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Homocysteine up-regulates ET_B receptors via suppression of autophagy in vascular smooth muscle cells

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ABSTRACT

The change of autophagy is implicated in cardiovascular diseases (CVDs). Homocysteine (Hcy) up-regulates endothelin type B (ET_B) receptors in vascular smooth muscle cells (VSMCs). However, it is unclear whether autophagy is involved in Hcy-induced-up-regulation of ET_B receptors in VSMCs. The present study was designed to examine the hypothesis that Hcy up-regulates ET_B receptors by inhibiting autophagy in VSMCs. Hcy treated the rat superior mesenteric artery (SMA) without endothelium in the presence and absence of AICAR, rapamycin or MHY1485 for 24 h. The contractile responses to sarafotoxin 6c (S6c) (an ET_B receptor agonist) were studied using a sensitive myograph. Levels of protein expression were determined using Western blot analysis. Punctate staining of LC3B was exanimated by immunofluorescence using confocal microscopy. The results showed that Hcy inhibited AMPK, and activated mTOR, followed by impairing autophagy, and increased the levels of ET_B receptor protein expression and the ET_B receptor-mediated contractile responses to S6c in SMA without endothelium. However, these effects were reversed by AICAR or rapamycin. Additionally, MHY1485 up-regulated the AICAR-inhibited ET_B receptor-mediated contractile response and the levels of ET_B receptor protein expression in presence of Hcy. In conclusion, this suggested that Hcy up-regulated ET_B receptors by inhibiting autophagy in VSMCs via AMPK/mTOR signaling pathway.

1. Introduction

Autophagy is a highly conserved and dynamic process of self-digestion, during which malfunctioning organelles, denatured proteins and a variety of macromolecules are degraded and recycled for cellular renovation (Mizushima and Komatsu, 2011; Choi et al., 2013). It plays a pivotal regulatory role in cellular homeostasis. Accumulating evidence demonstrated that autophagy regulates the metabolism, survival, and function of numerous cell types, including those comprising the cardiovascular system (Salabei and Hill, 2015). The change of autophagy is implicated in various vascular disease, including hypertension (Long et al., 2013), vascular aging (La Rocca et al., 2012), atherosclerosis (Martinet and De Meyer, 2009), and restenosis (Grootaert et al., 2015).

Imbalance of endothelin (ET) system consisting of ligands and their receptors plays an essential role in cardiovascular pathogenesis. Specifically, abnormal of ET receptor is a main cause dysfunction of ET system (Agapitov and Haynes, 2002). There are two ET receptor subtypes: endothelin subtype A (ET_A) and endothelin and subtype B (ET_B). ET_A receptors are present on vascular smooth muscle cells (VSMCs), where they mediate muscle contraction and regulating blood pressure. ET_B receptors are found on both endothelial and VSMCs. Normally, ET_B receptors are situated on vascular endothelial cells, and mediate vasodilation via release of nitric oxide and prostacyclin (Brunner et al., 2006) and clearance of endothelin-1 (ET-1) from the circulation (Kelland et al., 2010). Under pathological conditions [such as stroke (Vikman et al., 2006), coronary ischemic heart disease (Wackenfors et al., 2004), hypertension (Nilsson et al., 2008), and atherosclerotic plaque (Iwasa et al., 1999)], ET_B receptors are primarily located in

Abbreviations: AMPK, AMP-activated protein kinase; CVDs, Cardiovascular diseases; DAPI, 4,6-diamido-2-phenylindole dihydrochloride; DMEM, Dulbecco's modified Eagle's medium; DMSO, Dissolved in dimethyl sulfoxide; ET, Endothelin; ET-1, Endothelin-1; ET_A, Endothelin type A; ET_B, Endothelin type B; Hcy, Homocysteine; HHcy, Hyperhomocysteinemia; LC3B, Protein light chain 3B; mTOR, Mammalian target of rapamycin; PSS, Physiologic buffer solution; S6c, Sarafotoxin 6c; SMA, Superior mesenteric artery; VSMCs, Vascular smooth muscle cells

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VSMCs. ET_B receptors in VSMCs may mediate vasoconstriction in cardiovascular diseases (CVDs) (Dimitrijevic et al., 2009). As yet, it is unclear how autophagy is involved in regulation of ET_B receptors in VSMCs.

Hyperhomocysteinemia (HHcy) is a clinical condition characterized by increased levels of plasma homocysteine (Hcy) and a well-known risk factor for CVDs. Hcy is a sulfur-containing non-protein amino acid formed during the intracellular conversion of methionine to cysteine. Previous study demonstrated that HHcy impaired autophagy in primary astrocytes (Tripathi et al., 2016). But effect of HHcy on autophagy in VSMCs remains elusive. In addition, our previous study found that Hcy could up-regulate ET_B receptors in VSMCs (Chen et al., 2016a; Chen et al., 2016b). However, it is unclear whether autophagy is involved in Hcy-induced-up-regulation of ET_B receptor in VSMCs.

Based on these reports, our hypothesis is that Hcy up-regulated ET_B receptors via suppression of autophagy in VSMCs. The present study was designed to test our hypothesis. It may provide us with a new perspective for mechanism of Hcy-induced CVDs.

2. Materials and methods

2.1. Reagents

The selective ET_B receptor agonist sarafotoxin6c (S6c) (Fluka/Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 0.9% saline with 0.1% bovine serum albumin. AICAR (an agonist of AMP-activated protein kinase (AMPK) (5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside)(Fluka/Sigma-Aldrich, St. Louis, MO, USA), rapamycin [an inhibitor of mammalian target of rapamycin (mTOR)] (Rap) (Fluka/Sigma-Aldrich, St. Louis, MO, USA) and MHY1485 (an agonist of mTOR) were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO (vehicle) used was 1 μL/mL, which equals the volume of the inhibitor added to the organ culture. The DMSO concentration was the same in all test conditions, and it presented in the VSMCs or organ culture without the inhibitors to serve as the control. DL-Hcy (Fluka/Sigma-Aldrich, St. Louis, MO, USA) was diluted in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine (584 mg/L) containing 5.5 mmol/L D-glucose (Gibco/Invitrogen, Carlsbad, CA, USA) just before the experiments.

2.2. Tissue preparation and organ culture procedure

Male Sprague-Dawley rats (300–350 g) obtained from the Animal Center of Xi'an Jiaotong University, and were euthanized with CO_2. The superior mesenteric artery (SMA) was gently removed and freed from adhering tissue under a dissecting microscope. The endothelium was denuded by perfusion of the vessel for 10 s with Triton X-100 (0.1%, v/v). Then, physiologic buffer solution (PSS) (NaCl 119 mM, KCl 4.6 mM, $NaHCO_3$ 15 mM, NaH_2PO_4 1.2 mM, $MgCl_2$ 1.2 mM, $CaCl_2$ 1.5 mM, and glucose 5.5 mM) perfused the vessel for 10 s to wash out the Triton X-100. The vessels were then cut into 1 to 3 mm long cylindrical segments. The cylindrical segments were incubated at 37 °C in a humidified atmosphere of 5% $CO_2$and 95% air in DMEM with L-glutamine (584 mg/L)supplemented with penicillin (100 U/mL) (Life Technologies, Carlsbad, CA, USA), and streptomycin (100 mg/mL) (Life Technologies, Carlsbad, CA, USA). AICAR (500 μM), Rap (100 nM) and MHY1485 (10 μM) were added to the medium before incubation (Chen et al., 2016a; Chen et al., 2016b). The animal experiments in this study were approved by the Laboratory Animal Administration Committee of Xi'an Medical University and were performed according to the Guidelines for Animal Experimentation of Xi'an Medical University 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).

2.3. In vitro pharmacology

Incubated segments were immersed in temperature-controlled (37 °C) individual myograph baths (Organ Bath Model 700MO, J.P. Trading, Aarhus, Denmark) containing 5 mL PSS. The PSS was continuously aerated with 5% CO_2 in O_2, resulting in a pH of 7.4. The arterial segments were mounted for continuous recording of isometric tension with LabChart 7 Pro software (ADInstruments, Hastings, UK). A resting tone of 2 mN was applied to each segment, and the segments were allowed to stabilize at this tension for at least 1.5 h before exposure to a potassium-rich (60 mM K^+) buffer solution with the same composition as the standard solution, except that the NaCl was replaced by an equimolar concentration of KCl. The potassium-induced contraction was used as a reference for contractile capacity, and the segments were used only if potassium elicited reproducible responses over 1.0 mN. Concentration-response curves for S6c (10^{-11} M–10^{-7} M) were obtained by cumulative administration of the reagent (Chen et al., 2016a; Chen et al., 2016b).

2.4. Western blotting

The artery segments were lysed on ice for 1 h in RIPA buffer [Tris-HCl (pH 8.0) 50 mM, NaCl150 mM, 1% TritonX-100 (v/v), 1% deoxycholic acid (w/v), and 0.1% sodium dodecyl sulfate] containing 0.5 mM PMSF and protease inhibitors (Roche, Basel, Switzerland). The protein concentration was measured. Each sample was subsequently denatured by boiling for 5 min in Laemmle loading buffer. Equal amounts of protein were separated by 10% SDS-polyacrylamide gel electrophoresis and were transferred to PVDF membranes. After blocking with 5% bovine serum albumin or non-fat dried milk for 1 h at 37 °C, the membranes were incubated overnight at 4 °C with the following antibodies: anti-phospho-AMPKα antibody (Cell Signaling Technology, Danvers, MA, USA),anti-phospho-mTOR antibody (Cell Signaling Technology, Danvers, MA, USA), anti-AMPKα antibody (Cell Signaling Technology, Danvers, MA, USA), anti-mTOR antibody (Cell Signaling Technology, Danvers, MA, USA), anti-protein light chain 3B (LC3B) antibody(Beyotime Institute of Biotechnology, Haimen, China), anti-Beclin-1 antibody (Cell Signaling Technology, Danvers, MA, USA), anti-p62 antibody(Cell Signaling Technology, Danvers, MA, USA), anti-ET_B receptor antibody (GeneTex, Irvine, CA, USA), and anti-β-actin antibody (Abcam, Cambridge, MA, USA). After washing, the membranes were incubated using horseradish peroxidase-conjugated goat anti-mouse or -rabbit IgG (Thermo Fisher Scientific Pierce, Rockford, IL, USA) for 1 h at 37 °C, followed by enhanced chemiluminescence using the SuperSignal West Pico Substrate kit (Merck Millipore, Bedford, MA, USA). The protein bands were analyzed using the ChemiDoc-it HR 410 imaging system (UVP, Upland, CA, USA) (Chen et al., 2016a; Chen et al., 2016b).

2.5. Immunofluorescence examinations

The arterial segments were cut into 10 μm sections and mounted on slides. Following fixation, permeabilization, and blocking, the sections were incubated with rabbit polyclonal anti-LC3B antibody (Beyotime Institute of Biotechnology, Haimen, China) overnight at 4 °C. Subsequently, the sections were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG at room temperature for 1 h in the dark. Nuclei were labeled with 4,6-diamido-2-phenylindole dihydrochloride (DAPI) (Sigma Aldrich, MO, USA; final concentration of 1 μg/mL) Images were captured using a Leica TCS SP8 confocal microscope (Leica, Wetzla, Germany) and processed with Image J software (National Institutes of Health, DC, USA) (Chen et al., 2014).

2.6. Statistical analysis

All data are expressed as mean ± SEM. S6c-induced vasoconstriction was presented as a percentage of contraction induced by 60 mM K^+.