Effects of *Panax notoginseng* saponins on vascular endothelial cells *in vitro*

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ABSTRACT

AIM: To investigate the inhibition of endothelium-dependent *in vitro* vascular relaxation induced by the total saponins (gensenosides) from *Panax notoginseng* (PNS) and the effect of PNS on the cytosolic Ca^{2+} concentration on cultured bovine pulmonary artery endothelial cells. **METHODS**: The endothelial-dependent vascular relaxation was assessed using acetylcholine (ACh) or cyclopiazonic acid (CPA) induced relaxation in endothelium-intact rat aorta. Cytosolic Ca^{2+} level was assessed in real time using dynamic digital fluorescence ratio imaging. **RESULTS**: In addition to its direct relaxation of the smooth muscle cells at high concentrations, PNS, at 100 mg/L having little effect on smooth muscle, caused a marked inhibition of endothelium-dependent relaxation brought about by PNS. This inhibitory effect was due to its inhibition of elevation of cytosolic Ca^{2+}, which is required for the activation of NO generation and release from the vascular endothelial cells. Nifedipine has no effect on either the endothelium-dependent relaxation or the cytosolic Ca^{2+} level in the cultured endothelial cells. **CONCLUSION**: Our findings are consistent with the known action of PNS on receptor-operated Ca^{2+} channels and support our contention that PNS inhibits endothelium-dependent relaxation by preventing the increase of Ca^{2+} level in endothelial cells via the receptor-operated Ca^{2+} channels in the presence of ACh or the non-selective cation channels opened by CPA.

INTRODUCTION

Ginseng roots, as an effective tonic herbal medicine, have been widely used in traditional Chinese medicine for the past 20 centuries to enhance stamina and to recuperate from physical stress, particularly when the physical capacity is compromised. The use of ginseng products for health promotion has also increasingly won popularity in the Western countries as a result of the increasing public enthusiasm for herbal remedies and other non-conventional natural therapies under the newly evolving discipline in health care, the arena of complementary medicine. Despite wide use of ginseng by the public, the government regulatory bodies and the medical communities have not officially accepted the use of ginseng as a validated therapeutic measure. There are many legitimate reasons for lacking an official recognition of the health effects of this historically legendary wonder herb. The most compelling reason is the lack of systematic and credible research on its medical values and the lack of public education to differentiate fallacies from facts. For example, the ginseng plants grown in the Orient and the North America, *Panax ginseng* and *Panax quinquefolium*, respectively, are of different species. The practice of Chinese herbal medicine has clearly made the distinction in the use of these ginseng herbs; *Panax ginseng*, being of "hot/warm" nature (stimulatory), is generally recommended for enhancing blood circulation under Yin-dominant conditions. *Panax quinquefolium*, on the other hand, being regarded as "cold/cool" (sedative), should only be used moderately for individuals with yang-deficiency or yin-excess. In the West, on the other hand, many ginseng products usually do not identify the source, the species and the anatomical part of the herb used in the ginseng medicament. A third widely used ginseng species in China, *Panax notoginseng*, is also grown in the southwestern provinces of China. It is topically used as an ointment preparation to improve the microcirculation in the muscle and to dissipate physical bruises due to skeletomuscular injury in sport activities. Interestingly, all these ginseng species seem to work through their effects on the circulatory system.

A group of distinct glycosides referred to as ginseng saponins or ginsenosides, represents the major active ingredients with various pharmacological actions and biological effects[1]. The ginsenoside content also depends on the species of ginseng as well as the age and part of the ginseng plants used[2,3]. The crude saponins isolated from *Panax ginseng* (PGS) have been shown to cause vasorelaxation in some vascular segments, but vasoconstriction in others[4]. A standardized preparation of extract from the root of *Panax ginseng*, G-115, has also been shown to induce relaxation of contraction induced by phenylephrine (PE). PNG has long been shown to lower blood pressure in experimental animals via its direct vasodilatory effects and it selectively inhibited the contraction induced by phenylephrine (PE), but not that by KCl[5-7]. The fraction of total saponins from *Panax quiquefolius roots* (PQS), on the other hand, was shown to cause an enhancement of PE-induced contraction[8], but the crude saponins isolated from its leaves and stems cause partial relaxation of both PE- and KCl-induced contractions[9].

Endothelial cells are known to modulate vascular reactivity by releasing vasoactive substances, nitric oxide (NO) being most well known for its potent relaxant effects and its discovery is being recognized with a Nobel Prize for Medicine this year. Korean investigators have recently reported that PGS promoted NO release from blood vessels causing endothelium-dependent relaxation[10,11] (also see the work by Jeon *et al* in this issue), these ginseng saponins appears to act directly on the expression or the activation of the nitric-oxide synthase. It is not known whether ginsenosides from other ginseng species will also elicit a direct endothelium-dependent relaxant effect, particularly in light of the evidence for the Yin-Yang dichotomy on the vascular effects of different ginseng species. The primary aim of this work was to investigate the possible effect of PNS on the endothelium-dependent relaxation using rat aorta and to examine the effect of PNS on the change of Ca^{2+} distribution in cultured vascular endothelial cells using dynamic fluorescence ratio digital imaging technique.

MATERIALS AND METHODS

Animals and contractility studies   Aortic rings from the Wistar male rats (250 – 300 g body weight) were removed and placed in Krebs' physiological saline solution containing (in mmol/L): 115.5 NaCl; 4.6 KCl; 1.16 MgSO₄; 1.12 NaH₂PO₄; 2.5 CaCl₂; 21.9 NaHCO₃; 11.1 D-glucose, prepared in double distilled water. The solution was continually aerated with a 95 % O_2/5 % CO_2 gas mixture to maintain a pH near 7.4 at room temperature. After surrounding fats and connective tissues were removed, the arterial rings were then cut into ring segments 3 – 5 mm in width. Vascular rings were carefully suspended in the bath chamber containing 10 mL aerated Krebs' solution at 37 ℃ between two stainless steel hooks with a fixed bottom hook and an upper hook connected to a force transducer, which was connected to the Beckman R-611 recording dynograph. The optimal resting tension was set at 1 g. Following 90 – 120-min equilibration under the resting tension, recording of isometric tension development was made after 3 – 4 consecutive and consistent contractions which occurred in response to 100 mmol/L KCl. The endothelium layer of the vascular rings was removed, when required, by rubbing the inner surfaces against the teeth of the forceps. The effectiveness of the endothelial cell removal was functionally confirmed as the lack of relaxation following the application of 1 μmol/L acetylcholine (ACh) at the peak of the contraction to 10 μmol/L phenylephrine (PE). A 20 – 30 min period between two consecutive applications of stimulant was allowed for thorough washout of the drugs. A contraction to 100 mmol/L KCl was always obtained at the end of the experiment and this KCl-contraction was usually better than 90 % of the initial KCl-contraction in magnitude.

Cell culture and calcium imaging   Bovine pulmonary artery endothelial cells were obtained from ATCC (USA) and cultured in minimum essential medium (MEM from Gibco, Grand Island, NY) supplemented with 20 % fetal bovine serum, 0.1 % gentamicin and 0.1 % fungizone in a humid atmosphere of 95 % air/5 % CO_2 at 36 ℃. For digital Ca^{2+} imaging experiments, cells were exposed to 0.5 % trypsin to dislodge cells, which were then collected by low speed centrifugation and resuspended in HEPES-buffered saline solution containing 0.2 % bovine serum albumin and, in mmol/L, NaCl 126, KCl 6, glucose 10, CaCl₂ 1.5, MgCl₂ 0.3 and HEPES 10 with pH adjusted to 7.4 with NaOH. Cells were plated on coverslips and were about 40 % – 80 % confluent at the time of use. The cells were loaded with Fura-2 by incubating the cells for about one hour with 8 μmol/L Fura-acetoxymethyl ester (prepared in dimethyl sulfoxide as 1 mmol/L stock concentration). Since one run of each culture dish sample takes 15 – 30

min and a typical experiment contains 5 – 7 dishes, loading of cells with Fura-2 was staggered over the course of the experiment. The endothelial cells were mostly cobble-stone shape and remained firmly immobilized during the entire course of the measurement. Cytosolic distribution of Ca^{2+} concentration in single endothelial cells was monitored using a dynamic digital Ca^{2+} imaging system (Image-1/FL, Universal Imaging Corporation) coupled to a Zeiss inverted microscope (Zeiss IM 35) with a 100 × oil immersion lens and a numerical aperture of 1.25. Filter wheel held filters at 340 and 380 nm, which alternated and captured images on the first and second quadrant of the monitor screen. The ratio between these two wavelengths (340 nm/380 nm) was displayed on the third quadrant and the time event of the ratio changes at selected regions of the cells was displayed at the forth quadrant. Emitted fluorescence was detected with a 510-nm filter. Images were integrated and collected by a Pulnix camera (TM – 720) at a maximal speed of 3 sec/frame. Background values were obtained by defocusing. These cells were found to be quite Ca^{2+} tolerant and have functioinal Ca^{2+} channels and Ca^{2+} pumps[12].

RESULTS AND DISCUSSION

Fig 1 shows the effect of 100 mg/L PNS on the relaxation of endothelium-intact aortic rings precontracted by 10 μmol/L PE in response to 1 μmol/L ACh (a), 1 μmol/L cyclopiazonic acid (CPA; b). Fig 1c shows the control response when the relaxation was induced by 1 μmol/L of sodium nitroprusside (SNP) in an endothelium-independent way. Fig 1d shows another control using endothelium-denuded rings. PNS apparently inhibited the relaxation induced by either ACh or CPA, both are known to cause endothelium-induced relaxation via a Ca^{2+}-dependent manner.

In this experiment only 1 μmol/L CPA was used, since higher concentrations of CPA (> 10 μmol/L) would produced direct smooth muscle contraction by raising cytosolic Ca^{2+} level[13]. The relaxation induced by SNP following PE contraction was not endothelium-dependent and was also not affected by PNS. Addition of 100 mg/L PNS directly at the peak of PE contraction of the endothelium-intact rings was not able to induce relaxation. Increasing PNS concentration to 600 mg/L, however, a prominent relaxation was observed (Fig 1d) due to its direct action on the smooth muscle via inhibition of the putative receptor-operated Ca^{2+} channels[7]. We

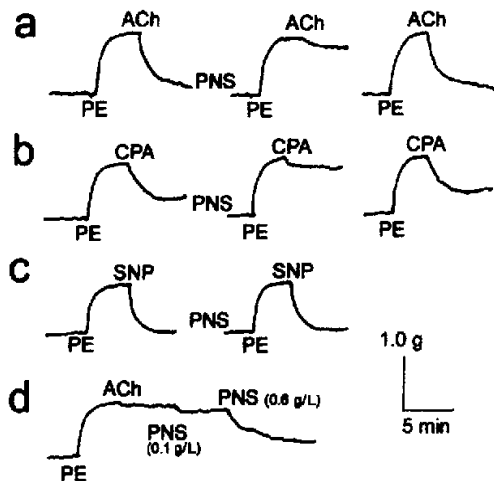Fig 1. Effect of 0.1 g/L PNS on the relaxation induced by 1 μmol/L Ach (a), 1 μmol/L CPA (b) and 1 μmol/L sodium nitroprusside, SNP (c) in endothelium-intact rat aorta precontracted with 10 μmol/L PE. In (a) and (b), the relaxation due to ACh or CPA was reversible following wash. In (d) using endothelium-denuded rat aorta, 1 μmol/L ACh or 100 mg/L PNS failed to induce relaxation, but 600 mg/L PNS caused prominent endothelium-independent relaxation.

have previously observed that such endothelium-independent relaxation was more effective for contraction at lower PE concentration (0.3 – 1.0 μmol/L) and was not effective for KCl-induced contraction[7].

These findings are consistent with the notion that PNS inhibited the putative receptor-operated Ca^{2+} channels in vascular smooth muscle and endothelial cells. In vascular smooth muscle cells, PNS acts by lowering the Ca^{2+} level causing direct relaxation, whereas in endothelial cells, PNS also acts by lowering the Ca^{2+} level, causing inhibition of the NO production and thus its release, resulting in the inhibition of endothelium-dependent relaxation induced by ACh and CPA. To test this hypothesis, we further study the direct effect of PNS on the Ca^{2+} level in cultured vascular endothelial cells by using digital fluorescence ratio imaging technique.

Fig 2 shows the Ca^{2+} imaging of the vascular endothelial cells at 340 nm, 380 nm and the 340/380 ratio. The cells had low resting Ca^{2+} levels in the physiological saline solution containing 2 mmol/L Ca^{2+} as indicated in panel 1 (also see the number 1 in the ratio-time profile showing ratio of ~ 0.6). However, the peripheral region of the cells showed a higher level of Ca^{2+} than the cytosolic space. Shortly after CPA was added (about
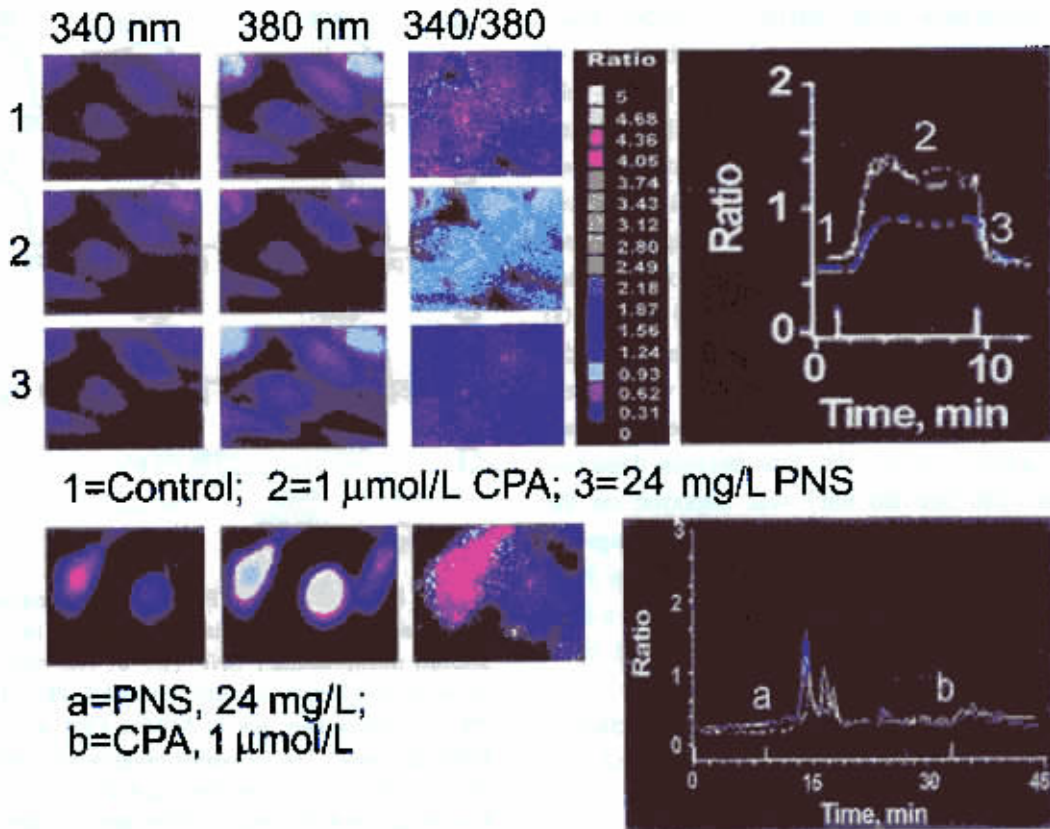
Fig 2. Digital fluorescence imaging of Fura-2 loaded vascular endothelial cells and the effects of 24 mg/L PNS (extracted from the leaves) on CPA-induced rise of Ca^{2+} concentration.

1.5 min after the 1st image being captured), the overall fluorescence ratio increased with the peripheral and nuclear regions higher (ratio ~ 1.3) than the cytosolic region (ratio ~ 0.8). The image shown was captured as indicated by number 2 of the ratio-time profile. Upon the addition of 24 mg/L PNS (from root), an immediate reduction of Ca^{2+} toward the resting level was observed (image captured at time point as indicated by number 3). Occasionally, the decrease of ratio was preceded by a burst of transient increase of ratio when PNS from the leaves/stems was used (see below). The lower panel shows the addition of PNS from the leaves/stems causing the bursting transient changes of the resting Ca^{2+} levels. It also prevented the elevation of Ca^{2+} induced by CPA. PNS from the root also shows such prevention of Ca^{2+} rise by CPA, but has no effect on the resting Ca^{2+} levels.

Although not shown in the results, we have also examined the effect of 0.1 – 1.0 μmol/L nifedipine and observed that it had no effect on the endothelial cell Ca^{2+} elevated by CPA. Although 0.1 μmol/L caused direct relaxation of rat aorta (endothelium intact) but the relax-

ation was incomplete. PNS did not modify the residual tone in the presence of nifedipine suggesting that nifedipine does not modify the vascular reactivity in an endothelium-dependent manner like PNS.

CONCLUSIONS

We have for the first time reported the inhibition of endothelium-dependent relaxation induced by PNS. This inhibitory effect of PNS was due to its inhibition of Ca^{2+}-activated NO generation and release from the endothelial cells, consistent with the known action of PNS on receptor-operated Ca^{2+} channels. We have further demonstrated directly by digital Ca^{2+} imaging that the increased Ca^{2+} level in endothelial cells in the presence of ACh or CPA was inhibited by PNS. Kimura et al[14] also showed in Fura-2 loaded human platelets that gensenosides inhibited Ca^{2+} influx and aggregation of human platelets. In a recent short review[15], Gillis presented evidence that some of the pharmacological profiles of gensenosides include their effects on the NO signaling pathway. Some of these findings are at variance with

those reported using PGS showing that it actually enhanced NO production and led to vascular relaxation[10,11,16], attesting to the Yin-Yang dichotomy of the effects of ginsenosides as we proposed earlier[17]. If we assume the lowering effect of Ca^{2+} level as a Yin characteristic, PNS from the roots would be dominated primarily by Yin ginsenosides, whereas PNS from the leaves/stems would have some prominent Yang ginsenosides in the pool of Yin ginsenosides. This study, together with our earlier studies[8,9] provides an interesting scientific basis for the Yin-Yang Principle of the Chinese art of healing. It also lays a sensible foundation for comparative identification of the Yin (inhibitory) and Yang (stimulatory) ginsenosides in the future.

The present results provided a logistic and mechanistic interpretation for the inhibitory action of PNS at the cellular level in relation to Ca^{2+} mobilization. However, the application of such interpretation for the stimulatory effect of PGS on endothelium-dependent relaxation, on the other hand, requires that PGS stimulates endothelial cells and activates NO synthase and releases NO. This interpretation seems intangible, because PGS directly inhibits the smooth muscle contraction, probably by way of inhibiting Ca^{2+} influx, like PNS. It is possible that PGS may activate the endothelial Ca^{2+} channels, but inhibit smooth muscle Ca^{2+} channels. Alternatively, PGS may have a direct stimulatory action on the NO synthase activity or act by removal of endogenous scavenger of NO or inhibitors of NO synthase.

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