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Homocysteine up-regulates endothelin type A receptor in vascular smooth muscle cells through Sirt1/ERK1/2 signaling pathway

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ABSTRACT

Sirtuin 1 (Sirt1) is a longevity gene that has protective effects in cardiovascular diseases (CVDs). The endothelin type A (ET_A) receptor is involved in pathogenesis of CVDs. The extracellular signal related kinases 1 and 2 (ERK1/2) signaling pathway is involved in regulation of the ET_A receptor induced by some CVD risk factors in vascular smooth muscle cells (VSMCs). Hyperhomocysteinemia (HHcy) is an independent risk factor for CVDs. The present study was designed to investigate the hypothesis that homocysteine up-regulates ET_A receptor through the Sirt1/ERK1/2 signaling pathway. In vitro experiments were performed in the rat superior mesenteric artery. The rat superior mesenteric artery was cultured with or without homocysteine (Hcy) in the presence and absence of Resveratrol (Res, a Sirt1 agonist), SRT1720 (a specific Sirt1 agonist) or U0126 (an ERK1/2 signaling pathway inhibitor) in serum-free medium for 24 h. In vivo, the rats received subcutaneous injections of Hcy in the presence of or absence of Res or U0126 for 3 weeks. The contractile response to ET-1 was studied using a sensitive myograph. In addition, the level of protein expression was determined using western blotting. Hcy significantly increased the expression of ET_A receptor and also increased the ET_A receptor-mediated contractile response induced by ET-1 in vitro. These effects were inhibited by Res, SRT1720 and U0126 treatment. In addition, Hcy down-regulated the level of Sirt1, and up-regulated the level of phosphorylated ERK1/2, which was reversed upon Res or SRT1720 treatment. In vivo results showed that HHcy results in the up-regulation of ET_A receptor expression, and elevated blood pressure in rats. However, Res and U0126 could block these effects, respectively. In conclusion, these results suggest that Hcy regulates ET_A receptor expression via the Sirt1/ERK1/2 signaling pathway in VSMCs.

1. Introduction

The endothelin system consists of the ligand ET-1 and its G-protein coupled receptors (GPCR), endothelin A (ET_A) and endothelin B (ET_B) receptor. This system is implicated in the pathogenesis of hypertension and cardiovascular diseases (CVDs) (Xu et al., 2010). Endothelin-1 (ET-1) is a potent vasoconstrictor, that induces strong and long-lasting vasoconstriction. ET-1 transmits signals through the ET_A and the ET_B receptors. The ET_A receptor is located on vascular smooth muscle cells (VSMCs) and mediates vasoconstriction (Davenport, 2002). Additionally, the ET_B receptor is primarily located in vascular endothelial cells and mediates vasodilatation (Schneider et al., 2007). ET_A is thought to be the predominant ET-1 subtype responsible for vasoconstriction in VSMCs (Evans et al., 1999). Previous studies have demonstrated that the up regulation of ET_A receptors in VSMCs resulting in vasoconstriction, which increases the risk of several CVD associated disorders, such as

Abbreviations: BP, Blood pressure; CVDs, Cardiovascular diseases; DBP, Diastolic blood pressure; DMEM, Dulbecco's modified Eagle's medium; DMSO, Dissolved in dimethyl sulfoxide; ERK1/2, Extracellular signal related kinases 1 and 2; ET-1, Endothelin-1; ET_A, Endothelin type A; ET_B, Endothelin type B; GPCR, G-protein coupled receptors; Hcy, Homocysteine; HHcy, Hyperhomocysteinemia; JNK, C-jun terminal kinase; MAPKs, Mitogen-activated protein kinases; mmLDL, Minimally modified low density lipoprotein; PSS, Physiologic buffer solution; Res, Resveroral; S6c, Sarafotoxin 6c; SBP, Systolic blood pressure; Sirt1, Sirtuin 1; SMA, Superior mesenteric artery; VSMCs, Vascular smooth muscle cells.

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hypertension (Cao et al., 2013), diabetes (Miyauchi et al., 2014), cigarette smoke (Cao et al., 2016; Cao et al., 2011), and minimally modified low density lipoprotein (mmLDL) (Li et al., 2013).

Homocysteine (Hcy) is a sulfur-containing amino acid formed during the transmethylation of methionine. Hyperhomocysteinemia (HHcy) (plasma Hcy ≥ 10 μM) is an independent risk factor for CVDs (Hankey and Eikelboom, 1999). Previous studies have described that the level of endothein-1 (ET-1) increased in hypertensive individuals with HHcy (Tousoulis et al., 2010). Moreover, HHcy has been shown to promote hypertension in mice (Familtseva et al., 2016). We have previously shown that HHcy elevates blood pressure (BP) by up-regulating ET_B receptor expression (Chen et al., 2016). However, the effect of HHcy on ET_A receptor expression in VSMCs, and its mechanism remain elusive.

Sirtuin 1 (Sirt1) is a nicotinamide adenine dinucleotide dependent histone deacetylase that affects a variety of cellular functions ranging from gene silencing, regulation of the cell cycle and apoptosis to energy homeostasis (Imai and Guarente, 2010; Michan and Sinclair, 2007). It is generally believed that longevity is mainly promoted by Sirt1. Previous studies demonstrate that Sirt1 may play a protective role in CVDs (Guarente and Franklin, 2011).

Mitogen-activated protein kinases (MAPKs) are an evolutionarily conserved serine/tyrosine kinase family. MAPKs are a key substance in signal transduction. There are three kinds of major MAPKs, extracellular signal related kinases 1 and 2 (ERK1/2), the C-jun terminal kinase (JNK) and p38 (Hazzalin and Mahadevan, 2002). One signal pathway that has garnered particular attention is the ERK1/2 signaling pathway which is believed to play a role in the regulation of ET_A receptor expression in VSMCs (Cao et al., 2013; Cao et al., 2016; Cao et al., 2011; Li et al., 2013).

However, it is not determined whether the Sirt1/ERK1/2 signaling axis is involved in regulation of ET_A receptor expression mediated by Hcy. Therefore, we hypothesized that Hcy up-regulates ET_A receptor expression in VSMCs through the Sirt1/ERK1/2 signaling pathway. The present study was designed to test our hypothesis in vitro and in vivo and may provide insight into the mechanism of homocysteine-induced CVDs.

2. Materials and methods

2.1. Reagents

Sarafotoxin 6c (S6c, a selective ET_B receptor agonist) (Fluka/Sigma-Aldrich, St. Louis, MO, USA) and ET-1 (an ET_A receptor and ET_B receptor agonist) (Fluka/Sigma-Aldrich, St. Louis, MO, USA) were dissolved in 0.9% saline with 0.1% bovine serum albumin. Resveratrol (Res, a Sirt1 agonist) (Fluka/Sigma-Aldrich, St. Louis, MO, USA), SRT1720 (a Sirt1 specific agonist) (Selleck Chemicals LLC, Houston, TX, USA) and U0126 (an ERK1/2 signaling pathway inhibitor) (Fluka/Sigma-Aldrich, St. Louis, MO, USA) were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO (vehicle) used in the experiments 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 was utilized in organ culture without inhibitors to serve as a 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) (Gibco/Invitrogen, Carlsbad, CA, USA) just before the experiments.

2.2. Tissue preparation and organ culture procedure

Male Sprague-Dawley rats (300–350 g), which were obtained from the Animal Center of Xi'an Jiaotong University, were euthanized with CO_2. The superior mesenteric artery (SMA) was gently removed and freed from adhering tissue under dissecting microscope. The endothelium was denuded by perfusion of the vessel for 10 s with Triton X-100 (0.1%, *v*/*v*) followed by another 10 s with a 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). The vessels were then cut into 1–3 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), streptomycin (100 mg/ml) (Life Technologies, Carlsbad, CA, USA). Res (100 μM) or SRT1720 (1 μM) were added to the medium before incubation (Chen et al., 2016; Xu et al., 2014; Jia et al., 2015).

The animal experiments in this study were approved by the Laboratory Animal Administration Committee of Xi'an Jiaotong University and performed according to the Guidelines for Animal Experimentation of Xi'an Jiaotong 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. Animal experimental protocol

Thirty-six male Sprague-Dawley rats (150–200 g) were obtained from the Animal Center of Xi'an Jiaotong University. They were randomly divided into the control group, Res group, U0126 group, HHcy group, HHcy + Res group, and HHcy + U0126 group for 3 weeks, six rats per group. All of the rats were maintained on a normal diet and allowed free access to food and water. This experimental protocol was approved by the University Animal Ethics committee at Xi'an Jiaotong.

Rats were treated with DL-Hcy to induce HHcy. The experiment protocol was as follows: DL-Hcy was dissolved in 0.9% NaCl solution (saline) (pH 7.4). Hcy solution was administered subcutaneously twice a day for 3 weeks. During the first, second, and third week of treatment, animals received 0.3, 0.4, and 0.6 μmol Hcy per gram of body weight, respectively (Chen et al., 2016; Kolling et al., 2016). Plasma Hcy concentration in rats subjected to this treatment reached levels similar to those found in severe HHcy patients (Streck et al., 2002). The rats in the control group were given subcutaneous injections of normal saline twice a day.

To investigate the participation of the Sirt1, Res was administered at a dose of 146 mg/kg/day, i.g. for 3 weeks to the rats in Res and HHcy + Res group (Dolinsky et al., 2013).

To investigate the participation of the ERK1/2, U0126 (10 μg/kg) was injected intravenously in the tail vein once a day to the rats in U0126 and HHcy + U0126 group (Grabauskas et al., 2011). U0126 was dissolved in DMSO, and was further diluted in saline immediately before the experimental injection. The injection time was 2 h before the first daily subcutaneous injection of DL-Hcy (Wang et al., 2016).

After 3 weeks, all of the rats were euthanized with CO_2. The SMA was gently removed and freed from adhering tissue under dissecting microscope. The endothelium was denuded by perfusion of the vessel for 10 s with Triton X-100 (0.1%, *v*/*v*) followed by another 10 s with a PSS. The vessels were then cut into 1–3 mm long cylindrical segments for in vitro pharmacological studies. In addition, other arteries were snap-frozen at −80 °C for western blot analysis.

2.4. BP measurement

After 3 weeks, the systolic BP (SBP) and diastolic BP (DBP) of all of the rats were measured via a noninvasive tail-cuff plethysmography method and monitored with the CODA 6 Non-Invasive BP System (Kent Scientific, Torrington, CT, USA). The highest and lowest readings were discarded, and the average of the remaining five readings was used (Li et al., 2007).

2.5. In vitro pharmacology

Fresh segments obtained from animal experiments or incubated segments were immersed in temperature controlled (37 °C) myograph individual baths (Organ Bath Model700MO, 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 the LabChart 7 Pro