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Losartan protects against cerebral ischemia/reperfusion-induced apoptosis through β-arrestin1-mediated phosphorylation of Akt

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ABSTRACT

Losartan, an angiotensin (Ang) II type 1 receptor blocker (ARB), has been revealed to protect against cerebral ischemia/reperfusion (I/R) injury. However, the mechanism by which losartan protect brain ischemia injury is still obscure. In this study, we investigated whether losartan protected against cerebral I/R injury by reducing apoptosis and the possible signaling pathways. Wistar rats were pretreated for 14 days with 5 mg/kg losartan, and then subjected to middle cerebral artery occlusion (MCAO) for 2 h followed by reperfusion. Meanwhile, PC12 cells pretreated with losartan were exposed to oxygen-glucose deprivation-reoxygenation (OGD/R), an in vitro model of cerebral ischemia. Our results showed that administration of losartan significantly inhibited the apoptosis by decreasing the number of apoptotic cells, decreasing the protein level of cleaved caspase-3, cytochrom C and Bax, and increasing the level of Bcl-2 both in vivo and in vitro. Moreover, losartan treatment markedly enhanced the phosphorylation of Akt and blockade of PI3K activity by wortmannin dramatically inhibited Akt phosphorylation and attenuated the anti-apoptotic effect of losartan. Furthermore, pretreatment with losartan significantly increased the protein level of β-arrestin1 and silence of β-arrestin1 by siRNA partly attenuated losartan-induced anti-apoptotic effect and the phosphorylation of Akt. These results suggested that β-arrestin1 modulated the activation of Akt in losartan-induced anti-apoptotic effect in cerebral I/R. Our data would provide a new molecular basis for further understanding of protective effect of losartan in cerebral I/R injury and may provide benefits of using losartan in the treatment of cerebrovascular disease.

1. Introduction

Acute ischemic stroke is one of the main causes of death and permanent disability in adults worldwide with a major health and economic impact, and greatly consumes medical resources (Go et al., 2014). While a complex sequence of pathophysiological events involved in stroke development and progression, angiotensin II (Ang II), the main effector of the renin-angiotensin system (RAS), is thought to play an important role in cerebral ischemic injury (Mateos et al., 2016; Mogi et al., 2014). Previous study showed that brain Ang II levels were increased 4–12 h after reperfusion following middle cerebral artery occlusion (MCAO) through the local generation of Ang II and the reduction of Ang II improved neurological outcome and reduced brain injuries in animal models of focal cerebral ischemia (Fu et al., 2011).

Ang II can activate a series of cell signaling pathways via two subtypes of G-protein coupled receptors (GPCRs), Ang II type 1 (AT_1) receptor and type 2 (AT_2) receptor (Horiuchi et al., 2010). The AT_1 receptor blockers (ARBs), such as losartan, have been confirmed to reduce the infarct size and cerebral edema and improved the outcomes from cerebral ischemia in animal experiments (Liu et al., 2012). Moreover, the protective effect of ARBs in acute ischemic stroke and the utility of this therapy for stroke were revealed by several clinical trials (Lindholm et al., 2002; Sandset et al., 2011; Schrader et al., 2003). However, the detailed mechanisms underlying the effects of ARBs protecting against cerebral ischemia/reperfusion (I/R) injury remain unclear.

As we all known, apoptosis is one of the major mechanism that lead to cell death after cerebral I/R. The phosphoinositide3-kinase/protein kinase B (PI3K/Akt) pathway is a central mediator in signal

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transduction pathways involved in cellular activation, inflammatory response, and apoptosis. The neuroprotective role of the PI3K/Akt pathway in cerebral ischemia has been widely studied (Hou et al., 2016; Tu et al., 2016). Our previous study has confirmed that losartan stimulated eNOS phosphorylation through PI3K/Akt signaling after MCAO-reperfusion in rat (Liu et al., 2012). However, it is unknown whether losartan can regulate apoptosis via the PI3K/Akt pathway in cerebral I/R.

β-arrestins (β-arrestin1 and β-arrestin2) were initially identified as the proteins desensitizing and internalizing GPCRs (DeWire et al., 2007). Moreover, recently β-arrestins have emerged as scaffold proteins to interact with other proteins and then influence intracellular signaling pathways dependent or independent of GPCRs (Chen et al., 2016; Tan et al., 2015). Wang et al. reported that β-arrestin1, but not β-arrestin2, protects against cerebral ischemic injury via prompting the autophagic process, and abolishing apoptosis and/or necrosis (Wang et al., 2014b). Arrestin-dependent signals regulating both ERK1/2 and Akt downstream of the angiotensin AT_{1A} receptor are critical mediators of GPCR-regulated protein translation (Ahn et al., 2009). Recently AT_1 receptor has been revealed to activate the PI3K/Akt pathways in a β-arrestin-dependent manner (Kendall et al., 2014). However, the role of β-arrestins1 in ARB mediated neuroprotection in cerebral ischemia is essentially uncharacterized. Here we demonstrated that losartan protected against cerebral I/R injury by reducing apoptosis via PI3K/Akt pathway regulated by β-arrestin1.

2. Materials and methods

2.1. Drugs and reagents

The losartan was purchased from Merck Sharp & Dohme (Hangzhou, China). All chemicals and reagents used in this study were of analytical grade and were purchased from Sigma (St Louis, MS, USA) unless otherwise mentioned.

2.2. Animal models for transient focal cerebral ischemia

Male Wistar rats (270–300 g) were purchased from Laboratory Animals Center of Shandong University and subjected to transient MCAO as described previously (Chen et al., 2014). All procedures were pre-approved by the Institutional Animal Use Committee. All surgeries were performed under sodium pentobarbital anesthesia and all efforts were made to minimize suffering. After 2 h of MCAO, reperfusion was initiated by the thread careful withdrawal.

Animals were randomly divided into the following groups: (1) sham group; (2) I/R group; Rats treated with vehicle (0.9% normal saline (NS)) orally for 14 days prior to MCAO; (3) I/R plus losartan group; I/R treated with 5 mg/kg per day losartan (dissolved in NS) orally for 14 days prior to MCAO. The dose of losartan was chosen based on our preliminary studies. (4) I/R plus wortmannin (PI3K inhibitor) group; (5) I/R plus losartan plus wortmannin group. Wortmannin was intraventricularly administered to rats with or without losartan 1 h before MCAO. The scalp was incised on the midline and the skull was exposed. Wortmannin (100 nM in 1% dimethyl sulfoxide in NS) or the vehicle (1% dimethyl sulfoxide in NS) (10 μl, 0.5 mm posterior and 1.4 mm lateral to the bregma and 3.1 mm from the duramater) was injected using a stereotaxic instrument and injector (Stoelting Co., Wood Dale, IL, USA). After 2 h, 8 h and 24 h of reperfusion, the rats were anesthetized, perfused with NS and then decapitated. The brains were immediately frozen in liquid nitrogen for the following studies.

2.3. Cell culture and treatment

PC12 cells were generously provided by Dr. Lihua Bao, Shandong University and were cultured in DMEM containing 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were subjected to the model of oxygen-glucose deprivation (OGD) (2 h of OGD followed by 6 h, 12 h, 24 h and 48 h of reoxygenation respectively) as described. Cells were pretreated with losartan 1 h before being exposed to OGD and incubated with losartan for an additional 24 h for reoxygenation.

2.4. Cell transfection

siRNA β-arrestin1 was purchased from Cell Signaling Technology (Beverly, MA, USA). siRNA AT_{1A} receptor was purchased from Hanbio Biotechnology (Shanghai, China). PC12 cells were seeded in culture plates 24 h before transfection. Transfection was performed using lipofectamine 3000 reagent (Invitrogen Corporation, Carlsbad, CA, USA).

2.5. Cell death/viability assessment

PC12 cells cultured in 96-well plate were pretreated with losartan (10 μM) for 1 h before being exposed to OGD for 2 h and incubated with losartan for an additional 24 h reoxygenation. OGD/R-induced cell death was determined by the cell counting kit-8 (CCK-8) (Dojindo China Co, Shanghai, China) assay.

2.6. Flow cytometry

Apoptotic cells were measured by flow cytometry using FITC-annexin V and propidium iodide (PI) staining. Briefly, approximately 1×10^5 cells were collected and washed with PBS twice, and subjected to FITC-annexin V and PI staining using annexin V-FITC/PI apoptosis detection kit (4A Biotech, Beijing , China). After staining, the assay was performed by a flow cytometry (Beckman coulter, USA).

2.7. TUNEL assay

Tissue sections or cultured cells were harvested for terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay using an in situ apoptosis detection kit (Roche Diagnostic , Indianapolos, IN) as described previously (Chen et al., 2014). TUNEL positive cells displayed brown staining within the nucleus and were counted in 3 non overlapping microscopic eye shots by a person blinded to the group assignment.

2.8. Western blot

Western blot was performed as described previously (Liu et al., 2015). Briefly, total proteins were isolated from mouse penumbral cortex and PC12 cells with RIPA (Beyotime, Haimen, China) buffer. Protein concentrations were determined using a BCA Protein Assay reagent kit (Beyotime, Haimen, China). The primary antibodies used in this study were: Bcl-2, Bax, cytochrome C, β-arrestin1 and GAPDH (Santa Cruz, CA , USA), p-Akt and total Atk (Cell signaling technology, Beverly, MA , USA), cleaved caspase-3, pro-caspase-3 (Proteintech, Wuhan, China). The signals were quantified by scanning densitometry and data within a linear range were quantified using Image Quant software (GE Amersham, Piscataway, NJ, USA).

2.9. Real-time PCR

RNA extraction and reverse-transcription were performed as described previously (Wang et al., 2014a). Real-time PCR analysis was performed with SYBR green (Invitrogen Applied Biosystems, Carlsbad, CA, USA) using a Bio-Rad iCycler system (Bio-Rad, Hercules, CA, USA). The mRNA levels of target gene were normalized by the housekeeping gene GAPDH. Relative expression was determined by the $2^{-\Delta\Delta CT}$ method. The specific primers are listed in Table 1.