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Valsartan attenuates intimal hyperplasia in balloon-injured rat aortic arteries through modulating the angiotensin-converting enzyme 2-angiotensin-(1–7)-Mas receptor axis

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ABSTRACT

The role of the Mas receptor in the activity of valsartan against intimal hyperplasia is unclear. Herein, we investigated the role of the angiotensin-converting enzyme 2 (ACE2)-angiotensin-(1–7)-Mas receptor axis on the activity of valsartan against intimal hyperplasiain balloon-injured rat aortic arteries. Wistar rats were randomized equally into the sham control group, injured group, and injured plus valsartan (20 mg/kg/d)-treated group. Valsartan significantly attenuated the vascular smooth muscle cell proliferation and intimal and medial thickening on days 14 and 28 after injury. The angiotensin-(1–7) levels as well as ACE2 and Mas receptor mRNA/protein expression were significantly decreased in the injured rats, compared to the uninjured rats; meanwhile, the angiotensin II level as well as the ACE and AT_1 receptor mRNA/protein expression were increased (all $P < 0.05$ or < 0.01). Additionally, the *p*-ERK protein expression was increased ($P < 0.01$). Treatment with valsartan significantly increased the angiotensin-(1–7) levels as well as ACE2 and Mas receptor mRNA/protein expression but decreased the angiotensin II level, ACE and AT_1 receptor mRNA/protein expression, as well as the *p*-ERK protein expression, compared to the injured group (all $P < 0.05$ or < 0.01). These results suggest that valsartan attenuates neointimal hyperplasiain balloon-injured rat aortic arteries through activation of the ACE2-angiotensin-(1–7)-Mas axis as well as inhibition of the ACE-angiotensin II-AT1 and *p*-ERK pathways.

1. Introduction

Percutaneous coronary intervention with or without stent placement is the most widely used therapeutic approach to treat coronary heart disease. However, restenosis remains one of the major limitations of this procedure. Approximately 30–50% of cases develop restenosis within 6 months after percutaneous transluminal coronary angioplasty [1]. In addition, in-stent restenosis occurred in 30–40% of cases after bare metal stent implantation [2]. Moreover, introduction of the drug-eluting stents successfully decreased the restenosis rate by 10% in some cases with relatively simple lesions [3], but its therapeutic effect and safety in more complex lesions needs to be verified in future studies. Currently, effective therapeutic strategies to prevent and inhibit restenosis are still insufficient.

Neointimal hyperplasia is a major pathological process of restenosis after angioplasty. Excessive vascular smooth muscle cell (VSMC) proliferation and migration are the main mechanisms involved in the process of restenosis after angioplasty [4]. The renin-angiotensin-aldosterone system (RAAS) participates in the development of restenosis. In addition, angiotensin II is the most important bioactive peptide in the RAAS. Angiotensin II promotes restenosis by accelerating neointimal hyperplasia mainly through the angiotensin II type 1 receptor (AT_1) [5,6]. Recently, the RAAS has been extended by the addition of a novel axis consisting of angiotensin-converting enzyme 2 (ACE2), angiotensin-(1–7), and the G protein-coupled receptor Mas [7]. ACE2 can convert

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angiotensin II into angiotensin-(1–7) through the Mas receptor. The ACE2/angiotensin-(1–7)/Mas axis can antagonize the classical RAAS pathway with the ACE-AT_1 receptor. Numerous studies implicate that angiotensin-(1–7) attenuates damage in other models of injury. These models range from oxidative injury to disruption of glucose homeostasis [8,9]. Taken together, it is suggested that upregulation of the ACE-2/angiotensin-(1–7)/Mas axis offers an alternative approach in the management of restenosis.

The mitogen-activated protein kinase (MAPK) pathways are implicated in the vascular response to injury. MAPKs participate in the process of neointimal hyperplasia via mediating endothelial cell and VSMC proliferation and migration. Therefore, MAPKs have been identified as promising targets in the treatment of restenosis [10]. Extracellular signal-regulated kinase (ERK) is an important member of the MAPK family. Activation of ERK activity leads to VSMC proliferation and accelerates cell migration [10]. Angiotensin II binds to the AT_1 receptor and stimulates ERK, leading to VSMC proliferation [11]. In contrast, angiotensin-(1–7) can partly antagonize the activation of MAPK induced by angiotensin II and inhibit MAPK-mediated VSMC proliferation and migration.

Valsartan is a widely used angiotensin II receptor blocker (ARB) [12]. Valsartan has reduced the development of in-stent neointimal hyperplasia by approximately 30% in animal studies [13,14]. Moreover, our previous findings have shown that treatment with valsartan significantly decreases neointimal hyperplasia of rat aortic balloon injury through upregulating ACE2 and angiotensin-(1–7) and downregulating the AT_1 receptor [5]; however, the role of the Mas receptor in the activity of valsartan against intimal hyperplasia is unclear. To the best of our knowledge, no previous study has addressed whether valsartan could target on the Mas receptor in balloon-injured rat aortic arteries. In this study, we aimed to investigate the role of the ACE2-angiotensin-(1–7)-Mas receptor axis on the activity of valsartan against intimal hyperplasia in balloon-injured rat aortic arteries.

2. Materials and methods

2.1. Drug and reagents

Valsartan was obtained from Beijing Novartis Pharma Ltd. Polyclonal anti-Mas, anti-ACE2, anti-AT_1, anti-ACE, anti-*p*-ERK, and anti-β-actin were purchased from Santa Cruz Biotechnology Inc. The angiotensin II radioimmunoassay kit was obtained from the Institute of Radioimmunoassay of Science and Technology Development Center of the People's Liberation Army General Hospital. Evan's blue dye was purchased from Sigma-Aldrich Co. The angiotensin-(1–7) enzyme-linked immunosorbent assay (ELISA) kit was obtained from Shanghai Xi Tang Biotechnology Co., Ltd., China. The Trizol reagent kit was from Invitrogen Co., and the reverse transcription kit was from Promega Co.

2.2. Animals and experimental protocol

Animal experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals from Qingdao University. Adult male Wistar rats (n = 36, 300–350 g) were purchased from the Qingdao Animal Center. All rats were kept under a 12 h light:12 h darkness cycle in a temperature-controlled room (22 ± 1 °C with 30–40% humidity) and fed with a standard diet of rat chow.

Thirty-six rats were randomized equally into three groups: sham control group, injured group, and injured plus valsartan-treated group. Intimal hyperplasia of aortic arteries was induced by endothelial denudation with a 2F balloon catheter. Aortic tissues were collected on days 14 and 28 post surgery, with 6 rats in each group at each time point. Rats in the valsartan group were administered with valsartan (20 mg/kg per day) by gastric gavage from 1 day before injury to day 14 or day 28 after injury. This dose was chosen according to methods described previously. Rats in the sham control group were exposed to surgery without inflation of the balloon. The surgical model was as follows: when the rat was injected with 0.1 mLof 0.9% normal saline, the 2F balloon catheter was entered into the left common carotid artery, then to the thoracic aorta and abdominal aorta in turn. Next, the balloon catheter was pulled back to the aortic arch, and saline was withdrawn. This process was repeated three times in order to denudate the aortic endothelia completely.

The Evan's Blue test was used to observe the success of aortic endothelial denudation. Briefly, rats were injected with 0.5% Evan's blue at a dose of 2 mL/kg in the left common carotid vein immediately after the procedure, then the animals were sacrificed after 1 h, and the aortic endothelial denudation was observed.

2.3. Morphometric analysis of aorta

At days 14 and 28 after injury, the rats were anesthetized with an intraperitoneal injection of chloral hydrate (10 mL/kg). The aorta was isolated, and approximately 4–5 cm of the thoracic aortic specimen was harvested under sterile conditions. Roughly 5 mm of the abdominal aorta near the aortic arch was fixed in 10% formalin and embedded in paraffin, and then it was cut into 5-μm tissue sections. Hematoxylin-eosin-stained sections were observed under a light microscope at 200 × magnification to detect VSMC migration, proliferation, and intimal hyperplasia. The intimal and medial thickness was determined by a JEDA801 series image analyzer (JEDA Science-Technology Development Co., China).

2.4. Determination of angiotensin II and angiotensin-(1–7) levels

The frozen aortic specimens (roughly 35 mg each) were homogenized and centrifuged at 3000 r/min for 20 min. The supernatant was collected to determine the angiotensin II levels by a radioimmunoassay kit, in accordance with the manufacturer's instructions. The angiotensin-(1–7) levels were detected by ELISA, following the manufacturer's protocols.

2.5. Determination of Mas, ACE2, ACE, and AT_1 receptor mRNA expression

Semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis was used to determine the mRNA expression of Mas, ACE2, ACE, and AT1 receptor, according to methods described previously [15,16]. Briefly, total RNA was extracted from individual aortic tissues using Trizol reagent, chloroform, and isopropyl alcohol. The RNA integrity was defined spectrophotometrically at a ratio of 260–280 nm greater than 1.7. The PCR primers used were as follows: Mas (100 bp) forward 5′-CAGATGTCACCGCCCCAAGCA-3′,reverse5′-GTGTTGCCATTGCCCTCCTGA-3′; ACE2 (263 bp) forward 5′-CAAAGTTCACTTGCTTCTTGG-3′, reverse 5′- TACTGTAAATGGTGCTCATGG-3′; AT_1 (444 bp) forward 5′-CACCTATGTAAGATCGCTTC-3′, reverse 5′-GCACAATCGCCATAATTATCC-3′; ACE (506 bp) forward 5′-TTCGTGGAGGAGTATGACC-3′, reverse 5′-TCAGAGTAGCCGTTGAGC-3′; β-actin (350 bp) forward 5′-GAGGGAAATCGTGCGTGAC-3′, reverse 5′-GGAGCCAGGGCAGTAATC-3′. PCRs were performed for 35 cycles of 1 min denaturation at 94 °C, 1 min of annealing at 58 °C, and 1 min of elongation at 72 °C, followed by 7 min of final extension. After 1.5% agarose gel electrophoresis, the PCR products were analyzed by a gel imaging analyzer. β-actin was used as an internal control. The mRNA expression of Mas, ACE2, ACE, and AT_1 receptor mRNA was