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Ginsenoside Rg_1 Stimulates Nitric Oxide Release in Pulmonary Artery Endothelial Cells in Culture

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Abstract : Considering the stimulatory effects of ginsenosides from *Panax ginseng* C. A. Meyer on the release of nitric oxide from bovine aortic endothelial cells *in vitro* and vasodilatation of rabbit pulmonary artery *in vivo*, the present study is designed to investigate the mechanism of nitric oxide release by ginsenosides in calf pulmonary artery endothelial cells. Nitric oxide release was determined in endothelial cells treated with ginsenosides and compared with those of the receptor-dependent agonists, bradykinin and ADP and the receptor-independent calcium ionophore A_{23187}. The results showed that total saponin and ginsenoside Rg_1, not Rb_1, stimulated nitric oxide release measured as conversion to L-citrulline. The nitric oxide releasing properties of total saponin and ginsenoside Rg_1 were different; total saponin stimulated only conversion to L-citrulline, like A_{23187}, while ginsenoside Rg_1 stimulated both L-arginine transport and conversion to L-citrulline, as bradykinin or ADP did.

Key words : A_{23187}, ADP, bradykinin, ginsenoside Rg_1, nitric oxide.

Isolated rings or strips of arteries relax in response to certain vasodilators including acetylcholine, substance P, bradykinin and adenosine 5'-triphosphate (ATP), only if the endothelial cell layer is present (Furchgott and Zawadzki, 1980; Furchgott *et al.*, 1981; Furchgott, 1983). This phenomenon also occurs in blood-perfused segments of coronory and femoral artery *in vivo* (Angus *et al.*, 1983; Angus and Cocks, 1984). The list of substances that require endothelial cells to relax arteries has been extended to include some substances considered to be vasoconstrictors of large arteries, namely noradrenaline and serotonin (Cocks and Angus, 1983). In this instance, the amines directly contract smooth muscle cells but this contraction is attenuated by the presence of endothelium. However, in all cases it is considered that the hormone stimulates the endothelial cell via a specific receptor to release a factor termed EDRF (endothelium derived relaxing factor), identified as nitric oxide, which locally inhibits the contraction of the underlying smooth muscle cells.

Panax ginseng has been described as having either hypotensive (rabbits) (Hsu,1956), hypertensive (dogs) (Chang, 1959) or biphasic effects on blood pressure of dogs and rats (Chen *et al.*, 1982a, b; Wood *et al.*, 1964). Total saponin from Panax ginseng showed heterogeneity of responses in different vessels (Chen *et al.*, 1984). It is suggested that the relaxant effect of total saponin in pulmonary and intrapulmonary arteries might be mediated through release of endogenous vasodilator substances. For example, the endothelial cell of the aorta contains acetylcholine, which can be released by injury (Furchgott and Zawadzki, 1980). Acetylcholine, acting on muscarinic receptors, stimulates formation of cyclic GMP which can then relax arterial smooth muscle (Furchgott and Jothianandan, 1983). In our previous study (Kim *et al.*, 1992), total ginseng saponin vasodilated pre-constricted lungs, which suggests the possibility of total saponin acting as cholinergic agonists. However, this possibility was eliminated by the fact that total saponin preserved acetylcholine-induced vasodilatation even after free radical injury, while acetylcholine caused vasodilatation after electrolysis-induced endothelial damage.

The present study is designed to compare the releasing properties of nitric oxide by ginsenosides with those by receptor-dependent agonist. bradykinin and ADP and receptor-independent calcium ionophore A_{23187} in pulmonry artery endothelial cells.

Materials and Methods

Chemicals

Chemicals were from the following sources : [^{14}C]-L-arginine (57.8 mCi/mmol), [^{14}C]-L-ornithine (58.0 mCi/mmol) and [^{14}C]-L-citrulline (53.7 mCi/mmol), NEN/

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Dupont (Willimington, USA); Dowex AG 50 WX-8, Bio-Rad Laboratories (Richmond, USA); Dulbecco's modified Eagle's medium (DMEM), L-glutamine, sodium pyruvate, Hepes, fetal calf serum, bradykinin, ADP, A_{23187}, N-ω-nitro-L-arginine and all other chemicals, Sigma Chemical Co. (St. Louis, USA). Immunocytochemical staining assay systems for angiotensin converting enzyme and Factor VIII, Zymed Laboratories (South San Francisco, USA). Ginsenoside Rg_1, Rb_1 and total saponin were provided from Korea Ginseng & Tobacco Research Institute (Taejon, Korea).

Endothelial cell culture

Calf pulmonary artery endothelial cells were purchased from ATCC (American Type Culture Collection). Endothelial cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 0.48 mM L-arginine, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM Hepes and 10% fetal calf serum. Endothelial cells were identified as such by their cobblestone-like appearance when seeded onto 6 well plates and by positive immunostaining for angiotensin converting enzyme (ACE) and Factor VIII.

Assay of nitric oxide synthase (NOS)

NOS activity was measured by monitoring the conversion of [^{14}C]-L-arginine to [^{14}C]-L-citrulline (Boulanger *et al.*, 1990) in a confluent monolayer of endothelial cells which were supplied fresh media for 30 min before addition of [^{14}C]-L-arginine (final concentration 850 nM, 0.05 μCi/ml). One min later, total ginseng saponin (final concentration, 10 μg/ml), ginsenoside Rb_1 or Rg_1 (final concentration 10 μM), bradykinin (0.1 μM), ADP (1 mM) or A_{23187} (10 μM) were added and incubated at 37℃ for 15 min. To inhibit the activity of NOS, nitro-L-arginine (100 μM) was added to cells 20 min before addition of [^{14}C]-L-arginine and ginsenoside Rg_1 or bradykinin. Cells were then washed with PBS, harvested in ice-cold Hepes/EDTA buffer (20 mM/2 mM, pH 6.0), sonicated (10 sec., three times) and centrifuged (10,000×*g*, for 15 min at 4℃) and the supernatant applied to a 1 ml Dowex 50 cation exchange column. [^{14}C]-L-Citrulline in the eluent and a subsequent 1 ml water wash was quantified by liquid scintillation spectrometry. The sole radioactive component was verified as [^{14}C]-L-citrulline by thin layer chromatography as described below (Fig. 1). Percent conversion, as an index of NOS activity, was calculated as dpm of [^{14}C]-L-citrulline /dpm of [^{14}C]-L-arginine ×100.

Identification of [^{14}C]-L-citrulline in cell eluents

The eluent (20 μl) from cation exchange column chromatography and radiolabelled authentic standards ([^{14}C]-L-arginine, [^{14}C]-L-ornithine, [^{14}C]-L-citrulline) were applied 3 cm above the bottom of the TLC plate (Whatman silica gel 150A) and migrated over a distance of 16 cm. The solvent system was composed of chloroform : methanol : ammonium hydroxide : water (0.5 : 4.5 : 2.0 : 1.0). After drying, the TLC plates were scanned with an Isomess model IM 3000 TLC linear analyzer. The R_f values for [^{14}C]-labelled L-arginine, L-ornithine and L-citrulline (0.53, 0.79 and 0.92, respectively) were identical to those of auhentic standards (Fig. 1).

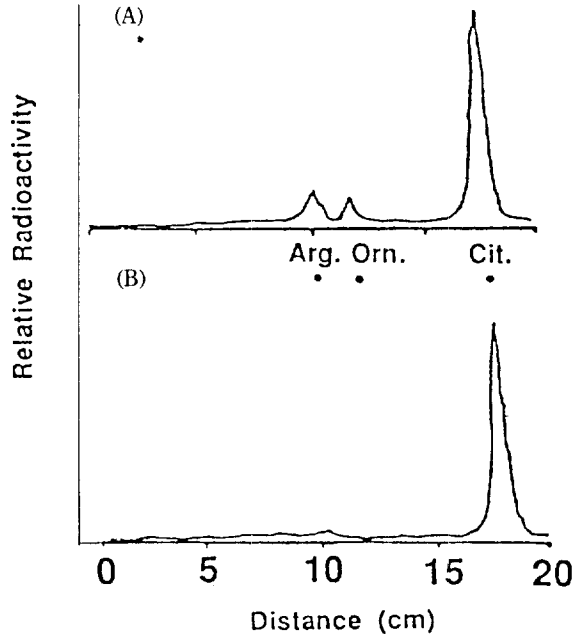

Fig. 1. Identification of [^{14}C]-L-citrulline from endothelial cell eluents. Samples (20 μl) were applied 3 cm above the bottom of the plate and migrated over a distance of 16 cm. Solvent system was composed of chloroform : methanol : ammonium hydroxide : water (0.5 : 4.5 : 2.0 : 1.0). TLC plates were scanned with TLC linear analyzer. A : a mixture of [^{14}C]-labelled authentic standards (Arg. : arginine; Orn : ornithine; Cit. : citrulline); B : endothelial cell eluents after cation exchange column chromatography.

Statistics

All values represent the mean± S.E.M. (n=10). Differences among groups were determined by one-way ANOVA with Newman-Keuls test (Zar, 1984). Values were considered significantly different if $p<0.05$.

Results

Effects of bradykinin, ADP and A_{23187} on nitric oxide release

[^{14}C]-L-Arginine (0.05 μCi/ml) was added to a confluent monolayer of endothelial cells seeded onto 6 well

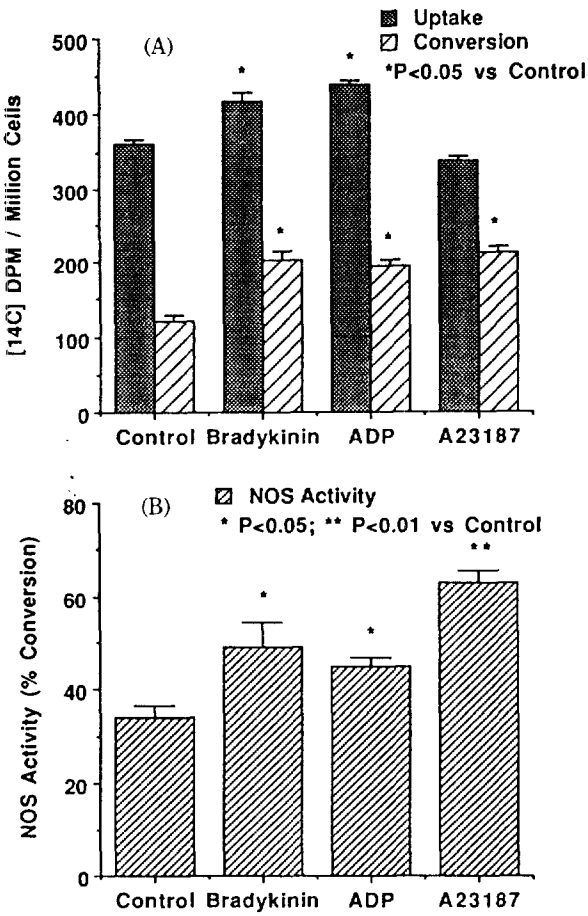

Fig. 2. Effects of bradykinin, ADP and A23187 on nitric oxide release. [^{14}C]-L-arginine (0.05 μCi/ml) was added to confluent monolayer of endothelial cells seeded onto 6 well plates (10^6 cells/well). One min later, bradykinin (0.1 μM), ADP (1 mM) and A_{23187} (10 μM) were treated and incubated at 37℃ for 15 min. NOS activity was expressed as % conversion, calculated as dpm of [^{14}C]-L-citrulline/dpm of [^{14}C]-L-arginine ×100.

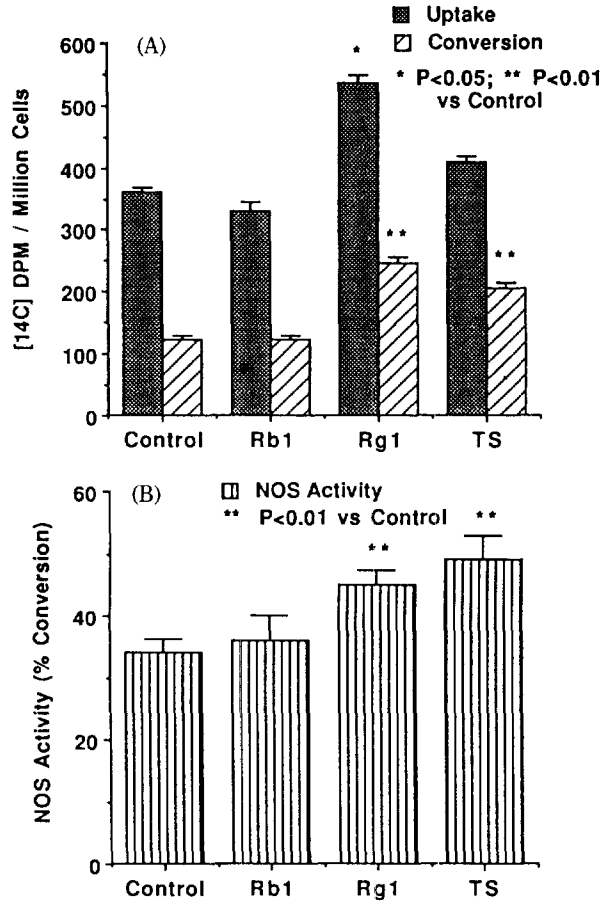

Fig. 3. Effects of ginsenoside Rb_1, Rg_1 and total ginseng saponin on nitric oxide release. Ginsenoside Rb_1, Rg_1 (10 μM) and total saponin (TS, 10 μg/ml) were treated to confluent endothelial cells 1 min after the addition of [^{14}C]-L-arginine and incubated at 37℃ for 15 min.

plates (10^6 cells/well). One min later bradykinin (0.1 μM), ADP (1 mM) and A_{23187} (10 μM) were treated and incubated at 37℃ for 15 min. [^{14}C]-L-Arginine uptake of bradykinin and ADP were significantly higher than that of the control; the dpm of [^{14}C] taken up by cells treated with nothing (control), bradykinin, ADP and A_{23187} were 361± 6.6, 416± 13, 438± 7 and 338 ± 5 dpm/10^6 cells, expressed as the mean± S.E.M. (Fig. 2A). Conversion to [^{14}C]-L-citrulline was stimulated by all three agonists; [^{14}C]-L-citrulline in cell eluents in the control, bradykinin-, ADP- and A_{23187}-treated cells were 122± 6.6, 203± 11.6, 195± 7.9 and 213± 8 dpm/10^6 cells, respectively. Nitric oxide synthase (NOS) activity was measured by monitoring the conversion of [^{14}C]-L-arginine to [^{14}C]-L-citrulline, which is a coproduct of nitric oxide. As shown in Fig. 2B, calcium ionophore A_{23187} stimulated NOS activity to 185% of control activity while the receptor-dependent agonist, bradykinin and ADP had 130~140% of the NOS activity shown in non-treated control cells.

Effects of ginsenoside Rb_1, Rg_1 and total ginseng saponin on nitric oxide release

Ginsenoside Rb_1, Rg_1 (10 μM) and total saponin (10 μg/ml) were given as treatment to confluent endothelial cells 1 min after the addition of [^{14}C]-L-arginine and incubated at 37℃ for 15 min. [^{14}C]-L-Arginine uptake and conversion to [^{14}C]-L-citrulline in ginsenoside Rb_1-treated cells were similar to those of the control cells. [^{14}C]-L-Arginine and [^{14}C]-L-citrulline of the control cells were 360± 10 and 122± 7 dpm/10^6 cells and those of ginsenoside Rb_1-treated cells were 331± 14 and 121± 15 dpm/10^6 cells. However, ginsenoside Rg_1 treatment showed 535± 12 dpm of [^{14}C]-L-arginine uptake and 247± 10 dpm of [^{14}C]-L-citrulline for 10^6 cells. Total saponin only significantly stimulated conversion to [^{14}C]-L-citrulline to a level of 205± 10 dpm/10^6 cells even though there was a tendency for an

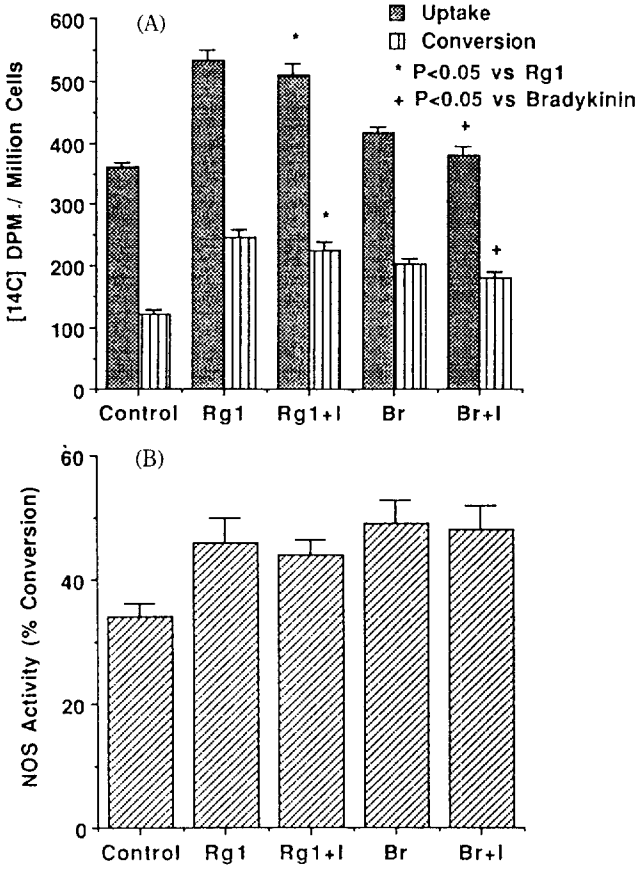

Fig. 4. Effect of nitro-L-arginine on bradykinin- and ginsenoside Rg_1-stimulated endothelial cells. Nitro-L-arginine (I, 100 μM) was added to the cells 20 min before addition of [^{14}C]-L-arginine and ginsenoside Rg_1 (10 μM) or bradykinin (0.1 μM). Confluent endothelial cells further incubated at 37℃ for 15 min.

increase in [^{14}C]-L-arginine uptake by total ginseng saponin (Fig. 3A). Ginsenoside Rg_1 and total saponin significantly increased NOS activities, to show 45± 2.4% and 49± 3.8% conversion ($p<0.01$ vs control), while control and ginsenoside Rb_1-treated cells showed only 34± 2.2% and 36± 4% conversion to [^{14}C]-L-citrulline (Fig. 3B).

Effect of nitro-L-arginine on bradykinin- and ginsenoside Rg_1-stimulated endothelial cells

Nitro-L-arginine (100 μM) was added to the confluent monolayer of endothelial cells 20 min before addition of [^{14}C]-L-arginine and ginsenoside Rg_1 (10 μM) or bradykinin (0.1 μM) (Fig. 4). Nitro-L-arginine inhibited both [^{14}C]-L-arginine uptake and conversion to [^{14}C]-L-citrulline ($p<0.05$, Fig. 4A), but NOS activity, calculated as dpm of [^{14}C]-L-citrulline/dpm of [^{14}C]-L-arginine ×100, was not changed by nitro-L-arginine (Fig. 4B). Even though nitro-L-arginine is known to be a potent inhibitor of the enzymatic transformation of arginine to nitric oxide, both substrate transport and NO release were suppressed by nitro-L-arginine in agonist-stimulated endothelial cells in the present study.

Discussion

The source of L-arginine for endothelial cell NOS can apparently be either intracellular or extracellular depending on factors such as the supply of L-arginine or its rate of conversion to nitric oxide. In arginine-deprived endothelial cells, L-arginine infusion can stimulate basal nitric oxide release and increase the nitric oxide release induced by A_{23187} (Palmer *et al.*, 1988). Agonists such as bradykinin and ATP, which stimulate nitric oxide release from vascular endothelial cells, also result in a rapid elevation in the uptake of L-arginine into the cell (Bogle *et al.*, 1991). These reports suggest that nitric oxide generation within the endothelial cell may be involved in the stimulation of L-arginine uptake, and an increase in L-arginine transport is closely associated with agonist-induced synthesis of nitric oxide, which provide a mechanism for increased substrate supply during NOS activation. In the present study, receptor-dependent agonist, bradykinin and ADP stimulated both L-arginine uptake and NO release, which was measured as conversion to L-citrulline. However, calcium ionophore A_{23187} significantly increased NOS activity rather than L-arginine transport, which differs from the report by Palmer *et al.* (1988). They used arginine-deprived endothelial cells that may increase the sensitivity to A_{23187} as compared with the arginine-supplemented endothelial cells in present study. The present study suggests that receptor-dependent agonist increases the substrate supply more than receptor-independent agonist during agonist-induced NOS activation in L-arginine-supplemented endothelial cells.

Ginseng saponin, a complex mixture of individual ginsenosides, has biphasic effects on blood pressure of dogs and rats (Chen *et al.*, 1982a, b; Wood, 1964) and produces different responses in different blood vessels (Chen *et al.*, 1984). Ginseng saponin lowered blood pressure in a dose-dependent manner in anesthetized rats. The relaxing effect on contraction induced by phenylephrine in rat and rabbit aorta was shown in ginseng saponin- and protopanaxatriol- (not protopanaxadiol) treated aortic rings (Kim and Kang, 1993). An endothelium-dependent vasodilation in perfused pulmonary artery of rabbit and stimulation of nitric oxide release in bovine aortic endothelial cells to ginseng saponin and ginsenoside Rg_1, not Rb_1, have been demonstrated (Kim *et al.*, 1992). The present study confirms the stimulatory effects of total saponin and ginsenoside Rg_1 on nitric oxide release by activating NOS activity in calf pulmonary artery endothelial cells,

but the properties of the release of nitric oxide are different between these two ginseng components. Ginsenoside Rg_1 increased L-arginine uptake into the cells in addition to NOS activation similar to that of bradykinin and ADP, while total saponin stimulated NOS activity rather than L-arginine transport. Since total saponin is a mixture of individual ginsenosides, a complex set of effects of the ginsenosides may be present in nitric oxide-mediated vasodilatation. Ginsenoside Rg_1 can cause membrane hyperpolarization in endothelial cells in addition to its effects on nitric oxide production, like bradykinin (Luckhoff and Busse, 1990) and this could account in part for the stimulatory effect of ginsenoside Rg_1 on L-arginine uptake.

Following inhibition of nitric oxide generation using a selective inhibitor of NOS, nitro-L-arginine, an analogue which does not inhibit endothelial cell L-arginine uptake, reduction in the stimulation of uptake induced by bradykinin was observed (Bogle *et al.*, 1991). Since the stimulatory effect of ginsenoside Rg_1 on L-arginine uptake and NO release, but not NOS activation, was inhibited by nitro-L-arginine, it may be concluded that ginsenoside Rg_1 could possibly act on cellular membrane like bradykinin, thus leading to an increase in L-arginine transport as well as stimulation of NO generation in pulmonary artery endothelial cells. L-Arginine transport in other cell types appears to be dependent upon the membrane potential (Rotoli *et al.*, 1991; Bussolati *et al.*, 1988) and further experiments are required to substantiate such a mechanism of ginsenoside Rg_1 by using bradykinin antagonists or Ca^{2+}-channel blockers.

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