Angiotensin II Chronic Infusion Induces B1 Receptor Expression in Aorta of Rats

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Abstract—We investigated whether angiotensin II infusion modulates in vivo the kinin B1 receptor expression and the mechanisms involved in this process. We also evaluated the role of the B1 receptor activation in aorta. Wistar rats received 400 ng/kg per minute of angiotensin II or saline (control rats) infusion during 14 days through an osmotic minipump. To investigate the role of superoxide anion in B1 receptor expression, rats received a reduced nicotinamide-adenine dinucleotide phosphate oxidase inhibitor in the drinking water during 14 days (60 mg/L of apocynin) simultaneously with angiotensin II infusion. Angiotensin II induced B1 receptor expression in the aorta and increased significantly systolic blood pressure, superoxide anion, and the nuclear factor κB activity. Apocynin treatment did not alter the blood pressure levels of angiotensin II rats and reduced the B1 receptor expression, superoxide anion generation, and nuclear factor κB activity to similar levels of the control rats. Vascular reactivity studies in isolated aorta reveal that B1 receptor agonist promoted endothelium-dependent dilation and increased the NO generation in aorta of angiotensin II rats. NO synthase inhibitor and B1 receptor antagonist inhibited the vasodilation and NO generation, which were not affected by B2 receptor antagonist or indomethacin. These results provide evidence that angiotensin II induces B1 receptor expression in aorta by superoxide anion generation, via reduced nicotinamide-adenine dinucleotide phosphate oxidase, concomitant to nuclear factor κB activation. We have also shown that B1 receptor agonist causes endothelium-dependent vasodilation through NO production in aortic rings, suggesting that the B1 receptor expression could be related with the vascular tonus control of angiotensin II rats. (Hypertension. 2007;50:756-761.)

Key Words: angiotensin II • B1 receptor • hypertension • superoxide anion • NF-κB

Kininergic effects due to the release of kinins are assigned to kinins.1 However, several studies have expressed constitutively and described to mediate most effects assigned to kinins. The B1R is an inducible receptor, and it was first described by Regoli et al.3 These authors showed that large rabbit arteries, which normally do not respond to des-Arg9-bradykinin (DABK), gradually increase their sensitivity to this agonist at least in part, because of B1 receptor (B1R) activation.2 The B1R is an inducible receptor, and it was first described by Regoli et al.3 These authors showed that large rabbit arteries, which normally do not respond to des-Arg9-bradykinin (DABK), gradually increase their sensitivity to this agonist when incubated in vitro in normal conditions. Various inflammatory stimuli4 and tissue injury5 are able to induce B1R, which seems to be related to the nuclear factor κB (NF-κB) activation.6

Previous study from our group demonstrated that hearts of renal hypertensive rats expressed B1R; however, we did not clarify whether this effect was because of either the elevated angiotensin II (Ang II) plasmatic levels or the high arterial blood pressure observed in this model of experimental hypertension.7

Therefore, the present study was undertaken to investigate whether infusion of Ang II leads to an increase in B1R expression in rat aorta. Other than the role of superoxide anion generation, NF-κB activation and the functional activation of B1R in isolated aorta of Ang II hypertensive rats were also evaluated.

Materials and Methods

Animals

Experiments were performed in male Wistar rats (n=32) weighting 180 to 200 g, obtained from the breeding stock of the Institute of Biomedical Sciences of the University of São Paulo. Rats were kept in a temperature-controlled room on a 12-hour light/dark cycle, 60% humidity, with standard rat chow and water ad libitum. All of the procedures used in this study were approved and performed in accordance with the guidelines of the ethics committee of the Institute of Biomedical Sciences of the University of São Paulo.

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**Induction of Hypertension by Ang II**

The rats were anesthetized with pentobarbital (50 mg/kg IP), and either 400 ng/kg per minute of Ang II for 14 days (Ang II group; n=16) or saline 0.9% (control group; n=16) were infused by an osmotic minipump (Alzet 2002) implanted subcutaneously. To assess the role of reactive oxygen species induced by Ang II in the B1R expression, apocynin (60 mg/L in drinking water), a reduced nicotinamide-adenine dinucleotide phosphate oxidase inhibitor, was simultaneously administered either with Ang II or saline in 8 rats per group (Ang II+apocynin and control+apocynin groups).

**Determination of Arterial Blood Pressure**

Systolic blood pressure was measured in conscious rats by indirect tail-cuff method (PowerLab 4/S, AD Instruments Pty Ltd) at days 0, 7, and 13 after the minipump was implanted as described previously and calculated as an average of 3 consecutives measurements.

**Aorta Excision**

On day 14 after minipump implantation, the rats were anesthetized with pentobarbital (50 mg/kg IP), the thorax was opened, and the descending aorta was excised and divided in 4 segments, which were used for measurement of superoxide anion and NO generation, immunohistochemical reaction to B1R determination, NF-κB activity, or vascular reactivity.

**Superoxide Anion Measurements**

Production of superoxide in the aorta was evaluated using the dihydroethidine (DHE) chemiluminescence method as described previously. Briefly, aorta segments, ~3 mm in length, (n=4 per group) were incubated in DHE (50 μmol/L) and kept at 37°C for 30 minutes in the dark. Aortic segments were homogenized in liquid nitrogen with pestle, suspended in acetonitrile, centrifuged, and the supernatants injected into a high-performance liquid chromatography system. DHE and DHE-derived products, such as 2-hydroxyethidium (EOH) and ethidium derived from oxidation of DHE by superoxide and by other reactive oxygen species, were determined using ultraviolet and fluorescence detection, respectively. Thus, the DHE-derived products were expressed as a ratio of EOH and ethidium generation per DHE consumed (initial DHE concentration minus remaining DHE).

**Immunohistochemical Reaction**

Aorta B1R expression was determined by immunohistochemical reaction. Aorta segments (n=4 per group) were fixed in paraformaldehyde 4%, mounted in tissue freezing medium and transverse sections (7 μm) were obtained and incubated with rabbit anti-B1Rs antibody (Santa Cruz Technologies). After that, sections were incubated with goat anti-rabbit antibody conjugated with biotin (Vector Laboratories). The reactions were amplified by the avidin-biotin-peroxidase complex (ABC Elite Vector) and visualized using 3,3′-diaminobenzidine. Images were collected with an optic microscope (Axioskop, Zeiss). The aorta B1R expression was analyzed by a computer system (KS-300 Software, Zeiss), and the mean optical density of the stain was measured in 5 different areas (~10 000 μm² each), located in 4 sections from each aorta. Parallel image acquisition of control and Ang II sections was performed with fixed parameters.

**Electrophoretic Mobility Shift Assay to NF-κB Consensus Oligonucleotide**

Nuclear extracts of aortas were prepared homogenizing aortas in cold phosphate buffer complemented with (in millimoles per liter) 0.1 EDTA, 0.5 dithiothreitol, 0.5 PMSF, and centrifuged. The pellets were resuspended in lysis buffer (in millimoles per liter): 10 HEPES (pH 7.9), 1.5 MgCl₂, 10 KCl, 0.1 EDTA, 0.5 dithiothreitol, 0.5 PMSF, and centrifuged. The protein concentration in the supernatants containing nuclear extracts was determined using the Bio-Rad protein reagent.

Electrophoretic mobility shift assay to NF-κB was performed by using the gel shift assay kit from Promega, as described previously. 32P-NF-κB double-stranded consensus oligonucleotide probe (5′-AGTTGAGGGCACTTTCCCCAGG-3′; 20 000 cpm) and nuclear extracts (10 to 15 μg) were used. DNA-protein complexes were separated by electrophoresis through a 6% nondenaturing acrylamide: bis-acrylamide gel. Gels were vacuum dried and analyzed by autoradiography. For competition experiments, NF-κB and transcription initiation factor IID (5′-GCAAGCATATAAGGTTAGGTAGG-3′) unlabeled double-stranded consensus oligonucleotide were included in 10- and 20-fold molar excess over the amount of 32P-NF-κB probe to detect specific and nonspecific DNA-protein interactions, respectively.

**Isolated Aorta Preparation**

Aorta segments, 4 mm in length, were cut and mounted between 2 steel hooks to measure the isometric tension as described earlier. Vessels were submitted to a tension of 1.5 g, which was adjusted during 60 minutes before the addition of a given drug. After that, the tissues were incubated with 10 μmol/L of enalaprilat for 30 minutes and then precontracted with phenylephrine (0.1 μmol/L), and, finally, a cumulative-concentration response curve to B1R agonist DABK (1 μmol/L to 100 μmol/L) was performed. In a second set of experiments, preparations were incubated during 30 minutes with enalaprilat and with HOE 140 (10 μmol/L) a B2 receptor antagonist; des-Arg⁹-Leu⁸-BK (DALBK; 10 μmol/L), a B1 antagonist; N⁰-nitro-l-arginine methyl ester (10μmol/L), an NO synthase inhibitor; or indomethacin (5 μmol/L), a cyclooxygenase inhibitor, for >30 minutes in the presence of enalaprilat. After that, cumulative-concentration response curves to DABK were done. The concentration of agonists and antagonists chosen were based on previous studies from our group.

Determination of NO production in endothelium rat aorta: NO was measured by 4,5-diaminofluorescein diacetate, an NO-sensitive fluorescent dye. The aortic cryosections (7-μm thick) were incubated with 4,5-diaminofluorescein diacetate (12.5 μmol/L), and the sections were stimulated with B1R agonist DABK (10 μmol/L) in the presence or absence of N⁰-nitro-l-arginine methyl ester (10 μmol/L) or B1R antagonist DALBK (10 μmol/L). After 1 hour, digital images were collected in a microscope (Axioskop, Zeiss) with ×40 objective lens, equipped for fluorescence (excitation at 485 nm; emission at 538 nm). The images were analyzed using image software (KS-300 Software, Zeiss) by measuring the mean optical density of the fluorescence observed in the endothelium. This fluorescence was evaluated in ≥3 locations in each image and in 4 aortas from different animals per group.

**Drugs and Reagents**

Ang II, DABK, and DALBK were purchased from Bachem Bioscience Inc. N⁰-nitro-l-arginine methyl ester, HOE 140, indomethacin, DHE, 4,5-diaminofluorescein diacetate, and phenylephrine were from Sigma.

**Statistical Analysis**

Data are presented as mean±SEM. Statistical analysis was performed using 1-way ANOVA (posthoc Tukey-Kramer multiple comparisons test), using SigmaStat, version 2.0 (Jandel Scientific Software). Values were considered statistically significant when P<0.05.

**Results**

**Blood Pressure**

There was no difference in the blood pressure levels between control and Ang II rats at day 0 (119±1.6 mm Hg versus
118±1.5 mm Hg; n=13). The Ang II infusion for 13 days resulted in a progressive increase in systolic arterial blood pressure in comparison with control group, measured at days 7 (114±2.1 mm Hg versus 156±6.7 mm Hg; n=8; P<0.05) and 13 (114±2.3 mm Hg versus 180±7.3 mm Hg; n=8; P<0.05). Treatment with apocynin did not interfere with the increase in arterial blood pressure observed in Ang II rats (day 7: 152±6.3 mm Hg; day 13: 186±7.9 mm Hg; n=5) during the 13 days of Ang II infusion. In control rats, the arterial blood pressure remained unaltered by apocynin treatment (day 7: 109±5.3 mm Hg; day 13: 108±6.3 mm Hg; n=5).

Superoxide Anion Generation in Aorta
A higher EOH concentration was observed in aortas of the Ang II group compared with the control group, and the apocynin treatment has only decreased the EOH concentration in aorta of Ang II. In contrast, no alteration in ethidium generation has been observed (Figure 1). These data suggested that superoxide anion might be the main reactive oxygen species in the Ang II aorta, because EOH is the DHE-derived product oxidated by superoxide anion.

Expression of B1R
In control rats, the aorta B1R expression was concentrated in the adventitia (Figure 2A). In Ang II, the stains were localized in the endothelium and vascular smooth muscle cells (Figure 2B). Aorta of Ang II rats presented an increased B1R expression when compared with control ones (Figure 2E). The apocynin treatment decreased the aorta B1R expression of the Ang II group but had no effect in aortas of the control group (Figure 2E).

NF-κB Activation in Aorta
The NF-κB nuclear binding activity was increased in aortas isolated from Ang II rats when compared with the control ones. Apocynin, a reduced nicotinamide-adenine dinucleotide phosphate inhibitor, decreased the NF-κB activity in Ang II aorta to a similar level found on those in control rats (Figure 3).

Functional Evidence of B1R in Aorta
DABK elicited a concentration-dependent relaxation in phenylephrine-preconstricted aorta rings with endothelium isolated from Ang II rats. However, no response to DABK was observed in aorta rings without endothelium isolated from Ang II and those with and without endothelium from control rats (Figure 4A).

The DABK-induced relaxation in Ang II rat aorta was inhibited by DALBK, a B1R antagonist, and by N^G-nitro-L-arginine methyl ester, an NO synthase inhibitor (Figure 4B). On the other hand, neither HOE 140, a B2 receptor antagonist, nor indomethacin, a cyclooxygenase inhibitor, had effect on the relaxation elicited by B1R agonist (Figure 4B).
Production of NO by DABK

DABK has almost duplicated the basal NO production in aorta isolated from Ang II rats. This effect was blocked by B1R antagonist and NO synthase inhibitor (Figure 4C). In contrast, DABK has not modified the NO basal production in aortic slices from control and Ang II+apocynin rats.

Discussion

In the present work we demonstrated for the first time that infusion of Ang II in Wistar rats led to an increase in B1R expression in aorta. In addition, the activation of this receptor, by an agonist of B1R, caused endothelial-dependent vasodilation and increased NO generation. Concomitant to the increase in B1R expression in aorta, there was an increase in superoxide anion generation and activation of NF-κB. We observed that the inhibition of reduced nicotinamide-adenine dinucleotide phosphate oxidase, by apocynin, decreased the aorta B1R expression in ANG II group, and we postulated that the B1R induction is because of the direct pro-oxidative effect of Ang II.

The hypertensive effect of Ang II could not be directly related to B1R induction in aorta of Ang II rats. In fact, apocynin treatment, used as a scavenger of superoxide anion, prevented the B1R expression without reducing the high blood pressure levels in Ang II rats. Furthermore, B1R induction is not observed in spontaneous hypertensive rats, a genetic model of hypertension. Altogether these findings give support to the hypothesis that induction of B1R is not secondary to arterial hypertension.

It is possible that B1R upregulation has been mediated by the activation of NF-κB induced by oxidative stress, because the reduction of superoxide anion levels in aorta by apocynin prevented the NF-κB activation. In fact, the oxidative stress is a potent activator of NF-κB that is known to induce B1R in several conditions. In functional studies we demonstrated that the activation of B1R in aorta of Ang II rats caused endothelial-dependent vasodilation and increased NO generation. We also observed that B1R vasodilatation was not sensitive to cyclooxygenase inhibition and, hence, is unlikely to involve prostaglandin.
release. These data are in agreement with others studies in human coronary arteries in which DABK-induced vasodilation is abolished by endothelium removal and inhibition of NO synthase but is unaffected by indomethacin. In contrast, B1R-mediated vasodilation of the rat coronary circulation involves the release of eicosanoids, in particular, prostacyclin.

On the other hand, B1R agonists can also cause vasoconstriction in some blood vessels as rabbit aorta. This effect is generally independent of an intact endothelium and involves a direct cooperation between protein kinase C and calcium release. According to Wohlffart et al, the complexity of B1R-mediated control of vasculature tone is highlighted by the fact that expression and pharmacology of the B1 kinin receptor-dependent response depends on the endothelial cell type. The discrepancies between the studies may depend mainly on species and experimental models.

The B1R expression in vasculature is poorly explored in hypertensive animals, and for the first time we have demonstrated that the B1R activation in aorta of Ang II-infused rats causes endothelium and NO-dependent dilation. These data suggest that B1R can participate in the control of aorta tonus in Ang II-infused rats.

The role of B1R expression in the cardiovascular system is still inconclusive. It has been related to either cardiovascular protection or injury. It has been suggested that the protective effect of B1R in cardiac tissue is associated with NO production and inhibition of mitogen-activated protein kinase and transforming growth factor-β1. Accordingly, these effects would compensate the progression of hypertensive cardiac remodeling. Moreover, it has been demonstrated that B1R contributes to the attenuation of neointima formation after balloon injury in rat artery and inhibits platelet-derived growth factor–stimulated mitogenesis in cultured vascular smooth muscle cells. On the other hand, Christopher et al demonstrated in culture of vascular smooth muscle cells that B1R induced by interleukin-1β is related to the mitogen-activated protein kinase activation suggesting that B1R in this case can induce cellular proliferation, which is a characteristic feature of vascular injury. To characterize the relevance of the effect of B1R in aorta of Ang II–infused rats, further studies will be required.

In conclusion, the present study provides new insight into the role of the vascular B1R in Ang II–dependent hypertension. We identified that chronically infused Ang II induces B1R expression in aorta by superoxide anion generation, via reduced nicotinamide-adenine dinucleotide phosphate oxidation, concomitantly to NF-κB activation. Moreover, the activation of B1R by DABK, a B1R agonist, induces endothelial NO-dependent vasodilation.

Perspectives

Further studies in other models of hypertensive animals can provide information about whether the B1R expression induced by Ang II is relevant and might indicate a potentially important therapeutic target for treating vascular alterations in hypertension.

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Disclosures

None.

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