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Research Article

Vascular activity of protopanaxadiol and protopanaxatriol, the major saponin fractions isolated from roots of *Panax notoginseng*

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ABSTRACT

Background: *Panax notoginseng* (PN) (Burk) F. H. Chen (Araliacea) is a Chinese herb commonly known in Asia for its medicinal potentials, antihypertensive included. The aim of this study was to investigate the vasorelaxant activity of protopanaxadiol (PDS) and protopanaxatriol (PTS), the major saponin fractions isolated from roots of PN, and identify the underlying mechanisms on isolated rat aortic rings. **Methods:** Radnoti Tissue-Organ bath instrument (ADInstruments) was used to perform Tissue-Organ bath experiments, and tensions produced on isolated rat aortic rings were recorded by the help of ADInstruments PowerLab 8/30 system (Model ML 870, Australia). **Results:** Both PDS and PTS (10^{-6}–10^{-3} µg/ ml) induced concentration-dependent relaxations in intact rat aortic rings pre-contracted with norepinephrine (1 µM).This activity was considerably reduced after removal of the endothelium and in intact rings pre-treated with N^G-nitro-L-arginine methyl ester (L-NAME, 100 µM), 1H-[1,2,4]-oxadiazole-[4,3-α]-quinoxaline-1-one (ODQ, 10 µM), and combination of L-NAME and ODQ. However, pre-treatment with indomethacin (10 µM) did not affect either PDS- or PTS-induced relaxations. Furthermore, both PDS and PTS (10^{-4} and 10^{-3} µg/ml) decreased $CaCl_2$- and potassium chloride (KCL)-induced vasoconstrictions in a dose-dependent manner. Our findings suggest that both PDS and PTS produced vasorelaxant effect that was mediated by NO-cyclic guanosine monophosphate in intact rings and by blockade of calcium influx in vascular smooth muscle. Cyclooxygenase pathway, on the other hand, had no apparent role. Among these fractions, PDS showed better effect with a slight difference. **Conclusion:** Findings from this study showed that both PDS and PTS fractions possess vasorelaxant activity. This supports the potential use of PN in management of cardiovascular diseases, especially hypertension.

KEY WORDS: Cardiovascular disease, *Panax notoginseng*, Protopanaxadiol, Protopanaxatriol, Vascular relaxation

BACKGROUND

Cardiovascular disease (CVD), which encompasses a spectrum of diseases including hypertension, is perennial among the leading causes of morbidity and mortality worldwide.[1-6] Many CVDs are pathophysiologically associated with deficiency of nitric oxide (NO) in the heart and blood vessels.[7] The regulation of vascular tone is one among therapeutic interventions used for the appropriate control of blood pressure as contraction and dilation of blood vessels directly control blood pressure.[8] Research aimed at finding better therapeutic options for CVDs is in progress in many laboratories around the world, and as a result, a large volume of data has been generated. Abnormal vascular reactivity, including impaired endothelium-dependent relaxation and enhanced sensitivity to vasoconstrictors, is a hallmark of hypertensive disease.[9,10] Therefore, the relaxation of aortic vascular smooth muscles is very crucial in hypertension and other CVDs. Therapeutically, antihypertensive drugs most used ultimately produce vasorelaxation either directly or indirectly.[11] Although hypertension can be pharmacologically managed, none of available drugs is without risk of adverse reactions. They instead tend to "control" the condition, while helping extend one's life.[12] Therefore, the discovery of new, effective, and safe natural vasodilators relevant

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to clinical use would be a great relieve to millions of people since CVD, especially hypertension, seems to represent a serious burden to the entire world.

Medicinal plants have since ages been used to manage hypertension with minimal side effects.[13,14] They have been of central focus for the screening of newer and better treatment of health problems, especially cardiac diseases.[15] In this regard, PN has been reported to be one of the promising medicinal herbs with antihypertensive potential.[16] Qualified as "the miracle root for the preservation of life," PN is among the most used herbs to prevent and treat CVD, either alone or in combination.[17-19] Clinically, PN has shown considerable potential in the treatment of coronary heart disease, stroke, inflammation, and immunological disease.[20-22]

PN and its components were found to possess several beneficial actions on the cardiovascular system including protection of cardiac ischemia,[23] calcium (Ca^{2+}) channel blockade,[24] and inhibition of platelet aggregation.[25] PN root is considered as unique herb for its distinct clinical usage[26] and for its high contents in Rb1, Rd, and Rg1 levels.[27]

Panax notoginseng saponins (PNS), the major components of PN,[28,29] include more than 30 different types of saponins,[30,31] among which ginsenosides Rb1, Rg1, Rd, and notoginsenoside R1 are found in the highest content.[32,33] Based on their chemical structures, Rg1, R1, and Re are classified as protopanaxatriol (PTS)-type saponins whereas Rb1 and Rd are categorized as protopanaxadiol (PDS)-type and they act through generation of NO inside cells.[34-37] Rg1 is reported to possess the ability to agonize glucocorticoid receptors which once activated can produce rapid NO in human umbilical vein endothelial cells (HUVECs).[38,39]

Hypotensive potential of PN total extract has been demonstrated in spontaneously hypertensive rats (SHR) by lowering blood pressure while ginsenosides Rb1 and Rg1 cause endothelium-dependent relaxation in mouse coronary arteries, through activation of NO in endothelial cells.[16] However, up to date vascular effects and mechanisms of either PDS or PTS, the two main saponin fractions from roots of PN have not yet been demonstrated. This study, therefore, investigated their vascular activities on rat isolated aortic rings and elucidated possible associated signaling pathways.

MATERIALS AND METHODS

All chemical reagents used were obtained from Sigma-Aldrich Inc., St. Louis, MO, USA. PDS and PTS fractions were provided by Zhejiang University, School of Pharmacology. Distilled water was used to dissolve all used drugs except indomethacin and 1H-[1,2,4]-oxadiazole-[4,3-α]-quinoxaline-1-one (ODQ) whose dissolution required dimethyl sulfoxide. The modified Krebs–Henseleit (K–H) solution was used as bath solution. Radnoti Tissue-Organ bath instrument (ADInstruments) was used to perform Tissue-Organ bath experiments, and tensions induced in isolated rat aortic rings were recorded by the help of PowerLab system (ADInstruments PowerLab 8/30, Model ML 870, Australia).

Preparation of Aortic Rings

Male Wistar rats (250–300 g) were executed by decapitation. Following decapitation, the descending thoracic aorta was carefully excised and placed in modified K–H solution pre-cooled at 4°C ($_{p}$H 7.4) from where the aorta was carefully cleaned of fat, connective tissues and cut into 2–3 mm ring segments (Mary, 1999). For the intact aortic rings, the endothelium remained intact and caution taken to avoid injury to EC. For the denuded rings, endothelium was removed by gently rubbing the luminal surface of the ring with a roughed polyethylene tube.

Measuring of Isometric Vascular Tone

The aortic rings were mounted between two stainless steel wires into a tissue bath (Radnoti Glass Technology) filled with 10 ml of modified K–H buffer which was maintained at 37°C (pH 7.4) and gassed continuously with a 95% O_2 and 5% CO_2 mixture. The rings were allowed to equilibrate for at least 60 min under the resting force of 2.0 g. During the equilibration period, the buffer was routinely changed every 15 min to prevent the accumulation of metabolites.[40] Tensions were recorded by the help of AD instrument PowerLab 8/30 system.

Experimental Protocol

After equilibration period, the viability of rings was checked by pre-treatment with KCL 60 mM. This was followed by washing out and stabilizing in modified K–H buffer followed by pre-contraction with norepinephrine (NE) 1 μM. At the plateau phase, the existence of intact endothelium was verified by the ability of acetylcholine (ACh) 10 μM to induce relaxation on 1 μM NE-contracted rings. Rings were considered to be functionally denuded of endothelium if vasorelaxation to ACh (10 μM) was <10%.[41] Pre-contracted rings (intact or denuded) were randomly exposed to cumulative concentrations of either PDS or PTS fractions to evaluate their relaxant effects.

Effect of Endothelium in PDS- and PTS-Induced Relaxations

To assess the role of endothelium on PDS- and PTS-induced relaxations, experiments were run on denuded rings.

Investigating Mechanisms underlying Vasorelaxant Effect of PDS and PTS

NO and cyclooxygenase (COX) pathways

To assess the role of NO and COX pathways, the two fractions (PDS and PTS) were pretreated with different inhibitors including indomethacin (10 μM), a COX inhibitor, a NO synthase inhibitor, and L-NAME (100 μM). Each of these inhibitors was incubated in the bath for 20–30 min before adding 1 μM NE to increase the tone.

NO/cyclic guanosine monophosphate (cGMP) pathways

The role of NO/cGMP pathways was investigated using either ODQ (10 μM) alone or a combination of both ODQ (10 μM) and L-NAME (100 μM).

Effect of PDS and PTS on KCL Responses

To investigate the impact of these two fractions on cumulative responses of KCL (7.5–60 mM), denuded tissues (rings) were incubated with either PDS or PTS (10^{-4} and 10^{-3} μg/ml) for 20 min and their effect was evaluated by comparing levels of KCL-induced contractility in the presence and absence of both PDS and PTS separately.

Effect of PDS and PTS Fractions on Extracellular Ca^{2+} Ions Influx

To assess the effect of PDS and PTS fractions on cumulative responses of $CaCl_2$ (10^{-3}–$10^{-1.5}$ M), denuded rings were bathed with Ca^{2+}-free, highly concentrated KCL (80 mM) Krebs solution then incubated with either PDS or PTS (10^{-4} and 10^{-3} μg/ ml) for 30 min and their effect was assessed by comparing contractions produced by $CaCl_2$ before and after incubation with either PDS or PTS.

Data and Statistical Analysis

All values are expressed as means ± standard deviation error. Relaxant responses are presented as percentage change in tension from pre-constriction levels. Data analysis was done by the help of one-way ANOVA followed by separation of means by Bonferroni *post-hoc* test (SPSS version 16.0). $P \leq 0.05$ was considered statistically significant. GraphPad Prism 5 was used for curve fitting.

RESULTS

Vascular Relaxant Effects of PDS and PTS

Both PDS and PTS induced dose-dependent relaxations in endothelium-intact rings.

To ascertain whether this activity was endothelium dependent, the same experiments were carried out on denuded rings and it was observed that endothelium removal significantly reduced PDS and PTS relaxations [Figure 1].

Effect of L-NAME, ODQ, L-NAME+ODQ, and Indomethacin on PDS-and PTS-Induced Vascular Relaxations

Our findings show that PDS- and PTS-induced relaxations were significantly reduced in endothelium-intact rings pre-treated with L-NAME (100 μM), ODQ (10 μM), and combination of ODQ with L-NAME. It was observed that L-NAME and ODQ combined further decreased these effects compared with L-NAME alone. However, PDS and PTS effect in intact tissues pre-treated with indomethacin persisted [Figures 1 and 2].

The Effect of PDS and PTS on $CaCl_2$- and KCL-Induced Vasoconstrictions

This study shows that pre-treatment of endothelium-denuded rings with PDS and PTS (10^{-4} and 10^{-3} μg/ ml) significantly reduced the potency of contractile responses to $CaCl_2$ and KCL [Figures 3 and 4].

DISCUSSION

This study has demonstrated that both PDS and PTS, the major saponin fractions isolated from roots of PN, induce vasorelaxation of NE pre-contracted rat aortic rings in a dose-dependent manner. This effect disappeared in the absence of functional endothelium. Our findings suggest that PDS and PTS dilate vascular smooth muscle through endothelium-dependent NO-cGMP pathway.

The vascular endothelium plays an important role in the regulation of cardiovascular functions, mainly in controlling vascular tone through synthesis and release of endothelium-derived relaxing factors, such as NO and prostacyclin (PGI_2). Impaired endothelial function, with reduced NO activity, is associated with CVDs, including hypertension.[42,43] In this regard, this study first investigated the role of functional endothelium in either PDS- or PTS-induced vasorelaxation and it was found that the removal of the functional endothelium abolished their relaxant effects [Figure 1b and c). Thus, we suggest that PDS- and PTS-induced relaxations were dependent on the presence of endothelium.

This study also investigated the involvement of NO release in relaxant effects of both PDS and PTS and it was found that their relaxant effects were significantly decreased in endothelium-intact rings pre-treated with L-NAME [Figure 2], indicating the contribution of NO in PDS and PTS effect which in particular supports the clinical use of PN in the management of CVD, especially hypertension in traditional Chinese medicine (TCM).These findings are consistent with a previous study that showed that both PN extract and the major compounds Rb1 and Rg1 increase NO

production in mouse coronary arteries.[44-48] Based on this, we can conclude that the vasorelaxant effect caused by both PDS and PTS is NO mediated.

It has been demonstrated that several stimulants activate soluble guanylyl cyclase (sGC) resulting in the formation of cGMP whose intracellular accumulation signals protein kinases, ion channels, and other effector systems causing smooth muscle relaxation.[49-53] In our experiments, ODQ, a specific sGC inhibitor, markedly attenuated vasorelaxant effect of both PDS and PTS and this was further decreased by combination of L-NAME and ODQ [Figure 2]. These findings indicate the involvement of NO and cGMP in PDS and PTS vasorelaxant activity, suggesting that their relaxant effect is induced through NO-cGMP pathway.

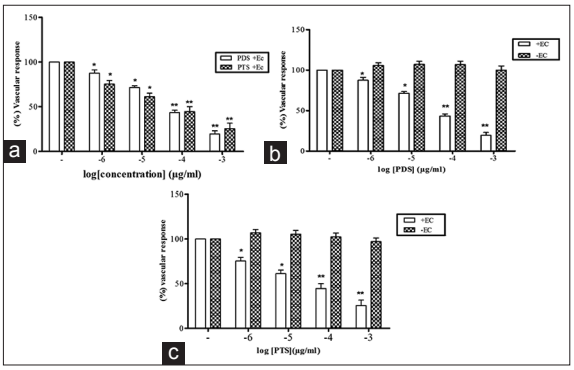

Figure 1: Vasorelaxant effects of protopanaxadiol (PDS) and protopanaxatriol (PTS) with or without endothelium. (a) PDS and PTS with endothelium, (b) PDS with and without endothelium, (c) PTS with and without endothelium. Data are shown as mean ± SEM of five independent experiments. SEM: Standard deviation error

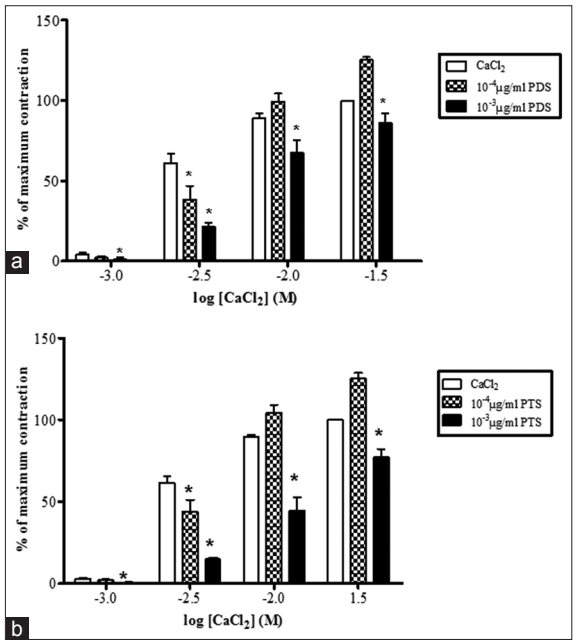

Figure 3: Effect of protopanaxadiol (PDS) and protopanaxatriol (PTS) on $CaCl_2$-induced contractions in endothelium-denuded rat aortic rings depolarized by 80 mM KCL. (a) Effect of PDS, (b) effect of PTS. Data are shown as mean ± SEM of five independent experiments. SEM: Standard error mean

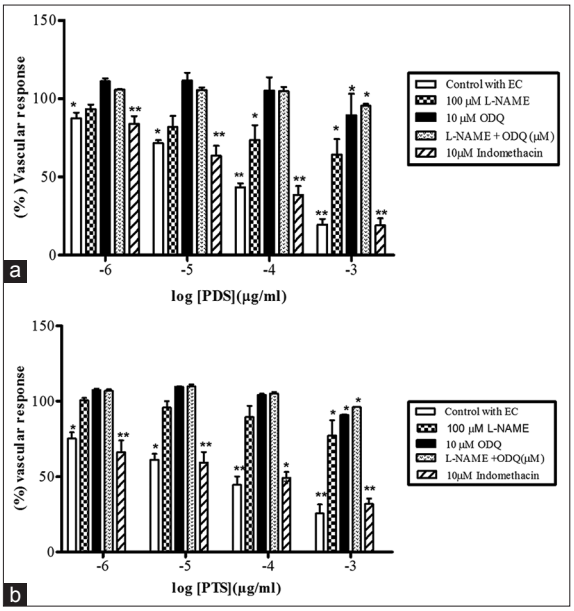

Figure 2: Effect of NG-nitro-L-arginine methyl ester (L-NAME), 1H-[1,2,4]-oxadiazole-[4,3-α]-quinoxaline-1-one (ODQ), L-NAME + ODQ, and indomethacin on protopanaxadiol (PDS)- and protopanaxatriol (PTS)-induced vasorelaxation. (a) Effects to PDS, (b) effects to PTS. Data are shown as mean ± SEM of five independent experiments. SEM: Standard error mean

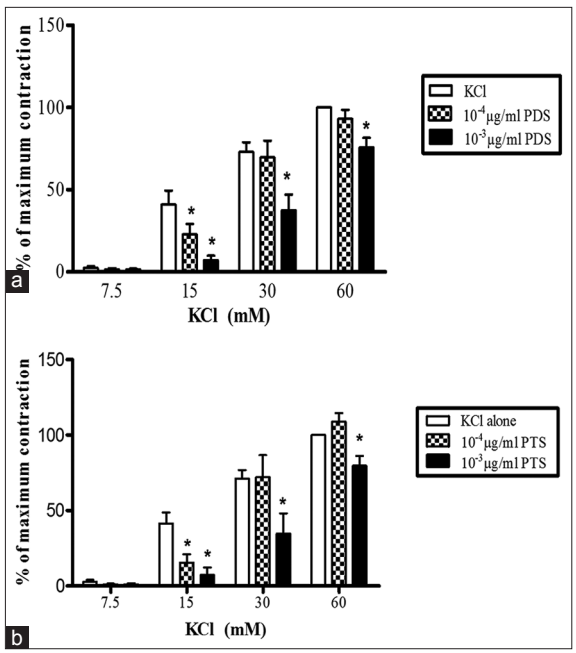

Figure 4: Effect of protopanaxadiol (PDS) and protopanaxatriol (PTS) on KCL-induced contractions in endothelium-denuded rat aortic rings. (a) Effect of PDS, (b) effect of PTS. Data are shown as mean ± SEM of five independent experiments. SEM: Standard error mean

The possible role of PGI_2 in PDS and PTS vasodilatations was also assessed in intact rings pre-treated with indomethacin. It was found that the presence of indomethacin does not affect the vasorelaxant effect caused by both PDS and PTS. Indomethacin is well known to possess the potential of blocking PGI_2 formation by inhibiting COX.[54] These findings, therefore, indicate non-contribution of vasoactive PGI_2 to PDS- and PTS-induced relaxations.

To further investigate possible mechanisms, we determined the role of Ca^{2+} in vasorelaxant effect of these fractions, and our findings demonstrated that both PDS and PTS significantly reduced Ca^{2+}-induced contractions of K^+-depolarized aortic rings and KCL-induced contractions. It has been confirmed that cytosolic Ca^{2+} increases during contraction of vascular smooth muscles either as a consequence of influx of Ca^{2+} from the extracellular space or release of Ca^{2+} from intracellular stores whereas relaxation is accompanied by a decrease in the cytosolic calcium influx level in smooth muscle cells.[53] Furthermore, it is also known that both potassium- and Ca^{2+} chloride-induced contractions of vascular smooth muscle are dependent on extracellular Ca^{2+} ions.[55-57] Therefore, findings from this study indicate that both PDS and PTS block the passage of extracellular Ca^{2+} ions across the vascular smooth membranes, suggesting that their vasorelaxant effect is mainly due to the inhibition of Ca^{2+} influx from the extracellular space.

CONCLUSION

This study has demonstrated that both PDS and PDS, major saponin fractions from roots of PN, cause endothelium-dependent relaxation through NO-cGMP, and in the vascular smooth muscle, they inhibit calcium influx from the extracellular space. COX pathway, on the other hand, had no apparent role. This suggests that their responses may be mainly due to the inhibition of voltage-dependent Ca^{2+} channels. Among these fractions, PDS shows better effect than PTS even though the difference is not statistically significant. These findings support the potential use of PN in the management of CVD, especially hypertension.

Further Directions

Further studies should assess possible involvement of K^+ channels in PDS- and PTS-induced vasorelaxation using various specific K^+ channel inhibitors such as charybdotoxin, iberiotoxin, or apamin.

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