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Angiotensin II type 1 receptor-associated protein regulates carotid intimal hyperplasia through controlling apoptosis of vascular smooth muscle cells

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ABSTRACT

Intimal hyperplasia is the main cause of restenosis after carotid artery injury, and the underlying mechanism involves the proliferation and migration of vascular smooth muscle cells (VSMCs). Angiotensin II Type 1 Receptor-Associated Protein (ATRAP) has been reported to withstand intimal hyperplasia by inhibiting VSMCs proliferation and migration; however, whether the beneficial effect of ATRAP associates with VSMCs apoptosis remains unclarified. We demonstrated that the adenoviral-mediated overexpression of ATRAP induced VSMC apoptosis, alleviating the balloon injury-induced neointima formation in rats. Under the condition of Angiotensin-II stimulation, ATRAP overexpression induced the apoptosis of rat VSMCs by depressing the PI3K-Akt signaling; whereas up-regulation of Akt by PTEN inhibitor abolished the apoptotic death. Thus, ATRAP regulates carotid intimal hyperplasia through controlling the PI3K-Akt signal-mediated VSMCs apoptosis.

1. Introduction

Atherosclerotic carotid artery stenosis accounts for 7–10% of transient ischemic attack and stroke [1]. Current clinical treatment of carotid stenosis includes percutaneous transluminal balloon angioplasty or carotid artery stenting (CAS) and carotid endarterectomy (CEA), supplemented by multifaceted medical therapy. However, severe restenosis or occlusion of the carotid artery was often observed after the revascularization with CAS and CEA, which might restrict the long-term efficacy of endovascular treatment and endarterectomy for preventing stenosis. Postoperative restenosis is an important problem looming over clinicians [2–4].

Primary pathological change of post-angioplasty restenosis is neointimal formation, which involves the proliferation, the migration toward the intima and the matrix accumulation of vascular smooth muscle cells (VSMCs) induced by the increased growth factors and the loss of growth-inhibitory factors [5,6]. The increase of Angiotensin II (Ang-II) in the injuried arteries contributes to endothelial dysfunction via vascular NADPH oxidase activation, production of reactive oxygen species and nitric oxide (NO) reduction. Moreover, Ang-II promotes VSMCs proliferation and migration by activating platelet-derived growth factors (PDGF), basic fibroblast growth factors (bFGF), transforming growth factor-β (TGF-β), and matrix metalloproteinases (MMPs) [7].

Angiotensin II Type 1 Receptor-Associated Protein(ATRAP) interacts specifically with the carboxyl-terminal cytoplasmic domain of Ang-II type 1 receptor(AT1R) but not with those of Ang-II type 2(AT2), M3 muscarinergic acetylcholine, B_2 bradykinin, endothelin B, and β_2 adrenergic receptors [8,9]. In vitro, overexpression of ATRAP induces the desensitization and/or internalization of AT1 receptor and markedly inhibits the phosphorylation of signal transducers and activators of transcription 3 (STAT3), Akt and extracellular signal–regulated kinase (ERK) [10,11]. ATRAP also attenuates the Ang-II-mediated increases in c-fos gene expression and TGF-β production, decreasing the proliferative response in vascular smooth muscle cells [12]. In vivo, the transgenesis of ATRAP inhibits neointimal formation after cuff-mediated vascular injury in mice, which is attributable to the reductions in cell proliferation, NADPH oxidase activity and p22phox [13]. These reports suggest that ATRAP as a negative regulator of AT1 receptor plays an inhibitory role in VSMCs proliferation both *in vitro* and *in vivo*, and thus in neointimal formation.

The dynamic balance of cell proliferation and cell death of

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VSMCs is important for vessel homeostasis in response to various external stress stimuli. A fundamental strategy for the treatment of neointimal formation is to inhibit VSMC proliferation and/or to promote VSMCs apoptosis [14]. Emerging evidence demonstrates that captopril inhibits neointimal formation by triggering the apoptotic death of neointimal VSMCs in the rat carotid artery balloon injury model [15]. However, the effect of ATRAP on VSMC apoptosis and the underlying mechanism remain largely elusive. Here we demonstrated that overexpression of ATRAP promotes VSMC apoptosis by inhibiting the phosphoinositide 3-kinase (PI3K)-Akt (PI3K-Akt) signaling pathway to prevent intimal dysplasia disorders. This finding suggests that ATRAP as a negative regulator of the PI3K-Akt axis, tilts the balance toward apoptosis of VSMCs.

2. Materials and methods

2.1. Agents

Angiotensin-II and PTEN inhibitor potassium bisperoxo (bipyridine) oxovanadate (bpv, phen) were purchased from Med Chem Express (USA). Rabbit monoclonal antibodies against FLAG (DYKDDDDK), caspase-8, caspase-3, cleaved-caspase-3, bax, Akt and phospho-Akt (Ser473) and total Akt (?) were purchased from Cell Signaling Technology, Inc (Germany). Mouse monoclonal antibodies against bcl-2 and GAPDH were purchased from Santa Cruz Biotechnology (USA) and Invitrogen (USA), respectively. Alexa Fluor® 790-conjugated goat anti-rabbit (mouse) antibodies were purchased from Abcam (UK).

Rat ATRAP and GAPDH primers were synthesized by Sangon (Shanghai, China). RNAiso Plus total RNA extraction kit and PrimeScript™RT Master Mix were obtained from Takara Biomedical Technology (BeiJing, China). SYBR Green real time PCR master mix were ordered from Toyobo BIOTECH CO., LTD (Osaka, Japan). In Situ cell death Detection Kit TMR red was purchased from Roche Applied Science (Switzerland).

2.2. Cell culture

VSMCs were obtained from thoracic aorta specimens of 5-week–old male Sprague-Dawley rat using a standard enzymatic dissociation technique as described previously [16]. VSMCs were cultured in the high glucose DMEM containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 g/ml streptomycin. Cultures were maintained in a humidified 5% CO_2 atmosphere at 37 °C. VSMCs in passage 4 through 7 were applied for the experiments.

2.3. Preparation of adenoviral vectors and transfection

Adenoviral vectors were prepared using cDNAs encoding FLAG-ATRAP (Adv.ATRAP) with a commercially available system (pHBAd-EF1-MCS-GFP Plasmid vector, Hanbio Biotechnology). Adenoviral vector expressing GFP (Adv.GFP) was used as a control virus. All experiments were performed 24 h after infection.

2.4. VSMCs apoptosis assay

The role of ATRAP in VSMCs apoptosis was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay. In brief, VSMCs were seeded in 96-well plates with 1×10^4 cells/well in DMEM supplemented with 10% FBS. After adhesion to the plate and at 50–70% confluence, VSMCs were infected with either Adv.ATRAP or Adv.GFP (200 MOI) for 24 h, and then the virus was removed by washing with PBS. After another 24 h in 0.1% FBS/DMEM, cells were either incubated with/without Angiotensin-II (100 nM) and with or without supplementation of bpv (200 nM) for 24 h. Subsequently, the cells were fixed in 4% paraformaldehyde for 1 h, and the apoptosis of VSMCs was measured by TUNEL analysis according to the manufacturer's protocol. The apoptosis index was calculated using the following formula: [(the number of TUNEL-positive cells)/(total cells) × 100%].

2.5. Real-time PCR

Total RNA was isolated from the pretreated VSMCs using a RNAiso Plus total RNA extraction kit, and the cDNA was synthesized using a PrimeScript™RT Master Mix. After reverse transcription of the RNA, cDNA was used as a template for PCR reactions with gene-specific primer pairs (Table 1). Real-time PCR was performed on an Applied Biosystems QuantStudio® 6 Flex (Applied Biosystems, USA). GAPDH was used as the endogenous control for mRNA expression. The fold changes of ATRAP expression were determined with the ΔΔCt method, and the relative expression of these genes was calculated by the $2^{-\Delta\Delta CT}$ method and normalized to GAPDH.

2.6. Western blotting analysis

When cells reach ~70–80% confluence, Adv.ATRAP or Adv.GFP (200 MOI) was added into the medium for 48 h. Then, the cells were lysed in RIPA buffer with a protease inhibitor mixture and centrifuged at 13,000 rpm for 20 min. The resulting supernatant was separated by 10% SDS–PAGE gel and electrotransferred to polyvinylidine difluoride membranes(PVDF). Membranes were blocked with 5% milk in 0.1% TBST before incubation with primary antibodies against caspase-3 (1: 1000), cleaved-caspase-3 (1: 1000), caspase-8(1: 1000), bax (1: 1000), bcl-2 (1: 200), FLAG (1: 1000) or GAPDH (1: 10,000) overnight at 4 °C, and followed by Alexa Fluor® 790-conjugated secondary antibodies (1:10,000) for 1 h. Fluorescent signals were collected using Odyssey LI-COR infrared imaging system (LI-COR, Lincoln, NE, USA). The quantification of western blots was done with Image Studio Lite Ver 3.1 (LI-COR).

2.7. Phosphorylation signal detection

The adenovirus transfected VSMCs were stimulated by Angiotensin-II with or without bpv. At indicated times (0, 15, 30, 60, 120, 240 min) after treatment, cell lysate was collected for determining Akt activity. Western blotting was done as mentioned-above. Blots were incubated with primary antibodies against phospho-specific Akt (1:1000), total Akt (1:1000) and FLAG (1:1000).

2.8. Animals preparation

Adult male Sprague–Dawley rats(24 animals, body weight ranging 350–500 g)were used. All rats were provided by Shanghai Municipal Laboratory Animal Center. The animals were housed in a room where lighting was controlled (12 h on, 12 h off) and the room temperature was kept at 25 °C. They were given a standard diet and water ad libitum. All surgical procedures and postoperative care were performed in accordance with guidelines of US National

Table 1
The primers for real-time PCR.

Gene	Sequence(5′-3′)	Sequence(3′-5′)
ATRAP	TGCTTGGGGCAACTTCACTATC	ACGGTGCATGTGGTAGACGAG
GAPDH	CTGGTGCTGAGTATGTCGTGGA	AGTTGGTGGTGCAGGATGCATT

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