Original Article

The effect of angiotensin II microinjection into the bed nucleus of the stria terminalis on serum lipid peroxidation and nitric oxide metabolite levels

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Abstract

Background: Overactivity of renin-angiotensin system is involved in the pathophysiology of renal and cardiovascular diseases. It is suggested that endothelial cells can release nitric oxide (NO) and reactive oxygen species in response to angiotensin II (Ang II). Angiotensin type 1 (AT1) receptor of Ang II has been found in the bed nucleus of the stria terminalis (BST). BST is involved in autonomic function. This study was performed to find the role of central Ang II in serum lipid peroxidation product and in releasing NO into circulation.

Materials and Methods: Twenty-one catheterized rats were placed in stereotaxic instrument. A hole was drilled above BST. In the control group, saline 0.9% (100 nl) was microinjected into the BST. In the second group, Ang II (100 μM, 100–150 nl) was microinjected into the BST. In the third group losartan (an AT1 antagonist) was microinjected (100 μM, 200 nl) before Ang II into the BST. Systolic blood pressure was recorded. The NO metabolite (nitrite) and malondialdehyde (MDA) were measured in the rat's serum.

Results: The data indicated that microinjection of Ang II into the BST produced a pressor response ($P < 0.0001$). It also increased MDA and nitrite levels of the serum significantly ($P < 0.001$, $P < 0.0001$). Pretreatment with losartan before Ang II microinjection attenuated serum's levels of MDA and nitrite ($P < 0.001$, $P < 0.0001$).

Conclusion: Our findings suggest that central effect of Ang II on blood pressure is accompanied with increased levels of MDA and nitrite in the circulation.

Key Words: Angiotensin II, bed nucleus of the stria terminalis, malondialdehyde, nitric oxide

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Received: 13.10.2015, **Accepted:** 22.12.2015

INTRODUCTION

Renin-angiotensin system (RAS) has an important role in electrolyte homeostasis and blood pressure regulation.[1] Over the activity of RAS is involved in the pathophysiology of some renal and cardiovascular diseases.[2-6] It has been established that there is a local RAS in the brain.[7] It has been shown that central RAS might be responsible for the pathogenesis of some cardiovascular diseases such as hypertension.[8] It is suggested that endothelial cells have angiotensin II (Ang II) type 1 (AT1) receptors. It is also suggested that endothelial cells can release nitric oxide (NO) and reactive oxygen species (ROS) in response to Ang II.[9,10] NO is an endogenous mediator which has various biological

Access this article online	
Quick Response Code:	Website: www.advbiores.net
	DOI: 10.4103/2277-9175.183667

How to cite this article: Kafami M. The effect of angiotensin II microinjection into the bed nucleus of the stria terminalis on serum lipid peroxidation and nitric oxide metabolite levels. Adv Biomed Res 2016;5:106.

actions, including vasodilation.[11] It is well known that NO plays important roles in the cardiovascular and nervous system.[11-13] Oxidative stress is resulted from high production of ROS and has an important role in the cardiovascular diseases.[14] The $NAD^+/NADP^+$ nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidases originated from smooth muscle cells of the vessels is one of the major sources of ROS.[15,16] Ang II treatment has been shown to increase superoxide production within the endothelium.[17,18] The development of oxidative stress has been confirmed during infusion of Ang II by increasing plasma levels of lipid peroxidation, and NADPH and NADH stimulated O_2 generation.[19] AT1 receptor is responsible for vasoconstriction effect of Ang II resulting in an increase in blood pressure.[20] Previous studies showed that enhanced AT1 receptor activation could be associated with some hormonal and metabolic disorders.[21] AT1 receptor was found in several regions of the brain. A high density of AT1 has been found in the solitary tract nucleus, paraventricular nucleus (PVN), rostral ventrolateral medulla (RVLM), caudal LM, amygdala and the bed nucleus of the stria terminalis (BST).[7,22]

BST is part of the limbic system located in the rostral forebrain. BST is involved in behavioral, neuroendocrine and autonomic functions, including cardiovascular regulation.[23] There are several studies investigated cardiovascular changes after chemical stimulation of BST.[24-26] Hypertension is a cardiovascular disease that is associated with additional risk factors such as RAS over activity and increase of ROS.[27-30] Despite well-known peripheral effects of Ang II on superoxide and NO production,[31-33] little is known about its central effects. In this study, we investigated the possible effects of central Ang II on the levels of lipid peroxidation and NO metabolite in serum. In addition, the possible mediatory role of the central AT1 receptor was also investigated.

MATERIALS AND METHODS

Animals

Twenty-one male Wistar rats, 8 weeks old (270 ± 10 g) obtained from the Animal Center, Isfahan University of Medical Sciences, Isfahan, Iran, were used in this study. 4–5 animals were housed in a standard cage at a temperature of 23–25°C with a 12 h light/dark cycle and free access to water and food. Animal handling and all related procedures were approved by the Isfahan Medical University Committee of Animal Research.

Experimental protocol

The experimental groups consisted of (1) control group, (2) Ang II group, (3) losartan - Ang II group. Rats were anesthetized with urethane (Sigma, 1.4 g/kg, i.p.). The trachea was intubated to ease ventilation. Catheters were implanted into the femoral artery and vein. Rats placed in stereotaxic instrument, and a hole was drilled above BST at coordinates of: 0.12–0.36 mm caudal, 1.1–1.7 mm lateral and 6.4–7.2 mm ventral to the bregma according to the atlas of Paxinos and Watson (2005). In the control group, saline 0.9% (100 nl) was microinjected into the BST using a glass micropipette. In the second group, Ang II (100 μM, 100–150 nl, Sigma)[34] was microinjected into the BST. Finally in the last group, losartan was injected (100 μM, 200 nl)[34] before Ang II microinjection. Systolic blood pressure (SBP) was recorded continuously, using a pressure transducer connected to a polygraph (HSE-Germany) and a computer program written in this laboratory by Nasimi. Each experiment lasted 3 h. The blood samples were obtained at the end of each experiment for measurement of nitrite and of metabolite and malondialdehyde (MDA) levels.

Biochemical measurement

The level of nitrite was measured by using Promega kit (Promega Corporation, USA). At first sulfanilamide solution (50 μl) was added to the samples. After 5–10 min incubation, 50 μl N-(1-Naphthyl) ethylenediamine solution was added, then the solution was incubated for 5–10 min again. Finally, the samples absorbance was measured by a microreader at the wavelength of 540 nm. The nitrite concentration was determined by comparison with the nitrite standard reference curve.[35]

MDA level of the serum was assessed by a manual method.[36] Briefly, 1000 μL of the sample was mixed with 2000 μL of the mixture of trichloroacetic acid, thiobarbituric acid, and hydrochloric acid. Then, it was incubated in a hot-water bath at the temperature of 100°C for 10 min. After centrifuging at 1000 g for 10 min, the absorbance of the supernatant was measured at 532 nm. The concentration of MDA was reported as μmol/L.

Histology

At the end of each experiment, the animal was sacrificed with a high dose of urethane and transcardially perfused with 100 ml of saline followed by 100 ml of formalin (10%). The brain was removed and placed in 10% formalin. After 3 days, the brains were sliced (100 μm), stained with cresyl violet 1%. The injection sites were determined under a light

microscope according to a rat brain atlas (Paxinos and Watson 2005). Data points outside of the BST were not included in the analysis [Figure 1].

Statistical analysis

Data are expressed as a mean ± standard error of the mean the serum levels of MDA and nitrite after Ang II microinjection and pretreatment with losartan among the groups were compared via Student's *t*-test.

RESULTS

Microinjection of vehicle (saline, 100–150 nl) did not affect SBP. The levels of nitrite and MDA in this group were 5 ± 1 μmole/l and 5 ± 0.6 μmole/l, respectively.

Effect of microinjection of angiotensin II and losartan after angiotensin II into bed nucleus of the stria terminalis on systolic blood pressure

Microinjection of Ang II (100 μM, 100–150 nl) increased SBP significantly compared to the control group (Student's *t*-test, $P < 0.0001$, $n = 7$). Microinjection of losartan before Ang II prevented the increase in SBP in response to Ang II, compared to the Ang II group (Student's *t*-test, $P < 0.0001$, $n = 7$) [Figure 2].

Effect of microinjection of angiotensin II and losartan after angiotensin II into bed nucleus of the stria terminalis on serum nitrite level

Microinjection of Ang II (100 μM, 100–150 nl) increased serum nitrite level compared to the control group (Student's *t*-test, $P < 0.0001$, $n = 7$). Pretreatment with losartan before Ang II injection prevented the increase in nitrite level compared to the Ang II group (Student's *t*-test test, $P < 0.0001$, $n = 7$) [Figure 3].

Effect of microinjection of angiotensin II and losartan after angiotensin II into bed nucleus of the stria terminalis on metabolite and malondialdehyde level

The serum level of MDA increased significantly (Student's *t*-test, $P < 0.001$, $n = 7$) in response to Ang II injection compared to the control group. Microinjection of losartan before Ang II injection prevented the increase in MDA level compared to the Ang II group (Student's *t*-test, $P < 0.001$, $n = 7$) [Figure 4].

Histology

Figure 1 shows the representation of the injection sites for Ang II into the BST. Injections outside the BST were not included in the data.

DISCUSSION

Most previous experiments examined the effect of circulating Ang II on the production of oxidative stress and NO in different tissues. A number of *in vivo* experiments have shown that ROS is able to cause hypertension by inactivation of NO in the vasculature or via a direct effect on the cardiovascular system,

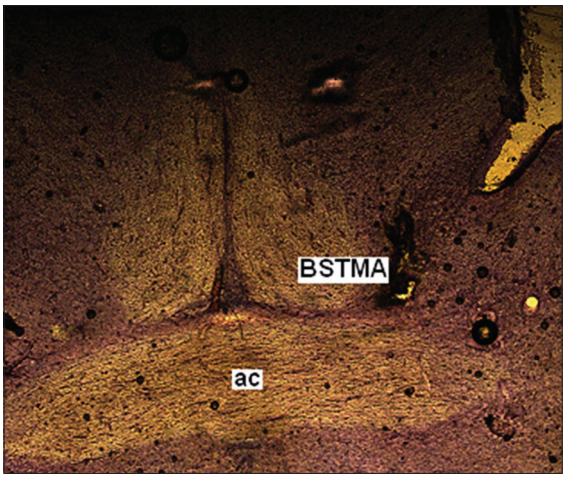

Figure 1: Photomicrograph of a Nissl-stained coronal brain section of the bed nucleus of the stria terminalis. ac: Anterior commissure, BSTMA: BST, medial division, anterior part

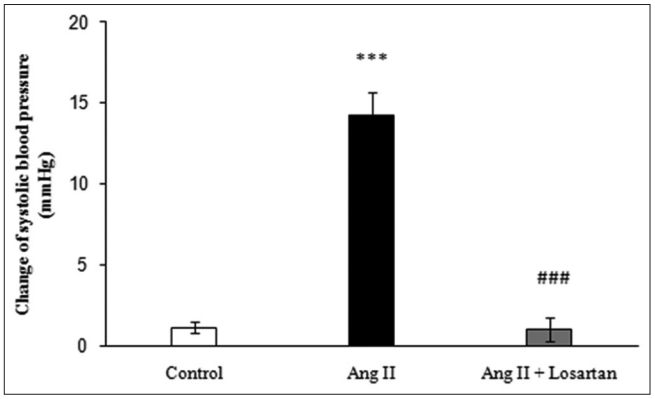

Figure 2: Effects of angiotensin II and angiotensin II after losartan on the systolic blood pressure. Microinjection of angiotensin II (100 μM, 100–150 nl) increased systolic blood pressure compared to the control group (Student's *t*-test, $^{***}P < 0.0001$, $n = 7$). Microinjection of losartan before angiotensin II prevented the increase in systolic blood pressure in response to angiotensin II compared to the angiotensin II group (Student's *t*-test, $^{###}P < 0.0001$, $n = 7$)

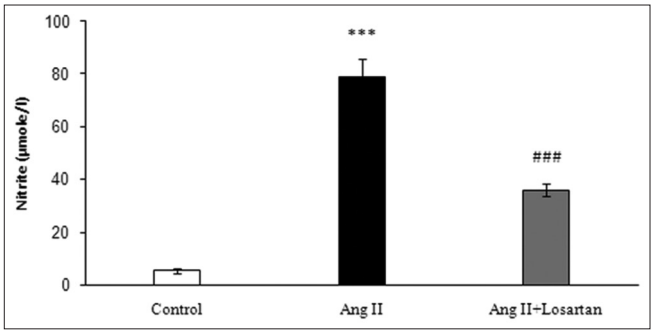

Figure 3: The serum level of the nitrite in the three experiment groups. Angiotensin II microinjection significantly increased serum level of nitrite compared to the control group (Student's *t*-test, $^{***}P < 0.0001$, $n = 7$). Losartan pretreatment prevented the increase in nitrite level compared to the angiotensin II group (Student's *t*-test, $^{###}P < 0.0001$, $n = 7$)

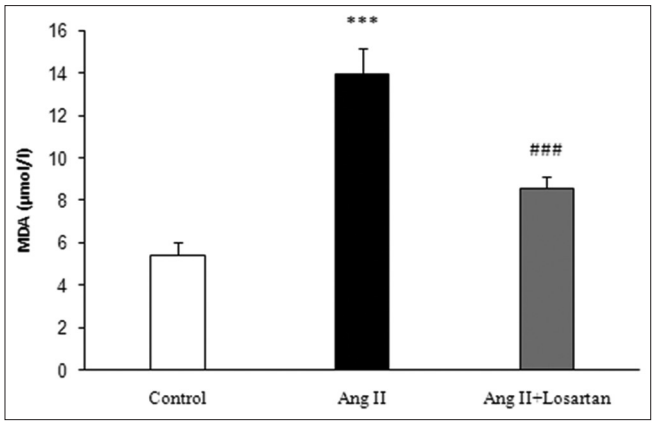

Figure 4: The serum level of the malondialdehyde in the four experiment groups. Angiotensin II microinjection significantly increased serum malondialdehyde level in the angiotensin II group compared to the control group (Student's *t*-test, $^{***}P < 0.001$). Losartan pretreatment prevented the increase in malondialdehyde level compared to the angiotensin II group (Student's *t*-test, $^{###}P < 0.001$, $n = 7$)

kidneys, and central nervous system.[37] Endothelial cells possess AT1 receptors that cause the release of NO and ROS in response to Ang II.[19,38] It has been shown that the increased level of ROS in some brain regions which controls blood pressure such as hypothalamic nuclei, and brainstem sites play an important role in the neurocardiovascular dysfunction observed in hypertension.[39] In this study, we found that microinjection of Ang II into the BST produced a pressor response. It has been shown that microinjection of Ang II into the BST increase blood pressure through sympathoexcitation and vasopressin release.[40] Sympathoexcitation in responses to microinjection of Ang II into the BST might be mediated through a cholinergic system of the BST as it was shown that microinjection of Ach into the BST produced a pressor response.[40,41] Microinjection of Ang II may disinhibit of vasopressin release by PVN. In support of this suggestion, it has been shown that BST send GABAergic projection to PVN that decrease the release of vasopressin.[42,43]

We found that microinjection of Ang II into the BST significantly increased MDA in serum. There is no similar study to compare with, however, Seifi *et al*. have shown that microinjection of Ang II into the PVN significantly increased MDA level in kidney.[44] In addition, there are some evidence that central ROS is involved in the action of Ang II in the regulation of cardiovascular activity and autonomic functions. For example, systemic infusion of Ang II in mice caused hypertension by elevation of O_2 production specifically in the subfornical organ.[45] It has also been shown that intracerebroventricular injection of Ang II increased local O_2 production in the RVLM.[46] These observations are consistent with the findings of our study, in which Ang II-induced production of the O_2 radical.

We found that microinjection of Ang II into the BST caused a significant increase in serum nitrite level following pressor effect. Although some previous reports investigating interaction of Ang II and NO suggested that oxygen species produced by Ang II inactivates and degenerates NO rather than producing it,[31,47] it has been reported that Ang II affects protein expression and NO production in rat's vascular smooth muscle cells.[48] It has been suggested that Ang II enhances NO production through an increase of cGMP and intracellular calcium.[49,50] Ang II acts as an activator of O_2 production.[31] In this study, we microinjected Ang II into the BST and it is unlikely that Ang II directly affects blood vessels. Considering that Ang II is composed by eight amino acids, which cannot pass the blood-brain barrier, the mechanisms underlying how the central Ang II induces oxidative stress and NO production in serum is unknown. In this study microinjection of Ang II caused a pressor effect, so an increase of MDA and NO might be due to an increase of shear stress on the vessels' endothelium following acute hypertension. There are studies showing that shear stress induces the release of NO from endothelial cells.[51,52] Furthermore, it has been shown elevated blood pressure and a mechanical stretch of a vessel wall induces ROS release.[53-55] It raises the possibility that high blood pressure itself increases ROS-independent of RAS activity. Acute hypertension causes a sudden increase in prostaglandin synthesis that leads to the generation of free oxygen radicals.[54] Another possible mechanism is the intervention of endothelial NO synthase (eNOS) or inducible NO synthase (iNOS). eNOS and iNOS have important functions in vasoregulation and inflammation.[38] eNOS synthesis low amount of NO and more is in physiological condition. In contrast, iNOS produces high amounts of NO as a defense mechanism. Thus, high levels of NO that was made by iNOS is toxic via the formation of reactive oxygen.[38] It has been shown that vascular expressions of eNOS and iNOS proteins in young spontaneously hypertensive rats (SHR) is up-regulated.[14] Recent studies have provided evidence for increased ROS activity in SHR.[56,57] Therefore, the associated increased ROS activity can enhance NO inactivation and reduce bioactive NO. This can, in turn, cause a compensatory up-regulation of NOS isotype expression.[58] NO could be increased by higher sheer stress due to increasing in blood pressure by Ang II.[59] Immediately after the onset of shear stress, there is an acute activation of the eNOS leading to NO release within seconds.[60]

We also found that the serum level of MDA increased after Ang II microinjection. It possible that iNOS is involved in increase of NO after acute elevation of blood

pressure. When eNOS is involved, the simultaneous increase of MDA is less possible.

Losartan is an antagonist that blocks AT1 receptor of Ang II. Pretreatment with losartan before Ang II prevented the increase of blood pressure MDA and NO levels. When there is no sheer stress resulted from elevated blood pressure, there is no production of NO and MDA in the vessels walls.

CONCLUSION

This study demonstrated that lipid peroxidation and nitrite are increased by microinjection of Ang II into the BST. It could be suggested that acute hypertension due to Ang II can increase peripheral production of ROS and NO. Further molecular studies are necessary for a better understanding of the relation between peripheral oxidative stress and central hypertension induced by Ang II.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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