Isoproterenol Inhibits Angiotensin II-Stimulated Proliferation and Reactive Oxygen Species Production in Vascular Smooth Muscle Cells through Heme Oxygenase-1

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Heme oxygenase (HO-1) is a well-known cytoprotectant against oxidative stress and exhibits an antiproliferative effect in vascular smooth muscle cells (VSMCs). The purpose of the present study was to test whether isoproterenol, one of the synthetic catecholamines having β-adrenergic activity, affected angiotensin II (Ang II)-induced cell proliferation and reactive oxygen species (ROS) production. Also, the presumptive underlying signaling pathways in VSMCs were studied. Aortic VSMCs from 11-week-old male Sprague-Dawley rats were used. Isoproterenol dose-dependently increased HO-1 expression through β2-adrenoceptor (AR) and protein kinase A (PKA) pathway, and isoproterenol concentration-dependently increased β2-AR mRNA expression. Isoproterenol attenuated Ang II-induced cell proliferation, as evidenced by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. This effect of isoproterenol was inhibited by pretreatment of the cells with β2-AR antagonist butoxamine, PKA inhibitor H-89 and HO inhibitor Tin Protoporphyrin IX (SnPP IX), respectively. Isoproterenol inhibited phosphorylation level of Ang II-induced extracellular signal-regulated kinase (ERK1/2). Isoproterenol significantly inhibited Ang II-induced ROS production through the ERK1/2 pathway. These findings suggest that isoproterenol, via induction of HO-1, inhibits Ang II-stimulated proliferation and ROS production in cultured VSMCs.

Key words heme oxygenase-1; isoproterenol; angiotensin II; reactive oxygen species; β2-adrenoceptor

Angiotensin II (Ang II) plays an important role in the regulation of vascular function, including cell growth, apoptosis, migration, inflammation and fibrosis. Previous studies indicate that Ang II activates reduced nicotinamide adenine dinucleotide (NADPH) oxidase in cultured vascular smooth muscle cells (VSMCs), inducing oxidative stress and cell proliferation through the induction of reactive oxygen species (ROS) and mitogen-activated protein kinases (MAPKs). ROS includes superoxide anions and hydrogen peroxide (H2O2) and are recognized as important signaling molecules for cardiovascular tissues. Excess ROS production is a likely initiator of atherosclerotic events, resulting in the increased synthesis of numerous mitogenic factors that contribute to the proliferation of VSMCs and vascular plaque formation.

Isoproterenol, a synthetic catecholamine, activates all subtypes of β-adrenergic receptors (AR). In cultured smooth muscle cells, activation of the β-AR pathway results in the inhibition of cellular proliferation. In addition, β-AR-mediated smooth muscle relaxation is involved in the activation of adenylate cyclase and cAMP dependent mechanisms. Recent evidence suggests that increased intracellular cAMP induces HO-1 expression via a protein kinase A (PKA)-dependent pathway.

HO-1 is a well-known cytoprotective agent against oxidative stress with an anti-proliferative effect in VSMCs. For example, VSMCs from HO-1-deficient mice display enhanced DNA synthesis and cell growth. Also, wire injury of femoral arteries results in greater intimal thickening in HO-1-deficient mice than in control mice. Conversely, the transfer of HO-1 gene to porcine arteries attenuates injury-induced proliferation of VSMCs and intimal thickening. In rat aortic VSMCs, overexpression of HO-1 decreases cell proliferation by stimulating programmed cell death and protects cells against oxidative injury. Moreover, functional expression of HO-1 gene within VSMCs induces an alternative modality to protect the vascular cells against active oxidative injury.

Therefore, the aim of this study was to test whether isoproterenol affected Ang II-stimulated cell proliferation and ROS production through HO-1 expression, and to identify the underlying signaling pathways in VSMCs.

MATERIALS AND METHODS

Materials Dulbecco’s modified eagle medium and fetal bovine serum were purchased from Thermo Scientific (South Logan, UT, U.S.A.). Pro-prep protein extract solution was purchased from Intron Biotechnology (Sungnam, Korea). The antibody against HO-1 was purchased from Stressgen Bioreagents (Ann Arbor, MI, U.S.A.). Anti-extracellular signal-regulated kinase (ERK), anti-phospho-ERK, anti-p38, anti-phospho-p38, anti-c-Jun N-terminal kinase (JNK) and anti-phospho-JNK antibodies were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). The oligonucleotides for polymerase chain reaction (PCR) primers of β2-AR (forward, 5'-CATCGTAGTGCCACGCTGTTG-3' and reverse, 5'-AATCGCAGCCTGGGTC-3'), β1-AR (forward, 5'-ACC-CTCTTCTGCTATCCA-3' and reverse, 5'-AGAGGGACCTGGGTATAC-3'), and β-actin (forward, 5'-TACCCATGAGGAGTCCATGCCG-3', reverse, 5'-AGAGGGACCTGGGTATAC-3') were obtained from Bioneer (Daejeon, Korea). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), isoproterenol, forskolin, propranolol, metoprolol, butoxamine, and β-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). H-89, Ang II, a specific inhibitor of Mitogen/ERK kinase (MEK)-1 (PD98059), MAPKs inhibitors (SB203580, JNK II) were purchased from Calbiochem (San Diego, CA, U.S.A.). Tin Proto-
porphyrin IX (SnPP IX) was purchased from Frontier Scientific (Logan, UT, U.S.A.). HO-1 siRNA, 2',7'-dichlorofluorescein diacetate (DCF-DA) and lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA, U.S.A.). Stock solutions of forskolin, SnPP IX, and DCF-DA were prepared in 100% dimethyl sulfoxide (DMSO). PD98059 and JNKII were dissolved in methanol. Further dilution was made with cell culture medium.

**Cell Culture** Aortic VSMCs were isolated from 11-week-old male Sprague-Dawley rats and were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) and 1% antibiotic–antimycotic (penicillin 10000 U/ml, amphotericin B 25 μg/ml, streptomycin 10000 μg/ml). Cells were maintained at 37°C and 95% O2/5% CO2 incubator. We used VSMCs from 4 to 8 passages at 70–90% confluence in 10 cm dishes and cell growth was arrested by incubation of the cells in serum-free DMEM for 16–24 h prior to use.

**Western Blot Analysis** Serum-starved VSMCs in 10 cm dishes were stimulated with Ang II and isoproterenol. Whole cell extracts were prepared by lysing the cells in pro-prep protein extract solution. The protein concentration was quantified with protein assay reagent from Bio-Rad (Hercules, CA, U.S.A.). Equal amounts of protein were mixed with sodium dodecyl sulfate (SDS) sample buffer and incubated for 5 min at 100 °C before loading. Total protein samples (30 μg) were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for 1 h 30 min at 100 V. The separated proteins were electrophoretically transferred onto a PVDF membrane for 1 h at 30 mA. The membranes were blocked with 5% non-fat milk in phosphate buffered saline containing 0.05% Tween 20 (PBS-T) for 2 h at room temperature. The membranes were then incubated with the primary antibodies at a dilution of 1:1000 in 5% skim milk in PBS overnight at 4 °C. The membranes were then washed with four changes of wash buffer (0.05% Tween 20 in PBS) and incubated for 1 h at room temperature in PBS-T containing anti-rabbit (Stressgen, Ann Arbor, MI, U.S.A.) or anti-mouse IgG (Santa Cruz, CA, U.S.A.) antibodies. After four times rinses with wash buffer, the membranes were exposed to ECL western blot detection reagents.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)** Total RNA was extracted from VSMCs by a single-step guanidine thiocyanate/phenol chloroform extraction procedure, using Trizol® reagent (Molecular Research Center, Cincinnati, OH, U.S.A.) according to the manufacturer’s instructions. RNA was reverse transcribed into single-stranded cDNA by incubation for 10 min at 30 °C, 30 min at 42 °C, and 5 min at 99 °C in a final volume of 20 μl, using a maxime RT premix kit (Intron Biotechnology, Sungnam, Korea). PCR amplification was carried out on cDNA equivalent to 100 ng of starting mRNA, using specific oligonucleotide primers for β1-AR, β2-AR, and β-actin. The cDNAs were heated for 5 min at 95 °C, then amplified using 28 cycles for β1-AR (94°C for 1 min, 61°C for 1 min, 72°C for 2 min) and β2-AR (94°C for 1 min, 55°C for 1 min, 72°C for 2 min), and 28 cycles for β-actin (94°C for 45 s, 55°C for 1 min, 72°C for 1 min), followed by 5 min of extension at 72 °C. The PCR products were separated on a 1.2% agarose gel and visualized by ethidium bromide (Et-Br) staining.

**MTT Assay** Cell proliferation was analyzed using the MTT assay. VSMCs were seeded on 24-well plates at a density of 1×10⁴ cells per well in DMEM supplemented with 10% FBS. After treatments with Ang II, isoproterenol, and inhibitors, 50 μl of 1 mg/ml MTT solution was added to each well (0.1 mg/well) and incubated for 4 h. The supernatants were aspirated, and the formazan crystals in each well were solubilized with 200 μl DMSO. An aliquot of this solution (100 μl) was placed in the 96-well plates. Cell proliferation was assessed by measuring the absorbance at 570 nm using a microplate reader. The experiments were repeated 3 times.

**Transfection of HO-1 DNA and siRNA** Transfection of VSMCs with DNA and siRNA were performed using lipofectamine 2000 reagent, according to the manufacturer’s instructions. Aliquots of 1×10⁴ cells were plated on 60-mm dishes on the day before transfection and grown to about 70% confluence. The cells were then transfected with 1 μg of HO-1 DNA + 2.5 μl of lipofectamine/2.5 μl HO-1 siRNA + 5 μl of Lipofectamine for 6 h in Opti-MEM® I reduced serum medium (Invitrogen, Carlsbad, CA, U.S.A.). Following an incubation period of 48 h, the HO-1 protein level was measured using Western blot analysis, while the cell proliferation was analyzed using the MTT assay.

**Measurement of Intracellular ROS Levels** The Ang II-induced ROS production was assessed using DCF-DA. Cells were grown in 60-mm dishes for 1 h following treatment with or without Ang II. After the indicated time period, the medium was replaced with phenol red-free DMEM containing 10 μM DCF-DA and then incubated in the dark for 30 min at 37°C. The cells were then washed twice and resuspended in 0.5 ml PBS for flow cytometry analysis. ROS in the cells cause oxidation of DCF-DA, yielding a fluorescent product DCF. The DCF fluorescence was measured using a FACS caliber flow cytometer and cellquest software. ROS levels are defined as the ratio between the mean fluorescence of treated and untreated cells.

**Statistical Analysis** All data are represented as the mean±S.E.M. Differences between data sets were assessed by analysis of variance (ANOVA) followed by Bonferroni’s t-test. A statistical difference was considered significant at p<0.05.

**RESULTS**

**Isoproterenol Induces HO-1 Expression through β-Adrenoceptor and Protein Kinase A Pathway in VSMCs** To examine the role of isoproterenol in the induction of HO-1, VSMCs were treated with isoproterenol at concentrations of 10, 25, 50 μM for 12 h. Isoproterenol concentration-dependently increased HO-1 expression (Fig. 1A). To assess the role of β-AR in the induction of HO-1, we tried the β-AR antagonist propranolol. Isoproterenol at a concentration of 50 μM effectively increased HO-1 expression and the increase was significantly inhibited by pretreatment of the cells with propranolol (Fig. 1B). The isoproterenol-induced HO-1 expression was inhibited by PKA inhibitor H-89 (Fig. 1C). We tested whether an adenylate cyclase activator forskolin could induce HO-1 expression in VSMCs. Forskolin significantly increased HO-1 expression and this effect was also inhibited by H-89 (Fig. 1D).

**β-Adrenoceptor Mediates Isoproterenol-Induced HO-1 Expression in VSMCs** To confirm subtype specificity of
β-AR, we used β1-AR antagonist metoprolol and β2-AR antagonist butoxamine. Isoproterenol-induced HO-1 expression was reduced by butoxamine, but not by metoprolol (Fig. 2A).

We tried to find out ligand-induced receptor upregulation during isoproterenol stimulation. Figure 2B shows that isoproterenol significantly increased β2-AR mRNA expression. On the other hand, the mRNA levels of β1-AR were undetected.

Isoproterenol-Induced HO-1 Expression Inhibits Ang II-Stimulated Proliferation in VSMCs

We investigated the effect of isoproterenol on Ang II-stimulated proliferation. As shown in Fig. 3A, Ang II concentration-dependently stimulated cell proliferation in VSMCs. Isoproterenol inhibited Ang II-stimulated proliferation, the effect of isoproterenol
was reduced by pretreatment of the cells with butoxamine, H-89 and HO inhibitor SnPP IX respectively (Fig. 3B). We used conventional concentrations of inhibitors to inactivate each enzyme. To knockdown HO-1, we transfected VSMCs with HO-1 siRNA and examined the effect of HO-1 siRNA on Ang II-stimulated proliferation. We found that HO-1 siRNA, but not non-targeting siRNA, inhibited isoproterenol-induced HO-1 expression and effect of isoproterenol to decrease VSMCs proliferation via HO-1 expression. Cell proliferation induced by Ang II-stimulation and that induced by HO-1 siRNA upon dual stimulation with Ang II and isoproterenol was not significant. (Figs. 3C, D).

Isoproterenol Induced HO-1 Attenuates Ang II-Induced Intracellular Reactive Oxygen Species Production

We tested whether Ang II increased ROS production in VSMCs. FACS analysis data in Figs. 5A and B show that Ang II increased ROS (control 50% vs. Ang II 94%), whereas pretreatment of the cells with an MEK-1 inhibitor PD98059 blocked Ang II-induced ROS production (94 to 53%). We then studied the effect of isoproterenol on Ang II-induced ROS production. Isoproterenol significantly suppressed Ang II-induced ROS production (85 to 54%). Also, this effect of isoproterenol was blocked by pretreatment with butoxamine, H-89 and SnPP IX, respectively (Figs. 5C, D).

DISCUSSION

HO-1 is now recognized as a beneficial molecule in protecting against oxidative stresses, vascular constriction as well as proliferation.13,14) We examined isoproterenol induced HO-1 expression in VSMCs. β-AR agonists was known to increase intracellular cAMP levels, and this subsequently led to the induction of HO-1, we anticipated that β-AR stimulation by isoproterenol might be involved in HO-1 expression in VSMCs. The present study demonstrated that β2-AR was involved in the induction of HO-1 by isoproterenol. In this study, high concentration of isoproterenol was tried to induce necessary and sufficient HO-1 expression in VSMCs. β2-AR is coupled with G-proteins to activate adenylyl cyclase. In line with this concept, forskolin, an adenylyl cyclase activator, increased HO-1 expression in VSMCs. We suppose that
increases in intracellular cAMP may lead to activation of cAMP-dependent PKA. Our findings supported this postulation, showing that the PKA inhibitor H-89 inhibited HO-1 expression after isoproterenol and forskolin treatments. These results suggest that the PKA played an important role in the expression of HO-1 in VSMCs.

It has been clearly demonstrated that the VSMCs proliferation is a crucial event in the development of hypertension and is activated by various growth stimulants and cytokines. Among the various extracellular growth stimuli, Ang II is a major signaling molecule involved in atherogenic stimuli. It is not only a vasoactive substance but also a mitogen that mediates the proliferative action of VSMCs. In the present study, isoproterenol inhibited Ang II-stimulated VSMCs proliferation. Pretreatment of the cells with butoxamine, H-89, SnPP IX and HO-1 siRNA attenuated the effect of isoproterenol on Ang II-stimulated VSMCs proliferation.

Ang II is also well known as an activator of MAPKs, which are implicated in cell growth and hypertrophy. The results of the this study showed that the Ang II-stimulated VSMCs proliferation was decreased by treatment of the cells with an MEK-1 inhibitor, while it was unaffected by a p38 or JNK inhibitor. The ERK1/2 pathway is a tyrosine kinase-dependent pathway normally stimulated by growth factor receptors that are involved in cell growth, proliferation and differentiation. Ang II also activates c-src dependent mechanism and c-src was suggested to control VSMCs proliferation and vascular contraction. Treatment with an MEK-1 inhibitor PD98059 reduced Ang II-induced high blood pressure in vivo and prevented the contractile effects of Ang II in isolated resistance arteries in vitro. Present study showed that isoproterenol selectively inhibited Ang II-activated ERK1/2 phosphorylation, this effect was restored by pretreatment with butoxamine, H-89 and SnPP IX individually. It was also found that overexpression of HO-1 by transfection of HO-1 DNA attenuated Ang II-activated ERK1/2 phosphorylation in VSMCs. HO-1 was reported to inhibit the proliferation of pancreatic stellate cells by repression of the ERK1/2 pathway.

Previous studies suggested that Ang II induced ROS production in various cell types, including cardiomyocytes, cardiac fibroblasts and smooth muscle cells. Furthermore, ERK1/2 activation by Ang II is dependent on ROS production although another study did not support this hypothe-
sis. Therefore, the relationship between Ang II-induced ROS production and ERK1/2 activation remains unclear and the role of the ERK1/2 pathway on ROS production has not yet been addressed. Our observations demonstrated that Ang II induced ROS production through ERK1/2 pathway in VSMCs. In addition, isoproterenol significantly inhibited Ang II-induced ROS production. A similar result was observed in vivo since α-lipoic acid, a free radical scavenger, was shown to reduce ERK1/2 activity in vascular tissue. According to the above study, the superoxide production by Ang II and NADPH oxidase activation was dependent on ERK1/2 and tyrosine kinase activation in cultured VSMCs, since superoxide production could be completely blocked by simultaneous treatments with PD98059, U0126, tyrphostin and genistein. The authors proposed that the superoxide anion could increase ERK1/2 phosphorylation and ERK1/2 could in turn increase superoxide production by activating NADPH oxidase. Thus, both the ERK1/2-MAPKs pathway and the superoxide anion production could mutually stimulate each other. All results taken together, isoproterenol inhibits Ang II-stimulated VSMC proliferation and ROS production through HO-1 expression, in which inhibition of the ERK1/2 pathway might play an important role.

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