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Signaling pathway of nitric oxide production induced by ginsenoside Rb1 in human aortic endothelial cells: A possible involvement of androgen receptor

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Abstract

Ginsenosides have been shown to stimulate nitric oxide (NO) production in aortic endothelial cells. However, the signaling pathways involved have not been well studied in human aortic endothelial cells. The present study was designed to examine whether purified ginsenoside Rb1, a major active component of ginseng could actually induce NO production and to clarify the signaling pathway in human aortic endothelial cells. NO production was rapidly increased by Rb1. The rapid increase in NO production was abrogated by treatment with nitric oxide synthetase inhibitor, L-NAME. Rb1 stimulated rapid phosphorylation of Akt (Ser473), ERK1/2 (Thr202/Thr204) and eNOS (Ser1177). Rapid phosphorylation of eNOS (Ser1177) was prevented by SH-5, an Akt inhibitor or wortmannin, PI3-kinase inhibitor and partially attenuated by PD98059, an upstream inhibitor for ERK1/2. Interestingly, NO production and eNOS phosphorylation at Ser1177 by Rb1 were abolished by androgen receptor antagonist, nilutamide. The results suggest that PI3kinase/Akt and MEK/ERK pathways and androgen receptor are involved in the regulation of acute eNOS activation by Rb1 in human aortic endothelial cells.

Keywords: Ginsenoside Rb1; Endothelial cells; Nitric oxide; eNOS; Androgen receptor; P13-kinase; Akt; ERK; MEK; Phosphorylation

Ginseng, the root of Panax ginseng C.A. Meyer (Araliaceae), is a well-known and popular herbal medicine used worldwide. Among more than 30 ginsenosides, the active ingredient of ginseng, ginsenoside Rb1 is regarded as the main compound responsible for many pharmaceutical actions of ginseng. The oral administration of ginseng caused a decrease in blood pressure in essential hypertension [1]. Intravenous administration of ginsenosides (a mixture of saponin from Panax ginseng C.A. Meyer) lowered blood pressure in a dose-dependent manner in anesthetized rats [2]. Although these reports suggest that ginsenosides could stimulate the production of nitric oxide (NO) by aortic vascular endothelial cells, the precise mechanisms of the ginsenoside actions have not been fully elucidated [3]. NO released from endothelial cells via the endothelial nitric oxide synthetase (eNOS) is a pivotal vasoprotective molecule. In addition to its vasodilating feature, endothelial NO has anti-atherosclerotic properties, such as inhibition of platelet aggregation, leukocyte adhesion, smooth muscle cell proliferation, and expression of genes involved in atherosclerosis [4].

The present study aims at investigating the signaling pathways involved in NO production by purified ginsenoside Rb1 in human aortic endothelial cells in vitro.

Materials and methods

Materials. Rb1, nilutamide, L-NAME (hydrochloride), Hanks' balanced salt solution (HBSS) were purchased from Sigma (St. Louis, MO,

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USA). ICI182780 was from Zeneca Pharmaceuticals (Macdesfield, UK). 4,5-diaminofluorescin diacetate (DAF-2 DA) was purchased from Daiichi (Daiichi Pure Chemicals Co., Ltd, Tokyo, Japan). PD98059, SH-5, wortmannin and Nitric Oxide Synthase Assay Kit were from Calbiochem (EDM Biosciences, Inc., La Jolla, CA, USA and Germany). L-[^{3}H]Arginine was purchased from Amersham (Amersham Biosciences, Uppsala, Sweden). Antibody of phospho-eNOS (Ser1177) was from upstate (Upstate Inc., Lake Placid, NY). Antibody for eNOS/NOS type III was purchased form BD Transduction Laboratories (BD Biosciences, Franklin Lakes, NJ, USA). All other antibodies were form Cell Signaling Technology (Beverly, MA, USA). LumiGLO Reserve Chemiluminescent Substrate Kit was from KPL (Gaithersburg, MD, USA). EBM-2 (endothelial cell base medium) was from Clonetics (Walkersville, MD, USA). Human aortic endothelial cells (HAECs) were from Cambrex (Cambrex BioScience Walkersville, Inc. Walkersville, MD, USA). Fetal bovine serum (FBS) was from CCT (Sanko Junyaku Co., Ltd, Tokyo, Japan), Fetal bovine serum charcoal stripped was from MultiSer (ThermoTrace Ltd., Melbourne, Australia).

Cell culture. HAECs were cultured in a 37 °C humidified atmosphere of 95% air/5% CO_2 in EGM-2 (endothelial cell grows medium 2) medium supplemented with 10% FBS. The EGM-2 medium consisted of 0.1% EGF, 0.04% hydrocortisone, 0.4% hFGF-B, 0.1% VEGF, 0.1% R^3-IGF-1, 0.1% ascorbic acid, 0.1% GA-1000, and 0.1% heparin. Experiments were performed with cells from passages 5 to 7. For all experiments, HAECs were plated at a concentration of 1×10^4/mL and grown to confluence. Then cells were serum-starved for 6 h in phenol red free EBM-2 containing 1% DCC-FBS, that was removed the steroid by processing it with dextran-coated charcoal (DCC-FBS). In some inhibitory experiments, the inhibitors were added to cells 60 min before the stimuli. DMSO was used as a solvent for Rb1, PD98059, wortmannin, SH-5, L-NAME, nilutamide, and DAF-2 DA present at equal concentrations (0.01%) in all groups, including the vehicle.

Western blot analysis. After treatment with reagents, confluent monolayers of cells were washed two times in ice-cold phosphate-buffered saline and lysed with buffer containing 20 mmol/L Tris–HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton-X, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/LNa_3VO_4, 1 μg/mL Leupeptin, 1 mM PMSF). For western blot analysis, total cell lysate was subjected to SDS–polyacrylamide gel electrophoresis (PAGE), and proteins were transferred to poly vinilidene difluoride (PVDF) membrane. The antibodies used in this study were anti-phospho-ERK1/2 (Thr202/Thr204), anti-ERK1/2, anti phospho-Akt (Ser473), anti-Akt, anti-phosph-eNOS (ser1177) and anti-NOS. Antibodies were detected by means of a horseradish peroxidase-linked secondary antibody and immunoreactive bands were visualized using LumiGLO Reserve Chemiluminescent Substrate Kit.

Endothelial NO synthase activity assay. Endothelial cell NO synthase (eNOS) activity was quantified by measuring the conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline by the use of a NO synthase assay kit.

Measurement of intracellular production of NO. Production of NO was assessed using the NO-sensitive fluorescent dye DAF-2 DA [5]. Briefly, confluent cells were serum-staved for 6 h. Because NOS generates O_2^- instead of NO in the absence of L-arginine, so L-arginine (100 μmol/L) was add 1 h prior to all solutions, except for the experiment with *N*-nitro-L-arginine methyl ester (L-NAME; a NOS inhibitor)-treated cells. Cells were loaded with DAF-2 DA (final concentration 5 μmol/L, 30 min 37 °C) and than rinsed three times with HBSS, kept in the dark, and maintained at 37 °C in 1% EBM-2 medium with a warming stage. After 30 min, cells were then treated with Rb1 or other stimuli. In some inhibitory experiments, the inhibitors were administered 30 min before loading with DAF-2 DA. Green fluorescence intensity was measured with a laser scanning confocal microscopy system (LSCM) (Bio-Rad Laser Sharp2000). The fluorescence image was obtained as a 512×512 pixel frame. Ex = 488 nm, EM = 510 nm. All other settings, including scanning speed, pinhole diameter, and voltage gain, remained the same for all experiments.

Statistics. Data are means ± SEM. Statistical comparisons were performed by Student's *T* test between two groups. A value of $P < 0.05$ was considered significant.

Results

Rb1 stimulates rapid production of NO in human aortic endothelia cells

We used the NO-specific fluorescent dye DAF-2 DA to evaluate the effect of Rb1 on NO production in HAECs. 5, 10, 15, 30, 60, 120 and 180 min after Rb1 treatment, cells were fixed and then viewed using a fluorescence microscope. Emission of green light (510 nm) from cells excited by light at 488 nm is indicative of NO production. A significant increase in green fluorescence was observed >15 min after the addition of Rb1 and lasted for 60 min in HAECs (Fig. 1A). Maximal stimulation of NO production was obtained at 30 min.

To verify that the rapid increase in green fluorescence in response to Rb1 treatment specifically reflected NO production, we compared results from HAECs treated with acetylcholine (1 μmol/L) or Rb1 (1 μmol/L) for 5 min. Reassuringly, treatment with either acetylcholine and calcium ionophore or Rb1 resulted in a increase in green fluorescence (Fig. 1B). We next examined the effects of the NOS inhibitor L-NAME to determine whether the NO increase was attributable to NOS derived de novo synthesis. As shown in Fig. 1C, the Rb1-induced DAF-2 DA fluorescence was completely suppressed by pretreatment with L-NAME (0.5 mmol/L). The results suggested that the rapid increase in NO production after Rb1 treatment was mediated by an increase in NOS activity.

Rb1 stimulates phosphorylation of eNOS (Ser1177) and increases NOS activity

To examine involvement of eNOS in the NO increase, the effect of Rb1 on eNOS phosphorylation at Ser-1177 was tested by Western blotting. As shown in Fig. 2, Rb1 induced rapid eNOS phosphorylation after 10 min of incubation, maximal eNOS phosphorylation by Rb1 was observed from 30 to 60 min of incubation. The relative magnitude of eNOS phosphorylation falls subsequently but is still significantly greater than control after 120 min of Rb1 incubation (Fig. 2A, upper blots). The acute effect by Rb1 on eNOS phosphorylation was concentration dependent (Fig. 2B, upper blots). Rb1 did not affect eNOS protein expression (Fig. 2A and B, lower blots).

To see whether Rb1 actually activates NOS in HAECs, we measured NOS activity after 30 min of treatment with Rb1. As shown in Fig. 2C, Rb1 significantly increased NOS activity in HAECs.

PI3-kinase/Akt and MEK/ERK pathways are involved in eNOS phosphorylation and NO production

Previous studies have demonstrated that PI3-kinase/Akt and MEK/ERK pathways are two important signaling cascades mediating eNOS activation by many stimuli in vascular endothelial cells [6,7]. Therefore, we examined