Effects of Homocysteine and Ginsenoside Rb1 on Endothelial Proliferation and Superoxide Anion Production

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Background. Homocysteine (Hcy) is an independent risk factor for cardiovascular disease by its multiple effects on vascular cells and thrombosis factors, which may be involved in oxidative stress mechanisms. Ginsenoside Rb1, a constituent of ginseng, bears various beneficial effects on the cardiovascular system. In the present study, we investigated the effect of Hcy on endothelial proliferation and a protective effect of ginsenoside Rb1 on the action of Hcy.

Methods. We initially incubated a mouse lymph node endothelial cell line (SVEC4-10) with increasing concentrations of Hcy or for different time periods and then assessed cell proliferation by using [3H]-thymidine incorporation. We then incubated SVEC4-10 cells with Hcy (50–200 μM) for 24 h with or without Rb1 (10 μM) to examine its inhibitory effect on the proliferation. These experiments were repeated in human umbilical vein endothelial cells (HUVECs). To explore the underlying molecular mechanisms, we measured superoxide anion, a reactive oxygen species (ROS), by using dihydroethidium (DHE) staining.

Results. SVEC4-10 cells treated with Hcy (50, 100, and 200 μM) for 24 h significantly reduced cell proliferation by 43%, 42%, and 40%, respectively, as compared with control cells (P < 0.01). SVEC4-10 cells treated with Hcy (50 μM) for 12 and 24 h showed a significant reduction of cell proliferation (P < 0.05). In HUVECs, Hcy (50 μM) significantly reduced cell proliferation by 55% as compared with control cells (P < 0.05). In the presence of Rb1, Hcy-induced inhibition of cell proliferation was effectively blocked in both SVEC4-10 and HUVECs. Furthermore, Hcy (50 μM) significantly increased superoxide anion production by 23% in SVEC4-10 as compared with control cells (P < 0.05). However, in the presence of Rb1, Hcy increased superoxide anion production by only 8%, showing that Rb1 almost completely blocked the effect of Hcy.

Conclusion. Hcy significantly inhibits endothelial proliferation with increased production of superoxide anion, which is effectively blocked by ginsenoside Rb1. This study provides some new aspects of Hcy-induced endothelial dysfunction, and suggests a potential role of Rb1 to block Hcy action, which may have clinical applications.

Key Words: homocysteine; ginsenoside Rb1; endothelial cells; cell proliferation; reactive oxygen species.

INTRODUCTION

Hyperhomocysteinemia is a common and independent risk factor for atherosclerosis and other cardiovascular diseases (CVDs) [1–4]. Animal experiments show that increased plasma homocysteine (Hcy) levels can induce atherosclerotic lesions [5, 6]. Some in vitro studies have shown that Hcy can suppress endothelial cell proliferation or viability by inhibiting the production of nitric oxide (NO) [7–9]. The inhibitory effect of Hcy on endothelial cells may be mediated by oxidative stress [10]. In fact, a strong correlation between plasma Hcy levels and reactive oxygen species (ROS) production, marked by plasma malondialdehyde, was identified in patients with CVD [11]. Therefore, the suppression of ROS may be a potential therapeutic strategy in preventing or retarding atherosclerosis in hyperhomocysteinemic patients.

Ginseng, a widely recognized herbal drug from Chinese medicine, is known to have pharmacological ef-
fects against certain chronic disease states [12]. Ginseng contains active saponin glycosides, such as ginsenosides. Approximately 30 of these compounds, such as ginsenoside Rb1, have been identified and appear to be responsible for most of the activity of ginseng. A number of studies have shown that ginsenosides can decrease blood pressure in both experimental animals and hypertensive patients by facilitating the endothelium-dependent relaxation of the blood vessels [13–16]. Other studies revealed that ginsenosides protect pulmonary vascular endothelial cells against free radical-induced injury and prevent neointimal hyperplasia in animal models [17, 18]. These findings indicate that ginsenosides may have a direct protective effect on endothelial cells but its precise molecular mechanisms are still ambiguous.

We hypothesized that Hcy could inhibit endothelial cell proliferation by oxidative stress, which could be blocked by antioxidant ginsenoside Rb1. A mouse lymph node endothelial cell line (SVEC4-10) was used in this study to generate preliminary data for performing further studies in mouse models in future. Human umbilical vein endothelial cells (HUVECs) were used to confirm some key data generated from SVEC4-10 cells such as effects of Hcy and Rb1 on cell proliferation. This study could advance our understanding about the pathogenesis of Hcy and suggest a new strategy to control this clinical problem.

MATERIALS AND METHODS

Chemicals and Reagents

DL-homocysteine (Hcy), dimethyl sulfoxide (DMSO), and ginsenoside Rb1 (from Panax quinquefolium root, dissolved in DMSO) were obtained from Sigma (St. Louis, MO). Dihydroethidium (DHE) was obtained from Molecular Probes (Eugene, OR). [3H]-thymidine was purchased from Amersham Biosciences (Piscataway, NJ).

Cell Culture

SVEC4-10 cells were purchased from ATCC (Manassas, VA). SVEC4-10 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 µg/mL) in a 37°C, 5% CO2, humidified incubator. The medium was replaced every 48 h. Before each experiment, the cells were cultured in DMEM with 0.5% FBS for 24 h for serum starvation.

HUVECs were obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD) and used between passages 3 and 8. HUVECs were cultured in Endothelial Basal Medium-2 (EBM-2) with growth factors and antibiotics (Clonetics EGM-2-MV BulletKit, Cambrex Bio Science, Walkersville, Inc.) supplemented with 10% FBS. The medium was replaced every 48 h. The cells were incubated in the EBM-2 medium with 1% FBS without addition of growth factors for 24 h for serum starvation before each experiment.

Cell Proliferation Assay

The effects of Hcy and Rb1 on the proliferation of SVEC4-10 and HUVECs were measured by the [3H]-thymidine incorporation assay. The cells were plated into the flat-bottomed 96-well plate (Falcon, Franklin Lakes, NJ) at the density of 2 × 104 cells/well in a final volume of 200 µL. After overnight seeding, the cells were starved as described previously for 24 h. The cells were then treated with various doses of Hcy or Rb1 for each time point. For the last 4 h of incubation, [3H]-thymidine (1 µCi/mL as final concentration) was added to the medium. After completion of the treatment, the cells were collected, and the levels of [3H]-thymidine incorporation were measured using Topcount (Perkin-Elmer, Shelton, CT).

DHE Staining Detected by Flow Cytometry

Cultured SVEC4-10 cells were treated with Hcy (50 µM) with or without Rb1 (10 µM) for 24 h. The treatment was halted by washing cells three times with PBS. One milliliter of DHE (3 µM) was added into each well of six-well plates (Falcon, Franklin Lakes, NJ) and incubated for 20 min at room temperature. Staining was stopped by washing cells three times with PBS and then analyzed by FACS Calibur (Becton Dickinson, San Jose, CA).

Statistical Analysis

Data are presented as mean ± SEM. Differences were analyzed by Student’s t test. A value of P < 0.05 was considered significant.

RESULTS

Inhibition of SVEC4-10 Cell Proliferation by Hcy

To observe the effect of Hcy on SVEC4-10 cells, we used various concentrations of Hcy for stimulation. We further incubated the cells for different time periods to determine the most effective condition. The SVEC4-10 cells were incubated with different concentrations of Hcy (0, 10, 50, 100, and 200 µM) for 24 h and the cell growth was assessed by measuring [3H]-thymidine incorporation. At concentrations of 50, 100, and 200 µM, the proliferation was reduced to 57%, 58%, and 60.2%, respectively, as compared with the control (100%; Fig. 1, n = 5, P < 0.01), showing a reduction by 43%, 42%, and 40%, respectively. SVEC4-10 cells treated with Hcy (50 µM) at different times (6, 12, 24, and 48 h)
showed a decrease of cell proliferation by 19%, 29%, 22%, and 12%, respectively, whereas the cultures at 12 and 24 h showed a statistical difference as compared with control cultures (Fig. 2, n = 4, P < 0.05).

Reversal of the Inhibitory Effects of Hcy on SVEC4-10 cells and HUVECs by Ginsenoside Rb1

To study the beneficiary effect of Rb1 on Hcy-treated SVEC4-10 cells, we assessed the cell growth after stimulation of Hcy with or without Rb1 at various concentrations. Serum-starved SVEC4-10 cells were incubated with Hcy (50 μM) with addition of Rb1 (0, 1, 5, and 10 μM) and were subjected to [3H]-thymidine incorporation assay. In SVEC4-10 cells, Hcy (50 μM) significantly inhibited proliferation by 35% (Fig. 3, n = 4, P < 0.01), but in the presence of Rb1 (10 μM), this inhibition was only 1%, as compared with controls (Fig. 3). Rb1 alone had no effects on cell proliferation (Fig. 3). In HUVECs, Hcy (50 μM) significantly reduced cell proliferation by 55% as compared with controls (Fig. 4, n = 6, P < 0.05), whereas Rb1 (10 μM) significantly blocked the action of Hcy (Fig. 4). These data demonstrated that ginsenoside Rb1 effectively blocked Hcy-induced inhibition of cell proliferation in both SVEC4-10 cells and HUVECs.

Inhibition of Superoxide Anion Production from SVEC4-10 Cells by Ginsenoside Rb1

To explore the molecular mechanisms of Rb1, we detected superoxide anion production from SVEC4-10 cells by staining them with DHE, a fluorescence dye for superoxide anion, and subsequent flow cytometry analysis. In SVEC4-10 cells, Hcy (50 μM) significantly increased superoxide anion production by 23% as compared with controls (Fig. 5A and B, n = 3, P < 0.01). However, in the presence of Rb1 (10 μM), Hcy increased superoxide anion production by only 8%, showing that Rb1 almost completely blocked the effect of Hcy.

**DISCUSSION**

Although ginseng and its active constituents, ginsenosides, have gained increased popularity worldwide for their various benefits, the underlying molecular mechanisms still remain to be elucidated. In particular, the information regarding the effects of ginseng on the vascular system is still limited. Our present work clarified the cellular mechanism as well as the functional influence of ginsenoside Rb1 in protecting endothelial cells from Hcy-induced damage. We found that Rb1 prevented Hcy-induced inhibition of endothelial proliferation and further showed that suppression of ROS production by Rb1 might be a potential mechanism.
This study demonstrates the beneficial role of ginsenoside Rb1 in preventing the detrimental effects of Hcy in endothelial cells.

Ginseng, a widely recognized herbal drug from Chinese medicine, has attracted much attention for its multiple pharmacological effects on the central nervous system, endocrine system, metabolic and immune system, and cardiovascular system [12]. The components of ginsenosides, the major constituents of ginseng, belong either to the protopanaxadiol or protopanaxatriol groups [19]. Ginsenosides Rb1, Rg3, and Rc represent protopanaxadiols, whereas ginsenosides Rg1 and Re are protopanaxatriols. Although Rb1 is known to enhance peripheral nerve regeneration and promote Schwann cell proliferation [20–22], our understanding of its effect on endothelial cells and vasculatures remains scarce. We previously showed that Rb1 could block Hcy- and HIV protease inhibitor ritonavir-induced endothelial cell dysfunction in porcine coronary arteries by reducing ROS production and upregulating endothelial nitric oxide synthase (eNOS) expression [23, 24]. Herein this study, we have clearly shown in vitro that Rb1 could block Hcy effect on endothelial cell proliferation and also present its potential underlying mechanism via ROS. Our results may shed new light on ginsenoside Rb1 as a therapeutic tool for CVD.

In human hyperhomocysteinemia, plasma Hcy levels are categorized as 15 to 30 μM (moderate), 31 to 100 μM (intermediate), and greater than 100 μM (severe) [4]. Nevertheless, Hcy-concentrations used in many in vitro studies are variable, ranging from 20 μM to 100 μM [7, 25, 26]. Herein, we used the concentration of 50 μM to reproduce the environment of the intermediate hyperhomocysteinemic status. We therefore believe that the concentration range of Hcy used in the current study is clinically relevant.

Hcy is known to stimulate ROS production from endothelial cells, which may be a potential mechanism of atherogenesis [27]. On the other hand, ginseng compounds protect endothelial cells from ROS-induced injury [17]. In the current study, we showed that Hcy (50 μM) significantly increased superoxide anion production by 23% in mouse endothelial cells and that this effect was almost completely blocked by gensenoside Rb1 (10 μM). Potency of the antioxidative property of gensenoside Rb1 also was demonstrated in our previous publications [23, 24]. The HIV protease inhibitor ritonavir significantly increased superoxide anion production by 151% in porcine coronary arteries, which level was returned to the control level when the cells were co-cultured with Rb1 [23]. Hcy-treated porcine coronary arteries also significantly increased superoxide anion production by 137%, which was almost completely blocked by Rb1 [24]. In addition to the mechanisms via ROS, Hcy can inhibit endothelial cell growth by inhibiting NO production from the cells [10, 28, 29]. Ginseng can protect vasculatures by inducing NO production from endothelial cells [30]. Our previous data also showed that Rb1 can block Hcy- and HIV protease inhibitor ritonavir-induced endothelial cell dysfunction of porcine coronary arteries by upregulating eNOS expression [23, 24]. Given these reports, it is conceivable that Rb1 may stimulate NO production in endothelial cells, thereby resulting in increase of the cell growth.

In our current study, major experiments were performed by using mouse lymph node endothelial cells (SVEC4-10) to generate preliminary data for performing further studies in mouse models in future. HUVECs were used to confirm some key data generated from SVEC4-10 cells.
such as effects of Hcy and Rb1 on cell proliferation. Superoxide anion experiments in HUVECs were not performed in this study, which could be a limitation. In addition, the experimental variability regarding cell proliferation in response to Hcy treatment such as Hcy (50 μM) for 24 h was observed. This variability may be generated from conditions of cell cultures and sensitivity of the assay and instrumentation. Thus, this could be another limitation of this study. However, we set a control for each experiment and data generated from this study were reliable and supported our general conclusions.

Although the data generated from SVEC4-10 and HUVECs exist some differences, they all support our conclusions that Hcy inhibits endothelial proliferation and that Rb1 effectively blocked this effect of Hcy on endothelial cells. Hcy at (50 μM), a clinically relevant concentration, was used in the study. However, it is not clear whether this concentration is optimal for both mouse and human endothelial cells in vitro. Further investigations are warranted.

In summary, biological effects of Hcy on endothelial proliferation may be mediated by multiple molecular mechanisms and pathways, which are not completely understood. Our current study has confirmed that Hcy at clinically relevant concentrations significantly inhibits endothelial cell proliferation through oxidative stress and that ginsenoside Rb1 effectively blocks this effect of Hcy by its antioxidative property. Since ROS changes are well correlated with Hcy-induced inhibition of cell proliferation in both Hcy treatment alone and Hcy plus Rb1 groups, our data are significant to demonstrate that oxidative stress may play a major role of Hcy in endothelial cells. This study suggests that ginsenoside Rb1 may have clinical applications in patients with hyperhomocysteinemia.

REFERENCES


