation of NADPH oxidase expression at renal tissue [41]. In a renal experimental model of hypertension, administration of losartan blunted increase in the activity and expression of NADPH oxidase [42]. In transgenic mice that overexpress rat angiotensinogen in their proximal tubule cells, perindopril treatment prevented the elevation of NADPH oxidase activity [43]. It has been observed that perindopril and losartan treatment reduced the level of NADPH oxidase at renal tissue in our study and this result was in agreement with the others. One of the major findings of our study was novokinin treatment provided reduction of NADPH oxidase level. Novokinin may reverse renal injury through down regulation of NADPH oxidase. In another study, it has been demonstrated that Compound 21 (C21), a selective angiotensin type 2 receptor agonist, was decreased aortic superoxide generation in SHR [44]. In obese Zucker rats, it has been determined that AT2 agonist CGP42112A reduced oxidative stress at kidney cortex [45]. These results and our findings confirmed the anti-oxidant effect of AT2 receptor.

Hypertension-induced cardiovascular and renal diseases are the most common cause of death worldwide. The RAAS has been a pivotal target. However, enough RAAS blockades cannot be achieved with ACE inhibitors or ARB because of counter regulatory mechanisms. Novel pharmacological approaches associated with RAAS have been attempted. AT2 receptor agonists such as novokinin may offer protection of target organs such as the kidney. AT2 receptor agonists could be combined with ACE inhibition or AT1 receptor blockade to inhibit NADPH oxidase and ADMA generation and their toxic effects. In this regard, further experimental studies are needed to exhibit potential benefits of novokinin in hypertension and end organ damage treatment for advanced clinical research.

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