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Ginsenosides-induced nitric oxide-mediated relaxation of the rabbit corpus cavernosum

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1 Ginsenosides, the active ingredients extracted from *Panax ginseng*, have been shown to promote nitric oxide (NO) release in bovine aortic endothelial cells. Since the endothelial cells and the perivascular nerves in penile corpus cavernosum contain NO synthase and an NO-like substance has been shown to be released from these cells which relaxes corpus cavernosum, the possibility that ginsenosides may relax corpus cavernosum by releasing endogenous NO was examined.

2 With an *in vitro* tissue superfusion technique, ginsenosides (250, 500 and 750 $\mu g\ ml^{-1}$) relaxed corpus cavernosum, concentration-dependently.

3 Using an *in vitro* tissue bath technique, acetylcholine (ACh)-induced relaxations were increased in the presence of ginsenosides (250 $\mu g\ ml^{-1}$).

4 Ginsenosides at 100 $\mu g\ ml^{-1}$ significantly enhanced the tetrodotoxin (TTX)-sensitive relaxation of corpus cavernosum elicited by transmural nerve stimulation.

5 The ginsenosides-induced, ACh-induced and ginsenosides-enhanced transmural nerve stimulation-elicited relaxations were significantly attenuated by N^G-nitro-L-arginine (100 μM) and oxyhaemoglobin (oxyHb; 5-10 μM), and were enhanced by superoxide dismutase (SOD; 50 u ml^{-1}).

6 The relaxations and their attenuation by N^G-nitro-L-arginine and TTX were associated with increase and decrease in tissue cyclic GMP levels, respectively.

7 It is concluded that ginsenosides may release NO from endothelial cells, and enhance NO release from endothelial cells elicited by other vasoactive substances and from perivascular nitrergic nerves in the corpus cavernosum. These endothelial and neurogenic effects of ginsenosides in inducing relaxation of the corpus cavernosum may account for the aphrodisiac effect of *Panax ginseng*.

Keywords: Ginsenosides; nitric oxide; rabbit corpus cavernosum; neurogenic vasodilatation; endothelium-dependent vasodilatation; aphrodisiac.

Introduction

It has been reported that ginsenosides, saponins extracted from *Panax ginseng*, relax pulmonary blood vessels (Chen *et al.*, 1984; Kim *et al.*, 1992) and that this relaxation is inhibited by N^G-nitro-L-arginine, a nitric oxide synthase (NOS) inhibitor (Chen *et al.*, 1993). Ginsenosides and one of its purified ingredients, Rg1, have also been shown to increase the conversion of L-arginine, a substrate for nitric oxide synthase (NOS), to citrulline, suggesting that ginsenosides enhance nitric oxide (NO) synthesis (Kim *et al.*, 1992). Since *Panax ginseng* is an essential constituent in traditional Chinese aphrodisiacs, and the relaxation of penile corpus cavernosum can be elicited by NO released from the endothelial cells and non-adrenergic, non-cholinergic (NANC) nerves innervating the corpus cavernosum (Ignarro, 1992; Bush *et al.*, 1992; Rajfer *et al.*, 1992), we examined the possibility that ginsenosides may relax the penile corpus cavernosum by modifying release of NO or a related substance from the endothelial cells and NANC nerves.

Methods

In vitro *techniques for recording tension changes*

Corpus cavernosum strips were prepared from penes of New Zealand white rabbits (3.5-4.4 kg) and mounted in tissue baths containing 10 ml Krebs bicarbonate solution at 37°C equilibrated with 95% O_2 and 5% CO_2 (Lee, 1982; Chen & Gillis, 1992). After 90 min equilibration, strips of corpus cavernosum were loaded with resting tensions of 2 g. Either a tissue superfusion technique or a tissue bath technique was used, with changes in isometric tension being measured by Grass FT03 transducers and recorded on a Grass Polygraph.

To detect the relaxation effect of ginsenosides and to avoid the potential foaming artifact generated by high concentrations of ginsenosides, a tissue superfusion technique (Chen & Gillis, 1992) was used. Krebs solution was superfused over strips of corpus cavernosum at 5 ml min^{-1} with a peristaltic pump. Experimental drugs were delivered with a second pump at 0.25 ml min^{-1} (1:10 dilution) for 2 min by direct application to the superfusate over the tissue. In the presence of active muscle tone induced by phenylephrine (3 μM), ACh (1 μM) was superfused for 2 min to verify the competency of the endothelial cells. At the end of each experiment, 1 μM 3-morpholino-sydnonimine (Sin-1), a NO-releaser, was superfused to induce a relaxation. The ginsenosides-induced relaxation was calculated as percentage of that induced by Sin-I.

To examine the effect of ginsenosides on ACh-induced relaxation, an *in vitro* tissue bath technique was used (Lee, 1982). After active muscle tone had been induced by phenylephrine (3 μM), ACh (0.3 μM-10 μM) was cumulatively applied to the bath. Thirty minutes after replacing the Krebs solution and in the presence of active muscle tone induced by phenylephrine (3 μM), ginsenosides (100, 250 or 500 $\mu g\ ml^{-1}$) were added 10 min before repeating the ACh concentration-response relationships. In some experiments, oxyhaemoglobin (oxyHb; 5-10 μM), prepared as described by Linnik & Lee (1989), was added to the tissue bath after the corpus cavernosum relaxation induced by ACh levelled off to detect the possibility of NO-mediated ACh-induced relaxation. The

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relaxations were calculated as percentage of active vessel tone induced by phenylephrine (3 μM).

To examine the effect of ginsenosides on the corpus cavernosum relaxation induced by transmural nerve stimulation, two parallel platinum electrodes, one on either side of the corpus cavernosum strips in the tissue bath were connected to a Grass 88 square wave pulse stimulator. This was coupled to a stimulus splitter (Med-Lab Stimu Splitter II) and two stimulus isolation units (Grass SIU5) to generate constant current biphasic square waves pulses (Lee, 1982). In the presence of active muscle tone induced by phenylephrine (3 μM) and with the current constantly monitored by an oscilloscope (BK Precision Model 1476), transmural nerve stimulation was delivered with a current of 200 mA and a pulse duration of 0.3 ms for 10 s at 2, 4, 8 and 16 Hz. Guanethidine (5 μM) and atropine (1 μM) were present (atropine was omitted when ACh was tested) throughout the entire experiment to eliminate the potential involvement of adrenergic and cholinergic components. Ginsenosides at different concentrations (100 μg ml^{-1} to 250 μg ml^{-1}) were added to the bath 10 min before repeating transmural nerve stimulation at the various frequencies. At the end of each experiment, TTX (0.3 μM), a sodium channel blocker, or N^G-nitro-L-arginine (50 μM), a NOS inhibitor, was added to the bath to verify the neurogenic origin or NO-mediated relaxation, respectively. Relaxation was calculated as a percentage of phenylephrine-induced active muscle tone.

Cyclic GMP assay

After maximum relaxation induced by various stimulants, corpus cavernosum strips were rapidly frozen in liquid nitrogen and stored at −70°C until assayed. Samples were pulverized by Wig-L-Bugamalgamator (Crescent Dental Manufacture Co. Lyons, IL, U.S.A.), homogenized, extracted in H_2O-saturated ether, and lyophilized to determine guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels by radioimmunoassay kits from Biochemical Technologies, Inc. (Stoughton, MA, U.S.A.) (Chen & Gillis, 1992).

Chemicals

Acetylcholine (ACh), atropine sulphate, guanethidine sulphate, phenylephrine, N^G-nitro-L-arginine, superoxide dismutase (SOD) and tetrodotoxin (TTX) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 3-Morpholinosydnonimine (Sin-1) was a gift from Hoechst AG (Frankfurt, Germany). Ginsenosides were extracted from *Panax ginseng* by C.A. Meyer as described by Shibata *et al.* (1965). Krebs bicarbonate solution consisted of (mM): NaCl 118.2, KCl 4.74, $CaCl_2$ 2.54, KH_2PO_4 1.19, $MgSO_4$ 1.19, $NaHCO_3$ 26.2, dextrose 11.1, indomethacin 0.006 and Na_2EDTA 0.023.

Statistics

Data were expressed as mean ± s.e.mean. Differences between the two groups were determined by Student's paired or unpaired *t* test and were considered significant if $P<0.05$.

Results

Ginsenosides-induced relaxation

Ginsenosides at 250, 500 and 750 μg ml^{-1} induced concentration-dependent relaxations. The induction of relaxation was immediate upon application of ginsenosides. These relaxations were significantly enhanced by SOD (50 u ml^{-1}) and were attenuated by N^G-nitro-L-arginine (50 μM) (Figure 1) and oxyHb (5 μM; $n=3$) (data not shown). N^G-nitro-L-arginine and oxyHb, but not SOD, significantly increased the resting muscle tone (data not shown). Ginsenosides at

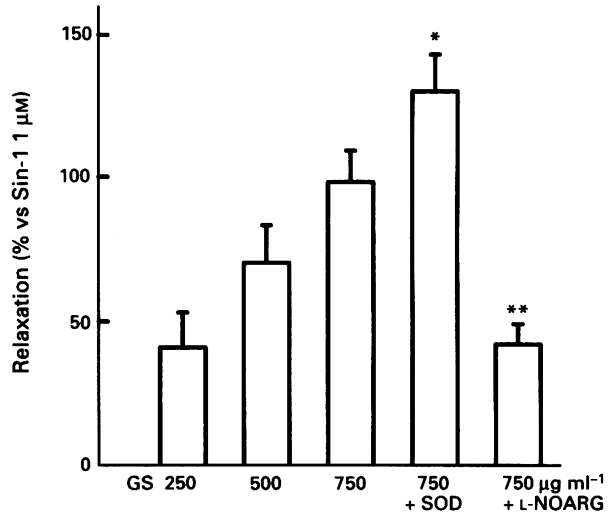

Figure 1 Ginsenosides (GS) induce relaxation of corpus cavernosum: GS (250, 500, 750 μg ml^{-1}) were superfused in Krebs solution over strips of corpus cavernosum for a period of 2 min each (for details, see methods). Superoxide dismutase (SOD) and N^G-nitro-L-arginine (L-NOARG) were superfused 3–5 min before superfusing GS. The relaxation induced by GS was concentration-dependent. The relaxation induced by GS at 750 μg ml^{-1} was equivalent to that induced by 1 μM Sin-1. This relaxation was significantly ($P<0.05$) enhanced by concomitant superfusion of SOD (50 u ml^{-1}), and was significantly attenuated by L-NOARG (50 μM). *$P<0.05$ and **$P<0.021$ indicate significant differences from GS 750 μg ml^{-1}, $n=6$ in each group.

750 μg ml^{-1} induced maximum relaxation which was equivalent to that induced by 1 μM Sin-1. Relaxation induced by Sin-1 (0.01–1 μM) was not affected by ginsenoside (250–500 μg ml^{-1}) (data not shown).

Ginsenosides-enhanced ACh-induced relaxation

In the presence of active muscle tone induced by phenylephrine (3 μM), the corpus cavernosum relaxed immediately upon application of ACh in a concentration-dependent manner (Figure 2). ACh-induced relaxation was blocked by atropine (1 μM), N^G-nitro-L-arginine (50 μM), and oxyHb (5 μM) (data not shown), and was enhanced in the presence of ginsenosides in a concentration-dependent manner.

Ginsenosides-enhanced transmural nerve stimulation-induced relaxation

Transmural nerve stimulation elicited relaxation of strips of corpus cavernosum in a frequency-dependent manner (Figure 3). The relaxation was significantly enhanced in the presence of 100 μg ml^{-1} ginsenosides. Higher concentrations of ginsenosides (250 μg ml^{-1}) did not further increase the relaxation elicited by transmural nerve stimulation at any frequency except 16 Hz. The enhanced-relaxation returned to normal after removing ginsenosides by washing with fresh Krebs solution. In the presence of TTX (0.3 μM), the relaxations elicited by transmural nerve stimulation at various frequencies were almost abolished while relaxations induced by ginsenosides and ACh persisted. The relaxations induced by transmural nerve stimulation at various frequencies were abolished by N^G-nitro-L-arginine (50 μM) (data not shown).

Effects of ginsenosides on cyclic GMP content

Relaxations of corpus cavernosum induced by ginsenosides (250 μg ml) and transmural nerve stimulation at 16 Hz were

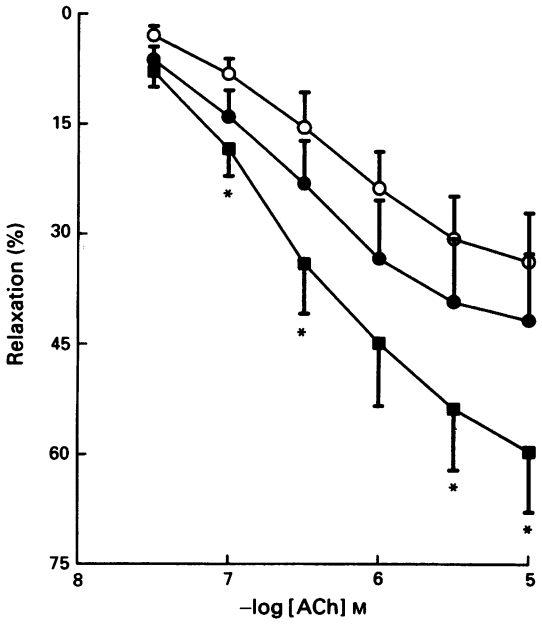

Figure 2 Ginsenosides (GS) concentration-dependently enhanced acetylcholine (ACh)-induced relaxation of corpus cavernosum. Ginsenosides at 250 μg ml^{-1} (■) but not at 100 μg ml (●) significantly enhanced the relaxation; (○) control. *$P<0.05$ indicates significant difference from the respective control, $n = 6$ in each group.

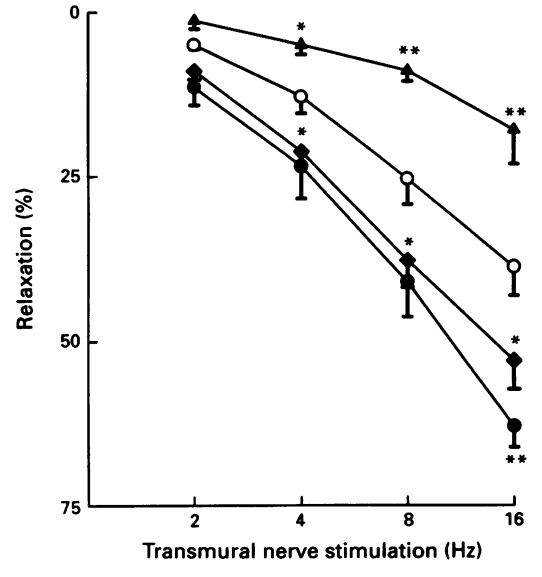

Figure 3 Ginsenosides (GS) enhanced transmural nerve stimulation-induced relaxation of the corpus cavernosum. Ginsenosides at 100 μg ml^{-1} (◆) enhanced transmural nerve stimulation-induced relaxation. At 250 μg ml^{-1}, (●) ginsenosides did not further enhance relaxation elicited by transmural nerve stimulation at various frequencies except 16 Hz. The relaxation was almost abolished by tetrodotoxin (TTX, 0.3 μM) (▲). Control (○). *$P<0.05$ indicate significant differences from the respective control, $n = 7$ in each group.

accompanied by significant increases in tissue cyclic GMP levels (Table 1). The increases were inhibited by N^G-nitro-L-arginine (50 μM) to a level significantly below the control values. In the presence of ginsenosides (250 μg ml^{-1}), transmural nerve stimulation at 16 Hz resulted in a greater increase in cyclic GMP content than that induced by ginsenosides or transmural nerve stimulation alone. This additive increase in cyclic GMP content was reversed by

Table 1 Effects of ginsenosides and transmural nerve stimulation (TNS) on cyclic GMP content in corpus cavernosum

	Cyclic GMP content (pmol g^{-1} wt)	n
Control	10.8 ± 1.2	4
Ginsenosides	18.4 ± 1.8*	4
TNS	18.6 ± 1.8*	4
Ginsenosides + TNS	29.3 ± 2.1**	4
Ginsenosides + TNS + TTX	16.0 ± 1.1*	4
Ginsenosides + N^G-nitro-L-arginine	4.7 ± 1.2*,†	4
Ginsenosides + TNS + N^G-nitro-L-arginine	4.8 ± 0.4*,†	4

Transmural nerve stimulation (16 Hz); ginsenosides (250 μg ml^{-1}); N^G-nitro-L-arginine (50 μM); tetrodotoxin (TTX, 0.3 μg ml^{-1}); n = number of experiments. *$P<0.01$ vs control, **$P<0.01$ vs ginsenosides or TNS, and †$P<0.01$ vs ginsenosides plus TNS indicate statistical significance (Student's unpaired t test).

TTX (0.03 μM) to the level equivalent to that induced by ginsenosides alone, while it was inhibited by N^G-nitro-L-arginine (50 μM) to a level significantly below the control values.

Discussion

In the present study, we have demonstrated that ginsenosides induce an endothelium-dependent relaxation, and enhance ACh-induced and transmural nerve stimulation-elicited relaxations in corpus cavernosum of the rabbits. These effects of ginsenosides appear to be mediated by release and/or the modification of release of NO from endothelial cells and perivascular nerves. This is based on the findings that both neurogenic and endothelium-mediated relaxations were enhanced by SOD, a scavenger of superoxide anion which is known to inactivate NO (Gryglewski *et al.*, 1986), and were blocked by N^G-nitro-L-arginine, a specific inhibitor of NOS (Rees *et al.*, 1990) and oxyHb which is known to trap NO (Martin *et al.*, 1985). Furthermore, relaxations of corpus cavernosum induced by transmural nerve stimulation, ginsenosides, or transmural nerve stimulation plus ginsenosides, and inhibition of these relaxations by N^G-nitro-L-arginine were associated respectively with increases and decreases in tissue cyclic GMP content.

It should be noted that the enhanced corpus cavernosum cyclic GMP content induced by ginsenosides and transmural nerve stimulation plus ginsenosides were decreased by N^G-nitro-L-arginine to a level significantly lower than that of the control, suggesting that there is a tonic release of endogenous NO in isolated corpus cavernosum. Furthermore, TTX, which almost abolished transmural nerve stimulation-elicited relaxation in the presence or absence of ginsenosides, decreased the tissue cyclic GMP content elicited by transmural nerve stimulation plus ginsenosides to a level equivalent to that induced by ginsenosides alone. These results provide further evidence that ginsenosides can modify the synthesis and release of NO in perivascular nerves and endothelial cells of the corpus cavernosum.

In the rabbit pulmonary and intrapulmonary arteries, ginsenosides have been shown to induce an endothelium-dependent, NO-mediated dilatation (Chen *et al.*, 1984; Kim *et al.*, 1992). It has also been shown that both ginsenosides (10 μg ml^{-1}) and Rgl (10 μM) significantly enhance the conversion of L-arginine to L-citrulline in bovine cultured aortic endothelial cells. This result suggests that ginsenosides can increase endothelial NOS activity and therefore NO production (Kim *et al.*, 1992). In the present study, although the ginsenosides-induced response in endothelium-denuded cor-

pus cavernosum was not examined due to difficulties in removing the endothelial cells, the corpus cavernosum vasodilator effect of ginsenosides qualitatively resembled that of ACh which is endothelium-dependent (Ignarro, 1992). Furthermore, ginsenosides enhance ACh-induced relaxation. These results suggest that the mechanism of ginsenosides-induced endothelium-dependent vasodilatation of corpus cavernosum is similar to that found in other vascular beds: both are mediated by activation of NOS and release of NO in the endothelium. Since the induction of relaxations by ginsenosides in corpus cavernosum and other vascular beds are immediate upon ginsenosides application (present study; Chen *et al.*, 1984; Kim *et al.*, 1992), it is most likely that ginsenosides activate an endothelial constitutive isoform of NOS.

In addition to the release of endothelial NO by ginsenosides, the present study, for the first time, demonstrated that ginsenosides enhanced neurogenic vasodilatation of the corpus cavernosum. It has been shown that electrical field stimulation of perivascular nerves of isolated corpus cavernosum of man and rabbits results in relaxation mediated by NO via activation of a constitutive isoform of NOS (Ignarro *et al.*, 1990; Moncada *et al.*, 1991; Bush *et al.*, 1992; Ignarro 1992). This NO-mediated neurogenic vasodilatation in the corpus cavernosum is supported by results of the present study. It is possible that ginsenosides may activate neuronal NOS activity similar to that observed in endothelial cells (Kim *et al.*, 1992). Thus, a given stimulation may increase synthesis and/or release of NO in the NANC nerves in ginsenosides-treated corpus cavernosum. This is supported by the finding of a greater increase in corpus cavernosum cyclic GMP content elicited by transmural nerve stimulation plus ginsenosides than the increase in cyclic GMP content induced by ginsenosides or transmural nerve stimulation alone. Ginsenosides enhancement of neurogenic vasodilatation is not due to modulation by ginsenosides of guanylate cyclase activity, since ginsenosides did not affect relaxation of the corpus cavernosum induced by Sin-1.

It is very likely that the enhanced neurogenic and endothelium-mediated vasodilatation in the corpus cavernosum induced by ginsenosides may contribute to the aphrodisiac effect of *Panax ginseng*, which is an essential constituent in the aphrodisiac prescription of traditional chinese medicine. It is interesting to note that the sensitivity of the corpus cavernosum to ginsenosides in inducing endothelium-dependent relaxation and enhancing transmural nerve stimulation-elicited relaxation appears to be different. The effective concentrations of ginsenosides ($250\,\mu g\,ml^{-1}$) in inducing relaxation and enhancing ACh-induced relaxation of corpus cavernosum is higher than that of ginsenosides ($100\,\mu g\,ml^{-1}$) in enhancing transmural nerve stimulation-elicited relaxation. These results suggest that ginsenosides have a preferential effect on neurogenic nitrergic vasodilatation in the corpus cavernosum. Although detailed pharmacokinetic data of ginsenosides in man remain to be determined, ginsenosides may exert a regional neurogenic vasodilatation in corpus cavernosum before inducing potential systemic hypotension through widespread endothelial mechanisms.

In summary, the results of the present study indicate that ginsenosides induce endothelium-dependent vasodilatation, enhance endothelium-dependent relaxation induced by ACh and increase neurogenic vasodilatation in the corpus cavernosum. The endothelial and neurogenic mechanism of ginsenosides in relaxing corpus cavernosum are predominantly mediated by NO and may, in part, account for the aphrodisiac effect of *Panax ginseng*.

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