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Mas receptor overexpression increased Ang-(1–7) relaxation response in renovascular hypertensive rat carotid

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ABSTRACT

Renin-angiotensin system (RAS) is an important factor in the pathophysiology of hypertension. Mas receptor, Angiotensin-(1–7) [Ang-(1–7)]-activated receptor, is an important RAS component and exerts protective effects in the vasculature. Ang-(1–7) vascular effects and Mas receptor expression in carotid from renovascular hypertensive (2K-1C) rats is not clear. In the present study we investigated Mas receptor vasodilator response activated by Ang-(1–7) in the carotid rings from sham and 2K-1C rats. Changes in isometric tension were recorded on organ chamber. Mas receptors expression was investigated in carotid by Western blot. Nitric oxide production was evaluated by 2,3-diaminonaphthalene (DAN) and eNOS expression and activity by immunofluoresce and western blot, respectively. Ang-(1–7) induced concentration-dependent vasodilator effect in carotid rings from sham and 2K-1C, which the hypertension increased vasodilatation response. In the 2K-1C carotid rings, A-779 (Mas receptor antagonist) reduced but not abolish the vasodilator effect of Ang-(1–7). Corroborating, Mas receptor protein expression was significantly increased in the 2K-1C rats. L-NAME and ibuprofen decreased Ang-(1–7) vasodilator response and L-NAME plus ibuprofen practically abolish the remaining vasodilatation response. Nitric oxide production is increased due increased of eNOS expression and *p*Ser^{1177} activity. Our results demonstrated that renovascular hypertension increased Mas receptors expression and nitric oxide production in the rats carotid which, consequently increased Ang-(1–7)-vasorelaxant response.

1. Introduction

Arterial hypertension is one of the causes associated with early death due to recurrence of stroke and cerebral edema. Hypertensive diseases usually induce brain edema, one of the most serious complications of abnormal cerebral hemodynamics and fluid dynamics [1,2].

The cerebral circulatory system and metabolic homeostasis contribute to health in the brain [1]. Blood supply to the brain originates from the common carotid arteries (CCA). The CCA come from the arch of aorta on the left and on the right side from brachiocephalic trunk and these arteries divide into an external carotid artery (ECA) and an internal carotid artery (ICA). The ECA supplies blood to the face and cranium wall and ICA supplies the large part of cerebral cortex via intracranial arteries. On the other hand, the vertebral artery (VA) and the basilar artery (BA), the vertebro-basilar system, supply blood to the occipital cortex, cerebellum, spinal cord and medulla oblongata [3,4]. The relative contribution of the ICA system and VA system to global cerebral blood flow at rest are estimated to be 75 and 25%, respectively [5]. Therefore, any modification in the cerebral circulation, especially during arterial hypertension, could contribute to early death due to recurrence of stroke and cerebral edema [2].

The renin-angiotensin system (RAS) is usually known as a hormonal and tissular system that release angiotensin II (Ang II) that is critically involved in the physiological blood pressure control, homeostasis, pathogenesis of hypertension and other cardiovascular diseases [6]. Previous studies have clearly shown that Ang II is an important peptide of the RAS and that Ang II-mediated activation of Ang II type 1 receptor (AT1) is the critical mechanism in the pathophysiology of 2Kidney-1Clip Goldblatt hypertension model (2K-1C) [7–9].

In recent years, increasing attention has focused on the role of the Ang-(1–7), a peptide formed from angiotensin I (Ang I) and from Ang II by the action of tissue peptidases such as endopep-

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tidases, carboxipeptidases and aminopeptidases [10,11]. Among them, the angiotensin-converting enzyme 2 (ACE2) seems to play an important role in the formation of this peptide through the hydrolysis of Ang II [12,13]. Many evidences indicate that the biological actions of Ang-(1–7) are mediated by the activation of the Mas receptor [14–20]. The G protein-coupled receptor Mas has been shown to bind Ang-(1–7) and is involved in many of its biological actions [21–23]. Mas receptor is blocked by the heptapeptide D-Ala(7)–Ang(1–7) [A-779], a specific and potent antagonist of Ang-(1–7) [24]. Many actions performed by Ang-(1–7) are mediated by nitric oxide (NO) [25–27] and prostacyclin [28].

Blood vessels are an important site for the formation and biological actions of Ang-(1–7) [29]. Many studies have shown that Ang-(1–7) has important physiological effects, including inhibition of vascular smooth muscle cells proliferation [30,31], natriuretic and diuretic action [32] and vasodilator effects [14,33–35].

The effect of Ang II in cerebral arteries is well recognized. However, the role of Ang-(1–7) in cerebral blood vessels is largely unknown. In this study, we aimed at investigating the vascular effects and underling mechanisms of Ang-(1–7)-Mas receptor activation in carotid artery from 2K-1C rats.

2. Methods

2.1. Animals

All experimental procedures were performed in accordance with guidelines for the humane use of laboratory animals and were approved by the animal ethics committee of the Federal University of Minas Gerais (protocol # 164/10). We used a total number of 46 male Wistar rats (23 sham and 23 2K-1C) provided by the animal facilities of the Biological Sciences Institute (CEBIO) of Federal University of Minas Gerais. Animals were housed in a temperature-controlled room and maintained on a 12–12 h light/ dark cycle with free access to water and food.

2.2. Experimental groups

Male wistar rats (180–200 g) were anesthetised using xylazin and ketamin (70:30 saline solution; 0.1 ml/Kg, i.p.) and submitted to a midline laparotomy. Then, renovascular hypertension (2K-1C) was produced by placing a silver clip (0.2 mm i.d.) on the left renal artery that remained in the animals for 6 weeks. Approximately, 75% of those animals became hypertensive (systolic arterial pressure above 160 mmHg) and were used in the experiments. Control rats (sham) were subject only to laparotomy. The systolic blood pressure was measured weekly in non-anesthetized animals by an indirect tail-cuff method during 6 weeks after surgery, time necessary to induce hypertension [36].

2.3. Vessel preparation

Following arterial pressure recordings, rats were anesthetized with halothane i.v. (700 μl) and killed. Carotid arteries were removed and immediately placed in Krebs solution (in mmol l^{-1}: 118.4 NaCl; 4.7 KCl; 1.9$CaCl_2$; 1.2 KH_2PO_4; 1.2 $MgSO_4{\cdot}7H_2O$; 25.0 $NaHCO_3$; 11.6 glucose; pH 7.4, at 37 °C). Carotid artery rings (4 mm) were mounted in organ chambers bubbled with a carbogenic mixture (95% O_2 and 5% CO_2), connected to force transducers (World Trade) stretched to a basal tension of 1 g and allowed to equilibrate for 60 min. Endothelial integrity was assessed by the ability of acetylcholine (ACh, 1 μmol/L) to induce more than 80% relaxation of vessels pre-contracted with phenylephrine (PE, 0.1 μmol/L). In some preparations, the endothelium was mechanically removed. The absence of a relaxant response to ACh was taken as evidence that the vessel segments were functionally denuded of endothelium.

2.4. Ang-(1–) and Mas receptor

Cumulative concentration-response curves were constructed to Ang-(1–7) (0.1 nmol/L–1 μmol/L) in vessels pre-contracted with PE (30 nmol/L–0.1 μmol/L, with or without functional endothelium, respectively) to the same tension level (∼1.0 g). The Mas receptor antagonist A-779 (10 μmol/L, 30 min.) was used to verify the participation of Mas receptor in vascular relaxation elicited by Ang-(1–7). To study the contribution of endothelium-derived factors in Ang-(1–7)-induced relaxation was used N_G-nitro-L-arginine methyl ester (L-NAME, 100 μmol/L, 30 min.) or ibuprofen (10 μmol/L, 30 min.) or L-NAME (100 μmol/L/L, 30 min.) plus ibuprofen (10 μmol/L, 30 min.).

2.5. Western blot analysis

The rats were anaesthetized with halothane i.v. (700 μl) and killed. The carotid arteries were removed an immediately stimulated with Ang-(1–7) (1 μmol/L, 10 min) in Krebs solution, at 37 °C. After this procedure, the arteries were frozen at −80 °C. The arteries were homogeneized in RIPA buffer (Trizma 50 mM; NaCl 150 mM; EDTA 1 mM; plus NP-40 1% and sodium deoxycholate 0.25%) and approximately 50 μg of protein from sham and 2k-1C were separate on a 10% gel via SDS-PAGE. Proteins were transferred onto a polyvinylidene fluoride membrane (Immobilon P; Millipore, MA) and blocked with 5% non-fat dry milk and 0.3% BSA in Tween-Tris-buffered saline for 24 h. The membrane was incubated (4 °C) overnight in 5% non-fat dry milk in Tween-Tris-buffered saline with antibodies anti-Mas receptor (1:500; Alomone laboratories, Jerusalem, Israel), anti-eNOS (1:500; Santa Cruz Biotechnology, CA, USA), anti- *p* -Thr^{495} (1:200; Santa Cruz Biotechnology, CA, USA), anti- *p* Ser^{1177} (1:200; Santa Cruz Biotechnology, CA, USA) and anti-β-actin (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA). After lavage, the membranes were incubated with a secondary antibody (1:4,000; Millipore) raised in rabbit (anti-Mas receptor) in goat (anti- *p* -Thr^{495} and anti- *p* -Ser^{1177}) and mouse (anti-eNOS and anti-β-actina) for 1 h. Blots were then washed and subjected to enhanced chemiluminescence with Luminol (Millipore).

2.6. Immunohistochemical analysis

Rats were anaesthetized, sacrificed and the vessels fixed in situ by constant pressure fixation with 4% paraformaldehyde through a 22-gauge butterfly angiocatheter in the left ventricle. The sham and 2K-1C carotid rats were embedded in 4% paraformaldehyde (24 h), alcohol 70% (48 h) and lastly in paraffin. After this procedure the tissue was cross-sectioned (5 μm) and fixed in glass slides.

Briefly, 5 μm-thick sections cut from paraffin blocks containing, deparaffinized, rehydrated, immersed in 10 mmol/l citrate buffer, pH 6.0, and submitted to heat-induced epitope retrieval using a vapor lock for 45 min. The slides were rinsed with PBS and immersed in 3% hydrogen peroxide for 20 min to block endogenous peroxidase. Non-specific protein binding was blocked with normal serum (Vectastain Elite ABC Kit, Universal, Vector Laboratories, Inc., Burlingame, CA, USA) for 30 min. The sections were then incubated with monoclonal primary antibodies specific for Mas receptor (dilution 1:300; Alomone laboratories, Jerusalem, Israel) overnight in a humid chamber. Following washes in PBS, a biotinylated pan-specific universal secondary antibody (Vectastain Elite ABC Kit, Universal, Vector Laboratories, Inc.) was applied for 30 min. Next, the slides were incubated with the avidin–biotin–peroxidase complex (Vectastain Elite ABC Kit, Universal, Vector Laboratories, Inc.) for 30 min and developed with the DAB Peroxidase Substrate