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The PI3K signaling-mediated nitric oxide contributes to cardiovascular effects of angiotensin-(1-7) in the nucleus tractus solitarii of rats

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ABSTRACT

Angiotensin-1-7 [Ang-(1-7)], acting via the Mas receptor in the central nervous system, is involved in the regulation of cardiovascular activity. Nitric oxide (NO) is implicated as an important modulator in the nucleus tractus solitarii (NTS), a key region involved in control of cardiovascular activity. The aim of the present study was to determine the role of phosphatidylinositol 3-kinase (PI3K) signaling in mediating the effect of Ang-(1-7) on NO generation in the NTS. In Sprague–Dawley rats, acute injection of Ang-(1-7) into the NTS significantly increased NO generation and neuronal/endothelial NO synthase (n/eNOS) activity, which were abolished by the selective Mas receptor antagonist D-Alanine-[Ang-(1-7)] (A-779), the PI3K inhibitor LY294002, or the Akt inhibitor triciribine (TCN). Western blotting analysis further demonstrated that Ang-(1-7) significantly increased levels of Akt/NOS phosphorylation in the NTS, and Ang-(1-7)-induced e/nNOS phosphorylation was antagonized by LY294002 or TCN. Furthermore, gene knockdown of PI3K by lentivirus containing small hairpin RNA in the NTS prevented the Ang-(1-7)-induced increases in NOS/Akt phosphorylation and NO production. The physiological (*in vivo*) experiments showed that pretreatment with the NOS inhibitor L-NAME, LY294002, or TCN abolished the decreases in blood pressure, heart rate, and renal sympathetic nerve activity induced by Ang-(1-7) injected into the NTS. Our findings suggest that nitric oxide release meditated by the Mas-PI3K-NOS signaling pathway is involved in the cardiovascular effects of Ang-(1-7) in the NTS.

1. Introduction

The renin-angiotensin system (RAS) plays a crucial role in regulating cardiovascular activities. Although angiotensin II (Ang II) is a major contributor to the pathogenesis of cardiovascular diseases, other peptides such as Ang-(1-7) have also been demonstrated to mediate the biological actions of the RAS [1]. Ang-(1-7) is formed from Ang I and Ang II by several endopeptidases and carboxypeptidases, including Ang-converting enzyme 2 (ACE2) [2]. Interestingly, the evidence suggests that Ang-(1-7) opposes many of Ang II-induced actions via the G-protein-coupled receptor Mas, producing several beneficial effects such as vasodilation, inhibition of cell growth and proliferation [3,4]. In the central nervous system, Ang-(1-7) has also been reported to be involved in cardiovascular regulation [5–7]. An enhancement in central ACE2/Ang-(1-7)/Mas axis significantly improves baroreflex and decreases blood pressure (BP) in the hypertensive state [8–10]. It is well known that the nucleus tractus solitarii (NTS), which contains the first synapse of the peripheral signaling afferents (e.g. baroreceptors) in the central nervous system, plays a key role in tonic and reflex control of cardiovascular activity [11,12]. ACE2 and the Mas receptors are expressed in the NTS [13,14]. Additionally, the ACE2-Ang-(1-7)-Mas receptor axis in the NTS is involved in modulating BP and baroreflex sensitivity [8,15,16]. However, the molecular mechanism by which Ang-(1-7) is involved in cardiovascular regulation in the NTS remains unknown.

Recent studies indicate that nitric oxide (NO) might play an important role in mediating the biologic effects of Ang-(1-7) in the

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brain [17,18]. NO, which is generated as a co-product during a conversion of L-arginine to L-citrulline by the NO synthase (NOS), exhibits many biological effects in the central nervous system [19,20]. It has been documented that NO derived from neuronal- or endothelial-NOS (nNOS or eNOS) is an important modulator of cardiovascular regulation in the NTS [21–25]. Interestingly, Yang et al. reported that Ang-(1-7) is capable of stimulating NO release in the cultured neurons, which is prevented by a Mas receptor antagonist (D-Alanine-[Ang-(1-7)], A-779) [26]. It has been demonstrated that brain-selective overexpression of human ACE2 increases nNOS and eNOS expression and NO production in the NTS [6]. The above evidence indicates that NO mechanism may be involved in mediating the cardiovascular effect of Ang-(1-7) at the level of NTS. However, much less is known about the signaling pathway responsible for this process. In the NTS, the phosphatidylinositol 3-kinase (PI3K) signaling pathway is also mentioned to play an important role in cardiovascular regulation [27–29]. The myocardial PI3K/Akt/nNOS signaling is suggested to be responsible for the ethanol-induced hypotensive effect [30]. Moreover, renin activates eNOS in the NTS via the AT1R/Ras/PI3K/Akt/eNOS signaling which induces a reduction of BP, whereas leaving several questions, such as the role of nNOS and Ang-(1-7)/Mas axis in the regulation of BP in the NTS, remain to be solved [31]. Therefore, this study was designed to determine the involvement of PI3K signaling in mediating the relationship between Ang-(1-7) and NO generation in the NTS of rats.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats weighing between 300 and 350 g were supplied by Sino-British SIPPR/BK Laboratory Animal Ltd (Shanghai, China) in these experiments. Rats were housed in a temperature-controlled room and kept on a 12 h:12 h light–dark cycle with free access to water. All procedures in this study were approved by the Institutional Animal Care and Use Committee of Second Military Medical University.

2.2. Microinjections of test agents and collection of tissue samples from the NTS

Under anesthetized with urethane (800 mg/kg ip) and α-chloralose (40 mg/kg ip), the rat was placed in a stereotaxic frame with the head inclined downward by 30°, and the dorsal surface of the medulla was exposed. According to an atlas of the rat brain [32], microinjection of drugs was made at 6 sites (0.1, 0.3, 0.5 mm rostral to calamus scriptorius, 0.3, 0.4, 0.5 mm lateral to midline, respectively, 0.5 mm deep to surface) in the bilateral NTS using a glass micropipette, as shown in Fig. 1. Test agents used in this study included Ang-(1-7) (25 pmol/site), A-779 (500 pmol/site), LY294002 (100 pmol/site), and TCN (500 pmol/site). The injection volume for each site was 100 nl. The doses of these agents were based on previous studies [8,21,29]. A microinjection of artificial cerebrospinal fluid (aCSF) served as the vehicle and volume control. The test agent was injected at each site for 10 s. Thirty min after injection of agents into the bilateral NTS, the rats were euthanized by an overdose of pentobarbital sodium (200 mg/kg, ip). The brains were quickly removed, frozen and stored at −80 °C until use. According to the rat brain atlas [32], tissues on both sides of the NTS (at the level of 0–0.5 mm rostral to calamus scriptorius) were obtained by the use of a punch-out technique with a cryostat.

2.3. Measurements of NO production and NOS activity

The rats were euthanized by an overdose of pentobarbital sodium (200 mg/kg, ip.) after received acute NTS injection of the test agents. Then they were perfused transcardially with phosphate buffered solution. The NTS tissue was obtained by the use of a punch-out technique with a cryostat according to the rat brain atlas [32]. NO production was photometrically quantified for the amounts of stable product nitrite produced in the tissue using the nitrate reduction method and Griess reagent according to the instructions for the NO assay kit (S0021, Beyotime Institute of Biotechnology, Haimen, China), and then the absorbance was measured at 540 nm using a microplate reader (Molecular Devices, USA).

Total tissue NOS activity was determined by following the instruction of the NOS assay kit (S0025, Beyotime Institute of Biotechnology, Haimen, China). The NOS activity was quantified by the NO amount in the use of a fluorescent probe, DAF-FM DA, in the presence of NADPH and L-arginine for 30 min at 37 °C. To measure iNOS activity, tissue homogenates were incubated with the above factors and the specific iNOS inhibitor, SMT(S-Methylisothiourea hemisulfate salt) (20 μM). Then the reaction mixtures were transferred to a fluorescence microplate reader (Synergy2, Bio-Tek, USA), and fluorescence was measured at 515 nm with an excitation wavelength of 495 nm. In the blank control, cell lysis buffer, instead of the tissue homogenates, was added into 0.1 ml assay mixture and reacted at 37 °C in the dark for 30 min. The fluorescence intensity was named as relative fluorescence unit 1 (RFU1). The value in any other group was named as RFU2. The final RFU was calculated with the formula, RFU = RFU2-RFU1. The value of NOS activity (RFU/mg protein) was expressed as 100% in vehicle control group, and the relative NOS activity in other groups was compared to control value.

2.4. Western blot analysis

Western blot analysis was performed to detect the protein expression of n/eNOS, phosphorylated Akt/NOS, and PI3K p110δ in the NTS, as described in our previous studies [33–35]. Briefly, the protein concentration was measured and loaded (20 μg) onto a 7.5% SDS-PAGE gel and then transferred to a polyvinylidene fluoride membrane. The membrane was probed with primary antibody (anti-nNOS, anti-eNOS, anti-Akt, phospho-eNOS Ser1177, anti-phospho-Akt Ser473, and anti-p110δ from Cell Signaling Technology; anti-phospho-nNOS Ser1416 from ABCAM) overnight at 4 °C in PBS buffer. The blots were washed three times and then incubated with secondary antibody. Immunodetection was accomplished using a Western Blotting Luminol Reagent (32106, Thermo, USA) for chemiluminescent detection. The protein bands were visually detected by Syngene Bio Imaging system (No 55000 Gene Company Limited) and quantified by GeneTools software (Gene Company Limited). The levels of target proteins were normalized to β-actin (Sigma), which served as a loading control.

2.5. Lentiviral construction and preparation

Class IA PI3K is composed of a heterodimer between a p85 regulatory subunit and a p110 catalytic subunit (designated p110α, β, γ or δ catalytic subunit). In this study, lentiviral-mediated RNAi targeting PI3K p110δ catalytic subunit was chosen for down regulation of PI3K in the NTS. As described in our previous study [36], the lentivirus containing small hairpin RNA (shRNA) was constructed and prepared by Invabio biotechnology Co., Ltd (Shanghai, China). The rat PI3K p110δ specific shRNA fragment (target sequence: GTAAACGACTTCCGCACTA, negative control sequence: