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Whey peptide Isoleucine-Tryptophan inhibits expression and activity of matrix metalloproteinase-2 in rat aorta

Irakli Kopaliani, Melanie Martin, Birgit Zatschler, Bianca Müller, Andreas Deussen*

Department of Physiology, Faculty of Medicine, Technische Universität Dresden, Germany

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ABSTRACT

Aortic stiffness is an independent risk factor for development of cardiovascular diseases. Activation of renin-angiotensin-aldosterone system (RAAS) including angiotensin converting enzyme (ACE) activity leads to overproduction of angiotensin II (ANGII) from its precursor angiotensin I (ANGI). ANGII leads to overexpression and activation of matrix metalloproteinase-2 (MMP2), which is critically associated with pathophysiology of aortic stiffness. We previously reported that the whey peptide Isoleucine-Tryptophan (IW) acts as a potent ACE inhibitor. Herein, we critically elucidate the mechanism of action by which IW causes inhibition of expression and activity of MMP2 in aortic tissue. Effects of IW on expression and activity of MMP2 were assessed on endothelial and smooth muscle cells (ECs and SMCs) *in vitro* and *ex vivo* (isolated rat aorta). As controls we used the pharmaceutical ACE inhibitor – captopril and the ANGII type 1 receptor blocker – losartan. *In vitro*, both ANGII and ANGI stimulation significantly ($P < 0.01$) increased expression of MMP2 assessed with western blot. Similarly, to captopril IW significantly ($P < 0.05$) inhibited ANGI, but not ANGII mediated increase in expression of MMP2, while losartan also blocked effects of ANGII. Signaling pathways regulating MMP2 expression in ECs and SMCs were similarly inhibited after treatment with IW or captopril. In ECs IW significantly ($P < 0.05$) inhibited JNK pathway, whereas in SMCs JAK2/STAT3 pathway, assessed with western blot. *In vitro* findings were fully consistent with results in isolated rat aorta *ex vivo*. Moreover, IW not only inhibited the MMP2 expression, but also its activation assessed with gelatin zymography. Our findings demonstrate that IW effectively inhibits expression and activation of MMP2 in rat aorta by decreasing local conversion of ANGI to ANGII. Thus, similar to pharmaceutical ACE inhibitor captopril the dipeptide IW may effectively inhibit ACE activity and prevent the age and hypertension associated rise of aortic stiffness.

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1. Introduction

During aging, elasticity of aorta is declining due to degradation of elastin and accumulation of collagen [1]. This process is largely accelerated during hypertension and results in development of aortic stiffness. Even though aortic stiffness was regarded as a hallmark of hypertension, it is now considered as an independent risk factor for development of cardiovascular diseases [2]. A stiffer aorta augments left ventricular afterload and systolic pressure, which may cause left ventricular hypertrophy and fibrosis [1]. Combined with impaired coronary perfusion this may progress to myocardial dysfunction and heart failure [1]. Overactivation of the renin angiotensin aldosterone system (RAAS) and its effector angiotensin II (ANGII), along with enhanced expression and activation of matrix metalloproteinase 2 (MMP2) has been strongly implicated in the pathophysiology of aortic stiffness [3,4]. ANGII and MMP2 activation is considered to result in the adverse remodeling of extracellular matrix (ECM), primarily degradation of elastin, largely mediated by action of MMP2, but also enhanced fibrosis and promotion of smooth muscle cell (SMC) hyperplasia resulting in vessel media hypertrophy [1,5]. Previous reports, including our own, established the RAAS and most importantly ANGII as a strong regulator of expression and activation of MMP2 in aorta [6–8]. In general, MMP2 is expressed and released into ECM as a latent 72 kDa pro-MMP2 and then proteolytically activated to 62 kDa MMP2 [9]. In a previous report [6] we demonstrated that both endothelial cells (EC) and smooth muscle cells (SMC) of rat

Abbreviations: ACE, angiotensin converting enzyme; ANGII, angiotensin II; ANGI, angiotensin I; AT1R, angiotensin II type 1 receptor; RAAS, renin-angiotensin-aldosterone system; ECM, extracellular matrix; MMP2, matrix metalloproteinase 2; EC, endothelial cell; SMC, smooth muscle cell; JNK, janus N-terminal kinase; JAK2, janus kinase 2; STAT3, signal transducer and activator of transcription 3.

* Corresponding author at: Department of Physiology, Faculty of Medicine Technische Universität Dresden Fetscherstr. 74, Germany.
E-mail address: Andreas.Deussen@tu-dresden.de (A. Deussen).

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aorta express MMP2. Furthermore, we showed cell-specific distinct molecular mechanisms of ANGII in regulation of MMP2. In ECs ANGII regulates MMP2 *via* mitogen activated protein (MAP) kinase pathway, specifically JNK → c-jun, whereas in SMCs *via* janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathway [6].

Animal studies using pharmaceutical ACE inhibitors conclusively demonstrate a beneficial role of RAAS inhibitors in development of arterial stiffness [10,11]. It is worth noting that the systemic RAAS is considered functionally distinct from that of local RAAS [12]. In this regard, intravascular ACE activity present on vascular endothelium throughout the circulation is considered as systemic ACE activity, which is mainly involved in blood pressure regulation and contributing to early development of hypertension [12]. In contrast, local ACE activity in vessel media is considered to be responsible for adverse remodeling of vessel wall ECM [13–15]. Most importantly, the benefit of pharmaceutical ACE inhibitors is believed to rely on the inhibition of local ACE activity along with systemic ACE inhibition. Therefore, direct local effects on vessel remodeling appear desirable rather than effects restricted to blood pressure control [12,13].

Even though antihypertensive therapeutic progress over the last few decades is evident, hypertension still remains a major worldwide health concern [16]. Targeting local ACE or angiotensin II type 1 receptor (AT1R) has been shown to be most effective in long term control of arterial blood pressure most likely through reversal of arterial remodeling [11,17]. However, a limitation of pharmacological treatment, even with ACE inhibitors, is that treatment starts after the manifestation of hypertension. At this time organ damage may already have occurred. This points to the importance of preventive treatment, starting in younger age. Animal studies support the potential effectiveness of this approach [11]. In addition to pharmaceutical ACE inhibitors, functional food additives have recently attracted attention in order to address this issue [18,19]. Previously, our group reported on the whey peptide Isoleucine-Tryptophan (IW), which effectively inhibits ACE activity [20] and lowers systolic blood pressure in spontaneously hypertensive rats (SHR) [21]. In this animal model, IW inhibits ACE activity both in plasma and aortic tissue. Our group also demonstrated that IW is well resorbed after oral uptake in humans and subsequently inhibits plasma ACE activity [22]. In SHR, along with effective inhibition of ACE in plasma and aortic tissue, IW treatment decreases expression and activities of MMP2 in aorta [21]. However, the available evidence does not conclusively demonstrate whether the inhibition of systemic (plasma) or local (in aorta) ACE activity is causal for decreased expression and activity of MMP2. Furthermore, some pharmaceutical ACE inhibitors (*e.g.* captopril, lisinopril) were shown to exert a direct inhibitory effect on MMP2 activity *in vitro* [23,24] pointing out the importance to clarify, whether IW exerts the same mode of action as pharmaceutical ACE inhibitors. The current study was undertaken to test whether the inhibitory effect of IW on expression and activity of MMP2 is specifically mediated by inhibition of tissue ACE activity or whether IW also exerts a direct inhibitory effect on enzymatic activity of MMP2.

2. Methods

2.1. Cell culture

Rat aortic endothelial and smooth muscle cells were purchased from Cell Applications Inc. (Cat. no. R304K-05a, R354K-05a,). The cells were cultured under sterile conditions according to a previous report and the user manual [6]. Briefly, ECs and SMCs were cultured and grown in T-75 flasks using rat aortic EC (Cat. no. R-211) and SMC (Cat. no. R-311) growth media, respectively. For experimentation, the cells were sub-cultured and grown in 12 well plates using a sub-culture kit. Stimulation of cells with ANGI and ANGII were performed in non-supplemented rat aortic EC (Cat. no. R-210) and SMC (Cat. no. R-310) media, respectively. The pharmaceutical ACE inhibitor captopril (CA) was used as a control to IW. Selective ANGII receptor type 1 (AT1R) block was performed by treatment with losartan as described before [6]. Cell lysates and supernatants were used for quantitative measurements.

2.2. Cell lysis

Cell lysis was performed using RIPA buffer (Cat. no. R0278, Sigma) according to the user manual. Briefly, after removal of non-supplemented media, the cells were quickly inoculated with 100 μl of RIPA buffer. The cells were shaken gently for 5 min and then scraped-off. The suspension was centrifuged to remove cell debris and supernatant was stored immediately at −80 °C until further experimentation.

2.3. Experiments on isolated rat aorta

Aorta was isolated from 10-week old male wistar rats ($n = 5$ per group). Use of animals was approved by the institutional ethics committee and by the local authorities (permission: 24-9168.24-1/2012-16). The rats were anesthetized with intraperitoneal injection of 1.3 g/kg BW urethane (ethyl carbamate). Under deep anesthesia the thoracic aorta was isolated. Aorta was divided in two 4 mm segments. One segment was left intact and from the other the endothelium was removed (denuded) as previously described [6]. Briefly, a cotton wire (with an outer diameter close to that of the inner lumen of the aorta) was gently moved back and forth 5–7 times. The successful removal of the endothelium was controlled by assessment of vessel relaxation in response to acetylcholine (see below). Removal of endothelium was also evidenced on sections of aorta stained with hematoxylin-eosin (see Fig. 1 in Ref. [30]). Stimulation of aorta with ANGI or ANGII with or without inhibitors was performed in non-supplemented Dulbecco's modified Eagle medium (DMEM, cat. no. 32430-027, Invitrogen). After stimulation, the segments were immediately frozen in liquid nitrogen and stored at −80 °C for protein extraction (see below).

2.4. Experiments on aortic ring

Efficient removal of endothelium from aorta without affected SMC function was assessed using a Mulvany myograph as previously described [25]. Briefly, aorta was pre-constricted with potassium-chloride (123.7 mmol/L KCl) and with noradrenaline (max. 10 μmol/L). On pre-contracted rings endothelium-dependent (with 10 μmol/L acetylcholine) and −independent (with 3.5 μmol/L sodium (Na) nitroprusside) relaxations were assessed, along with assessment of development of tension using noradrenalin (max. 10 μmol/L). Results are shown in Fig. 2 in Ref. [30]. Briefly, removal of endothelium largely abolished endothelium-dependent vasorelaxation in response to acetylcholine (10 μmol/L), but did not affect vasorelaxation in response to sodium-nitroprusside (3.5 μmol/L). Also, removal of endothelium did not affect development of tension in response to noradrenalin. Thus, sufficient removal of endothelium was achieved without affecting SMC function in aorta.

2.5. Protein extraction and assay

Proteins from aorta were extracted as previously reported with modifications [26]. Briefly, aorta was washed three-times in ice-cold phosphate buffered saline and then cut in small pieces followed with incubation in fresh, ice cold extraction buffer

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