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Ginsenoside-Rd, a new voltage-independent Ca^{2+} entry blocker, reverses basilar hypertrophic remodeling in stroke-prone renovascular hypertensive rats

Bing-Xiang Cai [a,1], Xiao-Yan Li [a,1], Jing-Hui Chen [b,1], Yong-Bo Tang [a], Guan-Lei Wang [a], Jia-Guo Zhou [a], Qin-Ying Qui [a], Yong-Yuan Guan [a,*]

[a] Department of Pharmacology, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou, 510089, People's Republic of China
[b] Department of Anesthesiology, the third affiliated hospital, Sun Yat-Sen University, Guangzhou, 510089, People's Republic of China

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ABSTRACT

The total saponins of *Panax notoginseng* have been clinically used for the treatment of cardiovascular diseases and stroke in China. Our recent study has identified ginsenoside-Rd, a purified component of total saponins of *P. notoginseng*, as an inhibitor to remarkably inhibit voltage-independent Ca^{2+} entry. We deduced a hypothesis that the inhibition of voltage-independent Ca^{2+} entry might contribute to its cerebrovascular benefits. Ginsenoside-Rd was administered to two-kidney, two-clip (2k2c) stroke-prone hypertensive rats to examine its effects on blood pressure, cerebrovascular remodeling and Ca^{2+} entry in freshly isolated basilar arterial vascular smooth muscle cells (BAVSMCs). Its effects on endothelin-1 induced Ca^{2+} entry and cellular proliferation were assessed in cultured BAVSMCs. The results showed that, *in vivo*, ginsenoside-Rd treatment attenuated basilar hypertrophic inward remodeling in 2k2c hypertensive rats without affecting systemic blood pressure.During the development of hypertension, there were time-dependent increases in receptor-operated Ca^{2+} channel (ROCC)-, store-operated Ca^{2+} channel (SOCC)- and voltage dependent Ca^{2+} channel (VDCC)-mediated Ca^{2+} entries in freshly isolated BAVSMCs. Ginsenoside-Rd reversed the increase in SOCC- or ROCC- but not VDCC-mediated Ca^{2+} entry. *In vitro*, ginsenoside-Rd concentration-dependently inhibited endothelin-1 induced BAVSMC proliferation and Mn^{2+} quenching rate within the same concentration range as required for inhibition of increased SOCC- or ROCC-mediated Ca^{2+} entries during hypertension. These results provide *in vivo* evidence showing attenuation of hypertensive cerebrovascular remodeling after ginsenoside-Rd treatment. The underlying mechanism might be associated with inhibitory effects of ginsenoside-Rd on voltage-independent Ca^{2+} entry and BAVSMC proliferation, but not with VDCC-mediated Ca^{2+} entry.

1. Introduction

The voltage-independent Ca^{2+} entry, which involves the Ca^{2+} entry requiring either stimulation of membrane receptors (receptor-operated Ca^{2+} channels, ROCC) or depletion of the sarcoplasmic reticulum Ca^{2+} store (store-operated Ca^{2+} channels, SOCC), has been demonstrated to play a crucial role in the functional regulation of intracellular Ca^{2+} handling and cell proliferation in many cell types, such as endothelial cells (Yao and Garland, 2005), T lymphocytes (Guan et al., 2006) and pulmonary vascular smooth muscle cells (Remillard and Yuan, 2006). And, it is possible that the inhibition of voltage-independent Ca^{2+} entry may provide a new target for treating some pathological processes involving cell-proliferation (Dietrich et al., 2006; Firth et al., 2007). For example, the augmented Ca^{2+} entry through SOCC has been found in proliferating pulmonary vascular smooth muscle cells during pulmonary hypertension. Inhibition of SOCC by antisense strategy attenuated the store-operated Ca^{2+} entry induced by cyclopiazonic acid and pulmonary artery smooth muscle cell proliferation (Golovina et al., 2001).

There is growing evidence that the cerebrovascular hypertrophy remodeling is a prominent feature of chronic hypertension and considered a major risk factor for stroke (Baumbach and Heistad, 1989). In a well-established two-kidney, two-clip (2k2c) stroke-prone hypertensive rat model (Zeng et al., 1998), our recent study has revealed that the basilar arterial media undergoes remodeling, with an increase in basilar arterial vascular smooth muscle cell (BAVSMC) size and cross-sectional area of the media, demonstrating this nongenetic hypertension model share similar morphological alterations in the cerebral basilar arterial media wall with those observed in genetic hypertension models (Shi et al., 2007). The research focusing on the relationship between Ca^{2+} entry and cerebral vascular remodeling in hypertension has attracted much interest, indicating

* Corresponding author. Department of Pharmacology, Zhongshan School of Medicine, Sun Yat-Sen University, No.74, Zhongshan 2 Rd, Guangzhou, 510089, People's Republic of China. Tel.: +86 20 8733 1857; fax: +86 20 8733 1209.
E-mail address: guanyy@mail.sysu.edu.cn (Y.-Y. Guan).
[1] These authors contributed equally to this work.

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the hypertensive remodeling of arterial smooth muscle is closely associated with an increase in the functional L-type Ca^{2+} channel and voltage-dependent Ca^{2+} entry (Simard et al., 1998), and resulting in an alteration in the vascular smooth muscle tone. However, it is unknown whether voltage-independent Ca^{2+} entry changes during hypertension. If the change does occur, it is also important to understand the contribution of such Ca^{2+} entry remodeling to hypertensive cerebrovascular remodeling.

As a well-known traditional Chinese herb medicine, *Panax notoginseng* has long been used as an effective hemostatic and analgetcic drug. Since 1982, the total saponins of *P. notoginseng* have been clinically used for the treatment of cardiovascular diseases and stroke in China. However, the underlying mechanism has not been fully understood. In our previous studies, we are the first to report that the total saponins from *P. notoginseng* have inhibitory effects on the contractile response and ^{45}Ca influx induced by activation of α_1-adrenoceptor without effects on Ca^{2+} release and the response to high K^+ in vascular smooth muscle (Guan et al., 1985, 1988, 1994). Recently, we have further identified that ginsenoside-Rd (Dammar-24(25)-ene-3,12,20(*S*)-triol-(20-*O*-β-D-gluco-pyranosyl)-3-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosid), a purified component from total saponins of *P. notoginseng*, remarkably inhibites voltage-independent Ca^{2+} entry without influencing voltage-dependent Ca^{2+} entry and Ca^{2+} release in vascular smooth muscle cells (Guan et al., 2006).

Given the emerging importance of voltage-independent Ca^{2+} entry in cell proliferation and tissue remodeling, we suggest a hypothesis that this mechanism may contribute to therapeutic effects of ginsenoside-Rd against stroke. In present study, we have examined the effects of ginsenoside-Rd treatment on the systolic blood pressure and structural remodeling of cerebral basilar artery during hypertension. The Ca^{2+} entries via SOCC, ROCC and VDCC have been examined respectively in BAVSMCs freshly isolated from 2k2c rats during the development of hypertension. To clarify the role of voltage-independent Ca^{2+} entry in vascular remodeling, we have also tested whether ginsenoside-Rd would inhibit the endothelin-1 induced cell proliferation and voltage-independent Ca^{2+} entry in cultured BAVSMC. Overall, the present study has provided *in vivo* evidence showing attenuation of hypertensive cerebrovascular remodeling after ginsenoside-Rd treatment. The underlying mechanism may be associated with inhibitory effects of ginsenoside-Rd on voltage-independent Ca^{2+} entry and BAVSMC proliferation, but not with VDCC-mediated Ca^{2+} entry.

2. Materials and methods

All the experimental procedures and protocols on animals were approved by the Sun Yat-Sen University Committee for Animal Research and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.1. Animal model

The healthy male Sprague–Dawley rats weighed 90–120 g were housed in a temperature-controlled environment (20–25 °C) with a 12 h light/dark cycle. All rats were held in Animal Research Center at the Sun Yat-Sen University. The rats used in *in vivo* studies (n=180) were randomly chosen for immunohistochemistry, electron microscopy and Mn^{2+} quenching experiments. Each group had 60 rats and was further divided into 5 groups: (1) sham-operated control group; (2) 2k2c hypertension group; (3) propylene glycol treated hypertensive group: rats were received intraperitoneal injection of 2 ml saline solution containing 20% propylene glycol per day after surgery. (4) ginsenoside-Rd group*:* rats were received intraperitoneal injection of ginsenoside-Rd (20 mg in 2 ml saline solution containing 20% propylene glycol/kg/day) after surgery. 2k2c stroke-prone renovascular hypertensive rats were prepared as described previously (Zeng et al., 1998). The animals were killed at the end of week 1, 4, 8 and 12 by the injection of pentobarbital (40 mg/kg).

2.2. Tissue preparation and immunohistochemistry

The rat brain was carefully removed and sections (8 μm) were prepared from freshly frozen rat basilar arteries as previously described (Shi et al., 2007). Each section was cut coronally at the level of the optic chiasm including middle cerebral arteries. Standard histological examinations were performed as previously described (Shi et al., 2007). Briefly, sections were exposed to α-smooth muscle actin monoclonal antibody (Sigma; dilution 1:400) at 4 °C overnight and then were treated with goat FITC-conjugated secondary antibody (Sigma; dilution 1:400) at room temperature for 30 min. The quantification of α-smooth muscle actin staining was captured and analyzed using confocal system (OLYMPUS, FV500-IX 81, magnification 400) and Image-Pro Plus 5.0. For each tested group at each time point, 12 rat brains were taken for the experiment, respectively. A total of 8 randomly selected sections from the area of basilar artery per brain were quantitated for each type of labeling.

2.3. Electron microscopy

Rats were deeply anesthetized with 10% chloraldurat and received intracardiac injection of heparin (500 U) and nitroglycerol (0.5 μg) in order to prevent coagulation and dilate blood vessels. Animals were then perfused transcardially with 0.1 M phosphate buffer, pH 7.4, the pressure was controlled ≈100 mmHg, followed by 4 °C fixative solution containing 4% freshly depolymerized paraformaldehyde, 0.25% glutaraldehyde and 15% saturated trinitrophenol in 0.1 M phosphate buffer at pH 7.4. The brain was removed, the tissue blocks containing the basilar artery at midpoint were cut into cubes of 1 mm×1 mm×3 mm, and then immersed into fixative solution at 4 °C, overnight. The tissue blocks were postfixed in 2% osmic acid for 2 h, dehydrated via graded alcohols and embedded in Epon 812. Ultrathin sections with thickness of 80–100 nm were prepared and stained with uranyl acetate and lead citrate before they were viewed under a transmission H-600 electron microscope (Hitachi, Japan). Care was taken to use only orthogonal sections based on visual examination of the sectioned block and section thickness (only the thinnest sections used).

2.4. Cell fresh isolation and culture

At the different time points during the development of hypertension, BAVSMCs were freshly isolated by enzymatic digestion as described previously (Shi et al., 2007). The basilar arteries were placed in a cold (4 °C), 95% O_2–5% CO_2-saturated solution containing (mM): 130 NaCl, 5 KCl, 0.8 $CaCl_2$, 1.3 $MgCl_2$, 10 HEPES and 5 glucose, pH 7.4. The arteries were cleaned of connective tissue and small side branches, cut into 0.2 mm rings and incubated in low Ca^{2+} solution (mM): 0.2 $CaCl_2$, 130 NaCl, 5 KCl, 1.3 $MgCl_2$, 10 HEPES and 5 glucose, pH 7.4, containing collagenase (Type II, 0.5 g/L), elastase (Type II-A , 0.5 g/L), hyaluronidase (Type IV-S, 0.5 g/L) and deoxyribonuclease I (0.1 g/L) for 1 h at room temperature. The rings were washed in fresh low Ca^{2+} solution containing trypsin inhibitor (0.5 g/L), deoxyribonuclease I (0.1 g/L) and then triturated gently for 15–20 times. The isolated cells were plated on glass coverslips in the above-mentioned buffer solution containing 0.8 mM $CaCl_2$ and fatty acid-free bovine serum albumin (2 g/L). The freshly isolated BAVSMCs should be used for experiments within 10 h.

BAVSMCs were cultured from rat basilar arteries by a modification of methods previously described (Guan et al., 2006). Briefly, Sprague–Dawley rats (180–200 g) were anaesthetized and decapitated. Basilar arteries were harvested rapidly and immersed in Kreb's solution (in mM) containing NaCl, 137, KCl 5.4, $CaCl_2$ 2.0, $MgCl_26H_2O$ 1.1,