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Angiotensin-(1-7) inhibits vascular calcification in rats

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ABSTRACT

Angiotensin-(1-7) [Ang-(1-7)] is a new bioactive heptapeptide in the renin–angiotensin–aldosterone system (RAAS) with potent protective effects in cardiovascular diseases, opposing many actions of angiotensin II (Ang II) mediated by Ang II type 1 (AT1) receptor. It is produced mainly by the activity of angiotensin-converting enzyme 2 (ACE2) and acts through the Mas receptor. However, the role of Ang-(1-7) in vascular calcification (VC) is still unclear. In this study, we investigated the protective effects of Ang-(1-7) on VC in an in vivo rat VC model induced by vitamin D_3 plus nicotine. The levels of ACE2 and the Mas receptor, as well as ACE, AT1 receptor, Ang II type 2 receptor and angiotensinogen, were significantly increased in calcified aortas, and Ang-(1-7) reversed the increased levels. Ang-(1-7) restored the reduced expression of lineage markers, including smooth muscle (SM) α-actin, SM22α, calponin and smoothelin, in vascular smooth muscle cells (VSMCs) and retarded the osteogenic transition of VSMCs by decreasing the expression of bone-associated proteins. It reduced alkaline phosphatase activity and calcium deposition in VC and alleviated the hemodynamic disorders of rats with VC. We provide the first in vivo evidence that Ang-(1-7) can inhibit the development of VC by inhibiting the osteogenic transition of VSMCs, at least in part by decreasing levels of the ACE/Ang II/AT1 axis. The increased expression of ACE2 and the Mas receptor in calcified aortas suggests the involvement of the ACE2/Ang-(1-7)/Mas axis during VC. Ang-(1-7) might be an efficient endogenous vasoprotective factor for VC.

1. Introduction

Vascular calcification (VC) is a common complication for patients with advanced atherosclerosis, diabetes mellitus or chronic kidney disease (CKD), especially those with end-stage renal disease (ESRD) on hemodialysis [1,18,25]. The metabolic disorders of altered phosphate, calcium, parathyroid hormone and vitamin D levels are involved in the pathogenesis of VC [19,27,33]. VC is an important contributor to cardiovascular morbidity and mortality and considered a strong prognostic marker [20,23,31].

Recent studies have revealed that VC is an active, cell-mediated and highly regulated process resembling bone mineralization [38]. The mechanisms of VC involve gain of osteogenic induction and loss of osteogenic inhibition. The disruption of the balance in favor of osteogenic promoters will induce changes in the phenotype of vascular smooth muscle cells (VSMCs) transforming them into osteoblast-like phenotype [8]. The phenotypic transformation of VSMCs is accompanied by decreased expression of contractile markers, including SM α-actin, SM22α, calponin and smoothelin, as well as increased expression of bone-associated factors such as alkaline phosphatase (ALP), bone morphogenetic protein 2 (BMP2), osteopontin (OPN) and osteocalcin (OCN), and transcription factors such as core binding factor α 1 (Cbfα1) and osterix [32,37,40]. Although the current treatment for VC is preventive and based on reducing hyperphosphatemia and hypercalcemia, new specific treatments for VC should be investigated. All the processes involved in VC, especially systemic and local factors that can promote or inhibit VC, are potential therapeutic targets [29].

In recent years, numerous studies, including our previous work, have shown that endogenous cardiovasoactive peptides are involved in arterial calcification. Some vasodilator peptides, such as intermedin, adrenomedullin and cortistatin, inhibit VC [5,6,22,30], whereas some vasoconstrictor peptides, such as endothelin-1 and angiotensin II (Ang II), promote VC [14,17,45]. Hence, further

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investigation of endogenous cardiovasoactive substances may reveal new therapeutic strategies for VC.

The heptapeptide angiotensin-(1-7) [Ang-(1-7)] is a new bioactive component in the renin–angiotensin–aldosterone system (RAAS) [34]. Angiotensinogen (AGT) is the precursor molecule of RAAS and its cleavage product is angiotensin I (Ang I). Ang I is a substrate of angiotensin converting enzyme (ACE) and yields Ang II, which is the major component of RAAS. Ang II binds 2 receptor subtypes, type 1 (AT1) and type 2 (AT2). Most Ang II actions are mediated by AT1, whereas AT2 mediates the beneficial effects counter-regulating those of AT1 [7]. Ang-(1-7) can be formed mainly from Ang II through the activity of ACE2 [34]. ACE2, a homolog of ACE, is a carboxypeptidase that catalyzes the conversion of Ang II to Ang-(1-7) [41]. Ang-(1-7) acts through binding to the specific Mas receptor, a seven-transmemberane G protein-coupled receptor encoded by *Mas1* oncogene [36].

Ang-(1-7) has been found to oppose many actions of Ang II in the heart, vessels, lungs, kidneys, brain and liver [10,35]. Thus, the RAAS is composed of 2 opposite arms: the ACE/Ang II/AT1 axis and the ACE2/Ang-(1-7)/Mas axis. The ACE2/Ang-(1-7)/Mas axis becomes the major counter-regulatory system against the ACE/Ang II/AT1 axis at both systemic and local levels [11,12,16]. As a strong vasoprotective factor, Ang-(1-7) exerts vasodilatation, anti-angiogenesis, anti-thrombosis, and anti-proliferation effects in vivo and in vitro [35]. However, the effects of Ang-(1-7) on VC still remain unclear and need to be explored. The aim of this study was to study the role of endogenous factors in VC. We examined whether Ang-(1-7) inhibits VC in a rat model of VC induced by vitamin D_3 plus nicotine (VDN) and investigated changes in major members of the ACE2/Ang-(1-7)/Mas and the ACE/Ang II/AT1 axes with calcification.

Table 1
Primers for quantitative real-time PCR.

Target		Sequence
AGT	Sense	5′-TCC ACC CCT TTC ATC TCC TCT-3′
	Antisense	5′-CTC GCA GGG TCT TCT CAT CC-3′
ACE	Sense	5′-ACG TCC CGG AAA TAC GAA G-3′
	Antisense	5′-GCA TCA GAG TAG CCG TTG AG-3′
ACE2	Sense	5′-ATC TAC CCA ACA CTT AAG CCA CC-3′
	Antisense	5′-TAC TTT CTC CTT TGC CAA TGT CC-3′
AT1	Sense	5′-CTC AAG CCT GTC TAC GAA AAT GAG-3′
	Antisense	5′-TAG ATC CTG AGG CAG GGT GAA T-3′
AT2	Sense	5′-ATCTGGCTGTGGCTGACTTAC-3′
	Antisense	5′-TTGCCAGGGATTCCTTCTC-3′
Mas	Sense	5′-TTGGTGGTGAAGATACGGAAGA-3′
	Antisense	5′-GCATGGGCATGGCAAAGAT-3′
BMP2	Sense	5′-TCA AGC CAA ACACAA ACA GC-3′
	Antisense	5′-TGA GCT AAG CTC AGT GGG-3′
Cbfα1	Sense	5′-GCC AGG TTC AAC GAT CTG AG-3′
	Antisense	5′-GAG GCG GTC AGA AAC AAA C-3′
OPN	Sense	5′-AGA CCA GCC ATG AGT CAA GTC A-3′
	Antisense	5′-TGA AAC TCG TGG CTC TGA TGT T-3′
OCN	Sense	5′-GGT GCA AAG CCC AGC GAC TCT-3′
	Antisense	5′-GGA AGC CAA TGT GGT CCG CTA-3′
β-Actin	Sense	5′-GAG ACC TTC AAC ACC CCA GCC-3′
	Antisense	5′-TCG GGG CAT CGG AAC CGC TCA-3′

AGT, angiotensinogen; ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme 2; AT1, angiotensin II type 1 receptor; AT2, angiotensin II type 2 receptor; Mas, the Mas receptor; BMP2, bone morphogenetic protein 2; Cbfα1, core binding factor α 1; OPN, osteopontin; OCN, osteocalcin.

2. Materials and methods

2.1. Animals and reagents

Male Sprague-Dawley (SD) rats (180–200 g) were obtained from the Animal Center, Health Science Center, Peking University (Beijing). All animal care and experimental protocols complied with the Animal Management Rule of the Ministry of Health, People's Republic of China (Document No. 55, 2001) and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Alzet Mini-Osmotic Pumps, model 2004, were from Durect Corp. (Cupertino, CA, USA). Synthetic Ang-(1-7) was from Phoenix Pharmaceuticals (Belmont, CA, USA). Vitamin D_3 and nicotine were from Sigma (St. Louis, Mo, USA). The ALP assay kit was from Nanjing Jiancheng Bioengineering Company (Nanjing, Jiangsu, China), the calcium assay kit was from Biosino Bio-Technology and Science (Beijing), the ACE activity assay kit was from Ningbo Ruiyuan Bio-Technology Co., Ltd (Ningbo, Zhejiang, China) and the ACE calibrator was from Audit Diagnostics (Cork, Ireland). Other chemicals and reagents were of analytical grade.

2.2. Rat model of VC and animal groups

The rat model of VC was performed as described previously [6,22,26] with minor modification. In brief, rats were randomly assigned to control group (Control), VC group (Cal), and Ang-(1-7) treatment group (Cal + Ang-(1-7)). 17 rats were given vitamin D_3 (300,000 IU/kg body weight, intramuscularly) plus nicotine (25 mg/kg body weight, in peanut oil, intragastrically) at 9 a.m. on day 1, and nicotine was re-administered at 6 p.m. on the same day. At 24 h later, 8 of these rats received Ang-(1-7) (24 μg/kg/hr) administered subcutaneously in saline through an Alzet Mini-Osmotic Pump, for 28 continued days. The dose and mode of delivery of Ang-(1-7) is performed as previously published studies [21,24] with minor modification. The remaining rats were the calcification control. An additional 8 rats were given saline as a vehicle control.

2.3. Measurement of hemodynamic features of rats

Hemodynamic features were measured by use of the Powerlab BL-420F Biological System (Tai-Meng Biotechnological Co., Chengdu, Sichuan, China). After rats were anesthetized intraperitoneally with pentobarbital sodium (45 mg/kg), a short PE-50 catheter filled with heparin saline (500 U/mL) was inserted to a depth of about 1.5 cm towards the heart via the right carotid artery. Hemodynamic features including heart rate (HR), systolic blood pressure (SBP), diastolic blood pressure (DBP), mean blood pressure (MBP), left ventricular systolic pressure (LVSP), LV end-diastolic pressure (LVEDP) and LV peak rate of contraction ($LV + dp/dt_{max}$) and relaxation ($LV - dp/dt_{max}$) were monitored. After rats were killed, the heart weight/body weight ratio (HW/BW) was measured, and aortas stripped of intima and adventitia were harvested. Samples were stored at −80 °C until use.

2.4. ALP activity assay

ALP activity in plasma and aorta was measured as described [6,22]. Abdominal aortic blood was collected and 2 mL of the sample was mixed with heparin (50 U/mL). Plasma was separated after centrifugation at 3000 rpm for 15 min at 4 °C. Aortic tissues were homogenized in ice-cold buffer (20 mmol/L HEPES, 0.2% NP-40, and 20 mmol/L $MgCl_2$, pH 7.4). After centrifugation at 8000 rpm for 10 min, supernatant was collected. The ALP activity of plasma and tissue supernatants was measured with use of the ALP assay kit. The