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Note

Sirt1-Mediated Anti-Aging Effects of *Houttuynia cordata* Extract in a High Glucose-Induced Endothelial Cell-Aging Model

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ABSTRACT: *Houttuynia cordata* (HC) is a herb widely used in traditional Asian medicine as an ingredient in complex prescriptions. HC is known for its anti-leukemic, anti-oxidative, and anti-inflammatory properties. However, its anti-vascular endothelial aging efficacy and the underlying mechanisms are not fully understood. In this study, we investigated the anti-aging effects of HC in a high glucose (HG)-induced endothelial cell (EC)-aging model. Treatment with HC (40 μg/mL) increased migration of ECs, and increased phosphorylation of extracellular signal-regulated kinases and p38 in a dose-dependent manner. Following HG treatment (30 mM), HC significantly decreased the number of senescence-associated β-galactosidase positive cells, which are the biomarkers for aging, in a dose-dependent manner. Based on levels of phosphorylation, HC (40 μg/mL) was shown to increase expression of sirtuin1 (Sirt1) and endothelial nitric oxide synthase (eNOS) by 74.4% and 328.2%, respectively. Furthermore, treatment of HG-induced senescent ECs with HC (40 μg/mL) significantly increased nitric oxide production ($P<0.05$). These results demonstrate that HC both increases EC migration and regulates the Sirt1/eNOS pathway, suggesting HC has potential for protecting ECs against HG-induced aging.

Keywords: anti-aging, endothelial cell, *Houttuynia cordata*, Sirt1/eNOS

INTRODUCTION

Senescence of vascular endothelial cells (ECs) can contribute to the development of atherosclerosis in humans (Minamino and Komuro, 2007; Wu et al., 2019). Many studies have shown that high glucose (HG) can induce EC aging (Matsui-Hirai et al., 2011; Hayashi et al., 2014; Lin et al., 2019). Moreover, EC dysfunctions can lead to nitric oxide (NO) deficiency (Thoonen et al., 2015). NO is synthesized from L-arginine by NO synthase, of which there are three important isoforms: the endothelial nitric oxide synthase (eNOS), neuronal NOS, and inducible NOS (iNOS). For example, eNOS has been shown to play a vital role in the development of diabetic cardiovascular complications by exacerbating oxidative stress (Kayama et al., 2015). In addition, sirtuin1 (Sirt1), a nicotinamide adenine dinucleotide (NAD^+)-dependent histone deacetylase, is involved in regulation of metabolism, cell survival, differentiation, and aging. Sirt1 is a key regulator of vascular EC aging. Previous studies have shown that Sirt1 activation can protect against vascular endothelial dysfunctions, whilst controlling eNOS generation (Hu et al., 2017).

Houttuynia cordata (HC) Thunberg, a member of the Saururaceae family, is a herb used for traditional healing in Southeast Asia. Recently, HC has been shown to be a rich source of naturally occurring polysaccharides and flavonoids (Lu et al., 2006a). Hence, HC is used for immune stimulation and chemotherapy in alternative medicine. Furthermore, HC exhibits various pharmacological properties, such as anti-leukemic (Chang et al., 2001), anti-oxidative (Hsu et al., 2016), and anti-inflammatory (Lu et al., 2006b) properties. Several studies have investigated HC, however only a few have evaluated the role of HC in preventing EC aging. To the best of our knowledge, this is the first study to show suppressive effects of HC on aging in a HG-induced aging model. This was achieved by using human umbilical vein ECs and evaluating the underlying mechanisms.

MATERIALS AND METHODS

Reagents

HC was obtained from Dr. Park at Kyungnam University, where it was extracted, separated, and subjected to qual-

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ity control, as described previously (Shon et al., 2014). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and gelatin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The senescence-associated β-galactosidase (SA-β-gal) kit was purchased from Abcam (Cambridge, UK) and the NO assay kit was purchased from Thermo Fisher Scientific (Vienna, Austria). p-p38, p-Sirt1, and p-eNOS antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA), and the β-actin and p-extracellular signal-regulated kinases (p-ERK) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit antibodies were purchased from GeneTex Inc. (Irvine, CA, USA).

EC culture

Human umbilical vein endothelial cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured at 37°C with 5% CO_2 in endothelial growth medium-2 (EGM-2; Lonza, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS). ECs were cultured for 48 h at 37°C with 5% CO_2 in EGM-2 (control) or HG 30 mM medium, with or without addition of different concentrations of HC (10～40 μg/mL).

Cell viability assay

Cells were cultured at 37°C for 72 h in EGM-2 medium supplemented with 2% FBS and various concentrations of HC, and were treated with MTT solution for 4 h. Resulting formazan deposits were dissolved with dimethyl sulfoxide, where the absorbance was measured at 570 nm using a VersaMax ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Scratch-wound migration assay

Cells were wounded, and then culture media was replaced with fresh media containing various concentrations of HC. Cells were maintained for 24 to 48 h. When at full migration, cells were imaged using a microscope (Olympus Optical Co., Ltd., Tokyo, Japan). The migrated cells were counted using an optical microscope at 200× magnification, and quantified manually.

SA-β-gal staining

To determine the number of senescent cells, SA-β-gal assays were performed using the SA-β-gal kit (Abcam), according to the manufacturer's instructions. Cells were fixed for 5 min in β-gal fixative, washed with PBS, and then stained using β-gal fixative solution at 37°C. This process was performed until β-gal staining was visible in either the experimental or control plate. SA-β-gal positive cells were observed via microscopy, with >500 cells counted using three independent fields.

Western blot analysis

Cells were harvested and lysed in protein-extraction solution (Intron Biotechnology, Inc., Gyeonggi, Korea) containing protease and phosphatase inhibitors (10 min at 4°C). Total protein concentrations in the supernatants were measured using Bradford assays. After heating at 95°C for 5 min, protein samples (30 μg) were separated by 8～12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis electrophoresis. Proteins were then transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA) at 100 V for 60 min. Membranes were incubated in 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) solution supplemented with 0.05% TBS with Tween-20 (TBST) (30 min at room temperature), then incubated in 5% BSA in TBST supplemented with primary antibodies (1:200 to 1:1,000) (overnight at 4°C). Next, membranes were inoculated with either HRP-conjugated goat anti-rabbit or anti-mouse antibodies (1:5,000) for 1 h, and protein bands were detected using an enhanced chemiluminescence detection kit (Intron Biotechnology, Inc.) and a LAS-1000 Imager (Fuji Film Corp., Tokyo, Japan). Semi-quantitative analysis of the densitometry results was performed using Image J (National Institutes of Health, Bethesda, MD, USA).

Measurement of NO production

NO concentrations were determined by detecting the concentration of nitrite using NO assay kits, according to manufacturer's instructions. Optical densities were determined at 560 nm using a microplate reader, and NO concentrations were calculated using a calibration curve.

Statistical analysis

Results are expressed as mean±standard deviation (SD). Statistical significance was determined by one-way analysis of variance (ANOVA). Post hoc comparisons between groups were performed using Bonferroni multiple comparisons tests. All calculations were performed using SPSS in Windows (v.23.0; IBM Corp., Armonk, NY, USA), and *P*-values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

HC cytotoxicity

To determine the cytotoxicity of HC on ECs, cells were incubated with various concentrations of HC (0 to 100 μg/mL) for 72 h. HC showed significant cytotoxicity ($P<0.05$) at concentrations >50 μg/mL at all-time points of treatment (Fig. 1). Therefore, HC concentrations of ≤50 μg/mL were considered non-toxic to ECs and were used in subsequent experiments.

HC increases cell migration.

The mitogen-activated protein kinase (MAPK) pathway regulates key processes involved in vascular EC development, such as cell proliferation, invasion, metastasis and survival, and angiogenesis (Pišlar et al., 2015). The MAPK involves ERK, c-Jun N-terminal kinase, and p38 (Kim and Choi, 2010). ERK and p38 are important signaling proteins involved in wound healing, whereas ERK plays an important role in controlling cell migration, proliferation, differentiation, and survival (Roskoski, 2012). In a recent study, the p38 MAPK signaling pathway was suggested to be involved in wound healing (Loughlin and Artlett, 2011; Kim et al., 2019; Wang et al., 2019). In the present study, we examined protein expression of ERK and p38 to identify the signaling pathways involved in HC-regulated EC migration. HC treatment (40 μg/mL) increased EC migration 1.5-fold relative to the control (P<0.05) (Fig. 2A). Western blot analysis showed that HC treatment (20 to 40 μg/mL) significantly increased phosphorylation of ERK in ECs (P<0.05). Furthermore, HC increased phosphorylation of p38 in a concentration-dependent manner; in particular, treatment with 40 μg/mL HC, increased activation of p38 >2.5-fold higher than the control (P<0.01) (Fig. 2B). These results show that

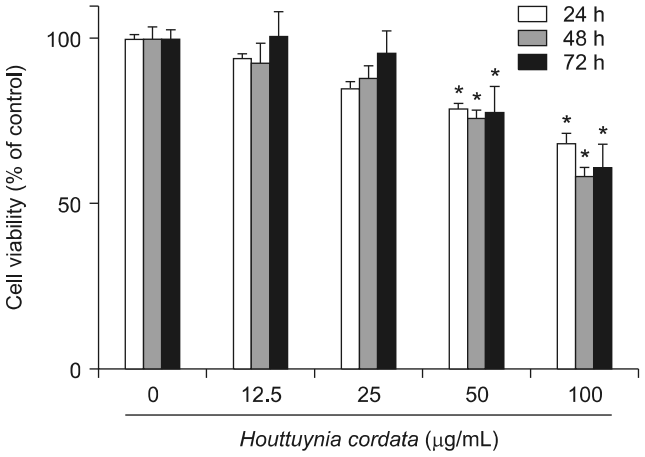

Fig. 1. Effects of *Houttuynia cordata* (HC) on cell viability. Cells were treated with HC (0 to 100 μg/mL) for 72 h. Cell viability was calculated as the percentage of viable cells cultured in the absence of HC. Results show mean±SD. *P<0.05.

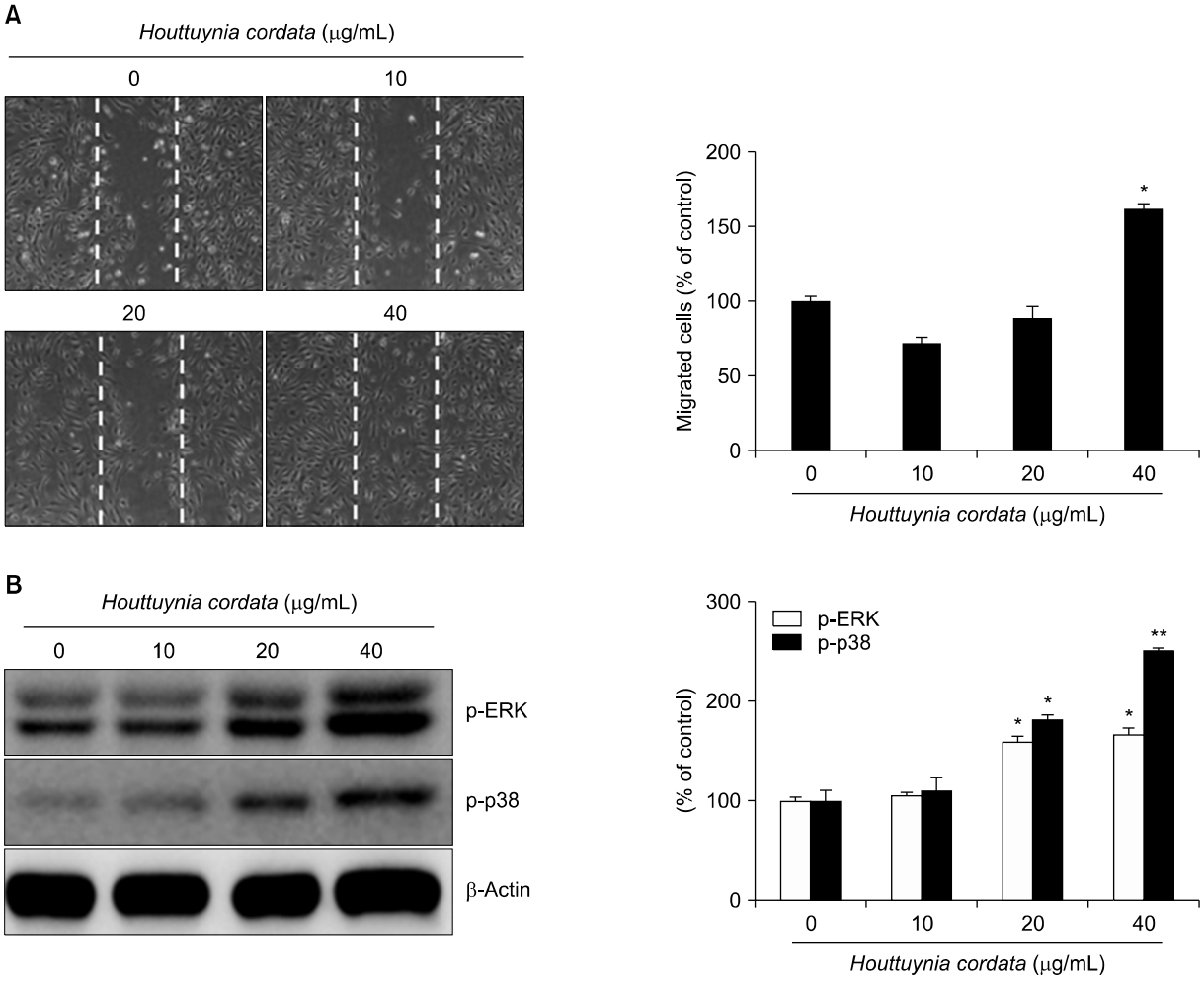

Fig. 2. Effects of *Houttuynia cordata* (HC) on cell migration. (A) Representative images show migration of control and HC-treated cells. Cell migration was expressed as the percentage of migration observed for control cells. (B) Representative blots of levels of p-extracellular signal-regulated kinases (p-ERK) and p-p38 phosphorylation. Quantitative analysis of p-ERK/β-actin and p-p38/β-actin. Results show mean±SD. *P<0.05 and **P<0.01.

HC regulates phosphorylation of ERK and p38 in a concentration-dependent manner. From these results of ERK and p38 MAPK activation, we concluded that HC induces EC migration and enhances its function in wound closure.

HC suppresses aging in HG-induced ECs.

To investigate the inhibitory effects HC on EC aging, cells were treated with glucose (30 mM) and various concentrations of HC (10 to 40 μg/mL). A trend of delayed aging was noted following treatment with increasing concentrations of HC. In particular, treatment with 40 μg/mL HC significantly decreased the abundance of the aging biomarker (SA-β-gal positive cells) by 10.2% compared with cells in the control group (31.5%) ($P<0.01$) (Fig. 3A). To determine if HC regulates expression of Sirt1, a protein that regulates aging, phosphorylation of Sirt1 and eNOS was investigated following treatment with HC. We showed HC (20 and 40 μg/mL) significantly increased the expression of Sirt1 by 58.5% and 74.4%, respectively, compared with cells in the control group ($P<0.05$). Following treatment with 40 μg/mL HC, expression of eNOS

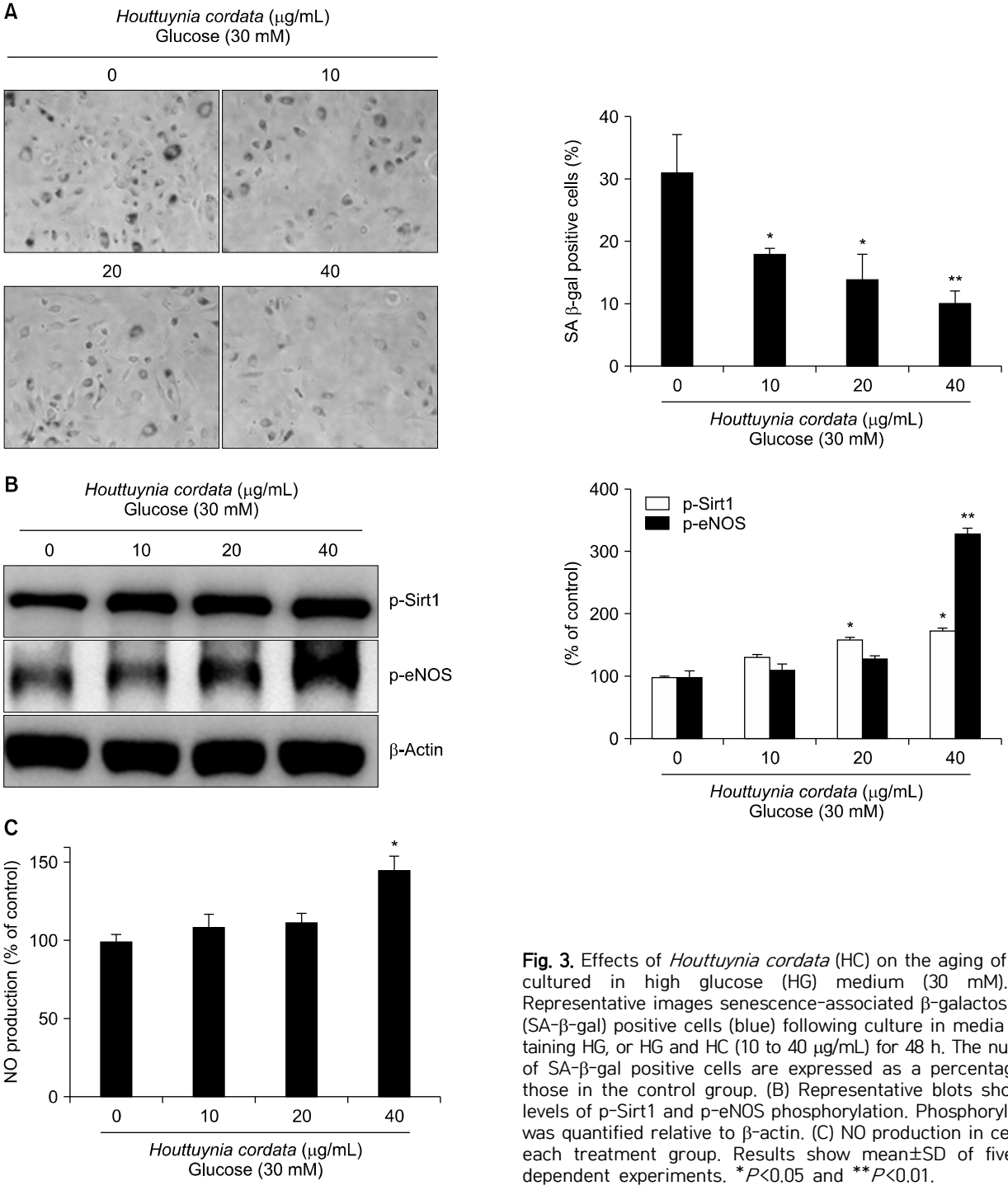

Fig. 3. Effects of *Houttuynia cordata* (HC) on the aging of cells cultured in high glucose (HG) medium (30 mM). (A) Representative images senescence-associated β-galactosidase (SA-β-gal) positive cells (blue) following culture in media containing HG, or HG and HC (10 to 40 μg/mL) for 48 h. The number of SA-β-gal positive cells are expressed as a percentage of those in the control group. (B) Representative blots showing levels of p-Sirt1 and p-eNOS phosphorylation. Phosphorylation was quantified relative to β-actin. (C) NO production in cells in each treatment group. Results show mean±SD of five independent experiments. *$P<0.05$ and **$P<0.01$.

was also significantly increased by over 3-fold HC compared with the control group ($P<0.01$) (Fig. 3B). Moreover, treatment with 40 μg/mL HC significantly increased NO production ($P<0.05$) in HG-induced aged ECs (Fig. 3C).

Following treatment with high concentrations of glucose, we observed a large number of ECs expressing the aging biomarker SA-β-gal, a major regulator of HG-induced aging (Sun et al., 2015). In previous studies, eNOS activation has been reported to control NO-mediated delays to cell aging in human ECs (Hayashi et al., 2008; Lee et al., 2017). Therefore, stimulating NO production may protect cells against oxidative stress and decrease the time to recovery. In this study, treatment of HG-treated ECs with 40 μg/mL HC significantly increased NO levels, which suggests that HC enhances to EC-mediated wound healing, and could therefore be used to treat aging in HG-induced ECs by mediating the Sirt1/eNOS pathway.

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AUTHOR DISCLOSURE STATEMENT

The author declares no conflict of interest.

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