PROCEEDING

The phytoestrogen ginsensoside Re activates potassium channels of vascular smooth muscle cells through PI3K/Akt and nitric oxide pathways

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Abstract : In vascular smooth muscle cells, large-conductance Ca^{2+}-activated K^+ channels (K_{Ca} channels) play a pivotal role in determining membrane potential, and thereby the vascular tone. Ginsenoside Re, a phytochemical from ginseng, is reported to activate this channel, but its precise mechanism is unsolved. Patch clamp studies showed that ginsenoside Re activates K_{Ca} channels in the arterial smooth muscle cell line A10 in a dose-dependent manner. The channel-opening effect of ginsenoside Re was inhibited by 1 μM L-NIO, an inhibitor of eNOS, but not by 3 μM SMTC, an inhibitor of nNOS, indicating that ginsenoside Re activated K_{Ca} channels through activation of eNOS. SH-6 (10 μM), an Akt inhibitor, and wortmannin, a PI3-kinase inhibitor, completely blocked activation of K_{Ca} channels by ginsenoside Re, indicating that it activates eNOS via a c-Src/PI3-kinase/Akt-dependent mechanism. In addition, the ginsenoside Re-induced activation of eNOS and K_{Ca} channel was blocked by 10 μM ICI 182,780, an inhibitor of membrane estrogen receptor-α, suggesting that eNOS activation occurs via a non-genomic pathway of this receptor. In conclusion, ginsenoside Re releases NO via a membrane sex steroid receptors, resulting in K_{Ca} channel activation in vascular smooth muscle cells, promoting vasodilation and preventing severe arterial contraction. J. Med. Invest. 54 : 381-384, August, 2007

INTRODUCTION

Potassium channels in vascular smooth muscle plays an important role in controlling vascular tone. Of these, ATP-sensitive K (K_{ATP}) channels and Ca^{2+}-activated K (K_{Ca}) channels are important because they are abundant on cell membranes and effectively control membrane potential as well as vascular tone. We have reported that ginsenosides increase nitric oxide (NO) production (1), and NO activates both K_{Ca} (2) and K_{ATP} (3) channels. However, precise mechanism of action has not been clarified. Recently, some phytochemicals such as epigallocatechin-3-gallate have been reported to increase NO production and dilate arteries through the PI3K/Akt and endothelial NO synthase (eNOS) pathways (4). In addition, this compound acts through membrane estrogen receptor (ER). Interestingly, the chemical structures of estradiol and some phytochemicals, especially phytoestrogens like ginsenosides, are similar to some degree. Medically, ginsenosides have been to protect against cardiac ischemia, a major cause of death in the West. In this study, we tested the effect of ginsenosides Re on K channels of vas-

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cular smooth muscle cells and clarified their intracellular mechanism and compared to those of insulin.

METHODS

Cell cultures

Embryonic rat thoracic aortic smooth muscle cells from DB1X rat (A10 cells) were obtained from American Type Culture Collection (ATCC, Manassas, VA). They were cultured at 37 ˚C in a humidified atmosphere with 95% air and 5% CO_2 by Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) containing 0.5 mg/mL gentamicin (Sigma), and 10% fetal bovine serum or calf serum (Invitrogen, Carlsbad, CA), respectively. The effects of a phytoestrogen, ginsenoside Re (Sigma Chemical Co. St Louis, MO), on K channels were studied using sub-confluent A10 cells cultured on cover slips. Ginsenoside Re-induced phosphorylation of Akt was measured by western blotting.

Patch-clamp experiments

Single K_{Ca} channel activities were measured by using the cell-attached patch-clamp recordings. A10 cells, subcultured on glass cover-slips, were placed in the experimental chamber filled with the buffered solution, containing (in mmol/L) KCl 140, $MgCl_2$ 1, $CaCl_2$ 0.1, D-glucose 5.5, HEPES 10, pH7.2. The buffered solution into the pipettes was containing (in mmol/L) KCl 140, D-glucose 5.5, HEPES 10, pH 7.2. The patch pipettes were made from the soft-glass capillaries (DRUMMOND SCIENTIFIC, Broomall, PA) by using an electrode puller (PP-830, Narishige, Tokyo, Japan). The resistance of the pipettes filled with the buffered solution was 7-10 MΩ. All drugs were added into the dishes. Inside of the pipette was voltage-clamped at +50 mV. All patch-clamp experiments were performed at 37 ˚C.

The currents of patched cell membrane were recorded with a patch clamp amplifier (L/M-EPC7, List-Medical, Darmstadt, Germany) and converted into digital files by using DigiData 1200 (Axon Instruments, Foster, CA). To remove the noises, we used a low-pass filter of 1 kHz. pClamp version 6 software (Axon Instruments) was used for recording the data and BIO-PATCH Ver.3.42 software (BIO-LOGIC, Claix, France) was used for analysis of the recording data. The open probability (NPo) of the ion channel was determined from current amplitude histograms and was calculated as follows :

$$NPo=\Sigma(n \times Pn)$$

where N means the number of channels in the patch and Pn means the integrated channel opening. The NPo of the KATP channels were determined from recordings during more than 120 s.

Cell preparation and Western blotting analysis

After preincubation in the HBS for 30 min, the cells were treated with the drugs. Before stimulation with 100 nmol/L insulin, the cells were pretreated with or without 100 nmol/L wortmannin or 100 nmol/L L-NAME for 10 min. The cells were lysed by cold cell lysis buffer containing (in mmol/L) NaCl 140, Tris-HCl 20, $MgCl_2$ 1, $CaCl_2$ 1, dithiothreitol 1, sodium vanadate 0.5, sodium pyrophosphate 20, phenylmethanesulfonyl fluoride 0.02, 10% glycerol (v/v), 1% Nonidet P40 (v/v). Total cell membrane lysates were obtained as described (5).

The cell lysates were electrophoresed in a sodium dodecyl sulfate (SDS) - polyacrylamide gel and transferred to the nitrocellulose membrane (Schleicher & Schnell Bioscience, Dassel, Germany). The membranes were incubated with the specific antibodies respectively as primary antibodies. Primary antibodies were detected with the appropriate horseradish peroxidase-conjugated secondary antibodies and immunoreactive bands were visualized using a enhanced chemi-luminescence substrate. We captured chemiluminescence images by using LAS3000-UVmini (Fujifilm, Tokyo, Japan). Blots were stripped by incubation in stripping buffer containing (in mmol/L) Tris-HCl 62.5, and 2-mercaptoethanol 100, 2% SDS, pH 6.8, at 50˚C for 40 min for a second round immunoblotting.

Statistical analysis

The statistical analysis of differences was estimated by the Student's t-test for unpaired samples. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Ginsenoside Re activates K_{Ca} channels through eNOS

Ginsenoside Re is reported to enhance potassium currents in cardiac myocytes in a NO-dependent manner (5). We found that in A10 aortic smooth muscle cells, ginsenoside Re also activated K_{Ca} channels dose-dependently with an EC50 of 4.1±0.3 μM (n= 15). In the presence of 100nM N^{ω}-nitro-L-arginine

(L-NAMA), a nitric oxide synthase (NOS) inhibitor, ginsenosides Re failed to activate K_{Ca} channels, suggesting the involvement of NO in this pathway. Treatment with 1 μM L-NIO, an inhibitor of eNOS, inhibited the K_{Ca} channel-opening effect of ginsenoside Re, whereas 3 μM SMTC, an inhibitor of nNOS, did not, indicating that this ginsenoside activates K_{Ca} channels via eNOS.

Ginsenoside Re Activates eNOS via PI3-Kinase/Akt Pathway

Recently, eNOS has been found to be activated via the PI3 kinase pathway by epigallocatechin, another phytochemical (4). We observed that SH-6 (10 μM), an Akt inhibitor, and wortmannin, a PI3-kinase inhibitor, completely blocked activation of K_{Ca} channels by ginsenoside Re, indicating that the ginsenoside Re activates eNOS via a c-Src/PI3-kinase/Akt-dependent mechanism.

Phosphorylation of Akt at Ser473 occurs when Akt is activated via a PI3-kinase-dependent pathway. We confirmed that ginsenoside Re induced phosphorylation of Akt in A10 vascular smooth muscle cells in a concentration-dependent manner. This increase in phosphorylation was inhibited by wortmannin and SH-6. These data further confirm that ginsenoside Re activates Akt via the PI3K pathway.

In addition, the ginsenoside Re-induced eNOS activation and K_{Ca} activation were blocked by 10 μM ICI 182,780, an inhibitor of membrane ER-α, suggesting that eNOS activation occurs via a non-genomic pathway of ER-α, in which c-Src, phosphoinositide 3-kinase, Akt, and eNOS are sequentially activated.

DISCUSSION

The present study provides convincing evidence to clarify a cadioprotective mechanism of ginsenoside Re in the cardiovascular system. We found that this phytochemical releases NO via a nongenomic pathway of sex steroid receptors resulting in K_{Ca} channel activation in vascular smooth muscle cells.

In our previous study, we found that K_{Ca} channels are controlled by various vasoactive substances, such as NO, endothelin, and angiotensin II. Using rabbit coronary artery, we found that K channels are very important in control of vascular tone and blocking them produces severe vasospasm (6, 7). Our present study indicates that ginsenoside Re activates the nongenomic pathway of sex steroid receptors to activate eNOS and release NO. Ginsenoside Re-induced K_{Ca} activation was blocked by inhibitors of c-Src, PI3-kinase, Akt, and eNOS, which are key signal molecules of the nongenomic pathway of sex steroid receptors, in which insulin also shares.

Hormone replacement therapy has been widely used in postmenopausal women for preventing cardiovascular events, osteoporosis, and other problems. There are, however, accompanying adverse effects : increased risk of estrogen-sensitive cancers (breast cancer and ovarian cancer). These adverse effects are considered to be mediated by genomic action of estrogen. Therefore, drugs which only act through nongenomic ER pathways are expected to avoid these problems. Ginsenoside Re seems to be a specific agonist for the nongenomic pathway of sex steroid receptors. In our previous studies, we found that ginsenoside Re does not activate the genomic pathway of sex steroid hormones, as it fails to recruit coactivators upon binding of ginsenoside to the sex hormone receptors (5). It also failed to promote growth of the cultured breast cancer cell line MCF-7 (5). Thus, ginsenoside is a naturally harvested, mechanism-specific agonist of sex steroid receptors. We expect that Panax ginseng has been successfully prescribed for health problems associated with the postmenopausal periods.

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