Endothelium-dependent vasodilatory effect of vitisin C, a novel plant oligostilbene from Vitis plants (Vitaceae), in rabbit aorta

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ABSTRACT

We investigated the pharmacological properties of vitisin C, a novel plant oligostilbene from Vitis plants. Vitisin C (1–10 μM) dose-dependently inhibited the contractile responses of endothelium-intact rabbit thoracic aorta induced by phenylephrine (1 μM). These inhibitory effects were abolished in the presence of Nω-nitro-L-arginine methyl ester (l-NAME; 300 μM), a potent inhibitor of nitric oxide synthase, but not atropine (1 μM), a non-selective muscarinic cholinoreceptor antagonist. In endothelium-denuded rabbit aorta, vitisin C was ineffective in attenuating phenylephrine-induced contraction. Moreover, vitisin C (10 μM) increased cGMP production in endothelium-intact, but not endothelium-denuded, aorta, and this increase was abolished in the presence of l-NAME (300 μM). To assess Ca2+ movement across the endothelial cell membrane induced by vitisin C, we further investigated 45Ca2+ influx into cultured rabbit aortic endothelial cells in the presence of vitisin C (3 μM), carbachol (1 μM) or A23187 (10 nM). Vitisin C and carbachol significantly enhanced 45Ca2+ influx, which was inhibited by nifedipine (10 μM), a blocker of L-type Ca2+ channels. In the presence of SK&F96365, a blocker of receptor-operated Ca2+ channels, 45Ca2+ influx induced by carbachol was significantly inhibited, whereas that induced by vitisin C was not affected. On the other hand, A23187 enhanced 45Ca2+ influx in the presence and absence of nifedipine and SK&F96365. These results suggest that vitisin C evokes endothelium-dependent vasorelaxation through enhancing nitric oxide release, which is facilitated by Ca2+ influx into endothelial cells via nifedipine-sensitive Ca2+ channels.

INTRODUCTION

Numerous oligostilbenes, consisting of oxidative oligomers of resveratrol, are biosynthesized as phytoalexins in a number of plant families, including Vitaceae, which contains the important species that are grown worldwide for table grapes, raisins and wine production [1]. In the last decade, we have extracted and purified various novel oligostilbenes that are tetramers of resveratrol from the genus Vitis [2–7]. Although some pharmacological properties of the tetramers of resveratrol, such as anti-inflammatory and/or hepatoprotective effects, have been reported, the vasorelaxant effects of these oligostilbenes are unclear [2,3,8]. Resveratrol (3,5,4′-trihydroxystilbene) is thought to be the active ingredient of red wines that has been shown to reduce heart disease [1]. Recently, Naderali et al. [9] reported that resveratrol induced vasorelaxation in the resistance arteries of the guinea pig by increasing nitric oxide (NO) production. In addition, Andriambeloson

Key words: endothelium, nifedipine-sensitive Ca2+ channel, oligostilbene, rabbit thoracic aorta, vitisin C.

Abbreviation: l-NAME, Nω-nitro-L-arginine methyl ester.

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et al. [10] have demonstrated that polyphenolic extracts, including stilbenes, from red wines induce endothelium-dependent vasorelaxation. However, the detailed mechanisms responsible for these vasorelaxant effects are still unknown.

Vitisin C extracted from *Vitis* plants (Vitaceae) is a tetramer of resveratrol. The chemical structure of vitisin C has been determined [4] and is shown in Figure 1. Although the pharmacological properties of vitisin C have not been reported, we expect it to have resveratrol-like vasorelaxant effect because of its structural similarity to resveratrol. The aim of our present study was to demonstrate vasorelaxant effects of vitisin C, one of the tetramers of resveratrol, using rabbit thoracic aortic rings. Furthermore, we investigated the detailed mechanism underlying the vasorelaxant action of vitisin C using cultured rabbit endothelial cells.

**METHODS**

**Reagents**

Phenylephrine, A23187, SK&F96365 and atropine were obtained from Sigma-Aldrich Fine Chemicals. Nifedipine, DMSO and *N*-nitro-l-arginine methyl ester (l-NAME) were from Wako Pure Chemical Industries. All chemicals used were of the highest purity commercially available. All solutions were made fresh in sufficiently high concentrations so that only very small aliquots were added to the assay tubes or the culture medium. A23187, SK&F96365 and vitisin C were freshly dissolved in DMSO. The final concentration of DMSO in the bath never exceeded 0.4 % (v/v), and this had no effect on the cells or assays.

**Isolation of vitisin C**

Isolation of vitisin C was performed using a method described previously [4]. Briefly, dried stems of *Vitis* plants (Vitaceae) were extracted with methanol at room temperature to yield the methanol extract. This methanol extract was partitioned with ethyl acetate and water to give the ethyl acetate-soluble fraction. This fraction was chromatographed over silica gel, and was eluted with *n*-hexane/ethyl acetate (1:4, v/v). The *n*-hexane/ethyl acetate eluting fraction was chromatographed over silica gel, and was eluted with chloroform/methanol (10:1, v/v) to obtain vitisin C as an amorphous powder. The structure of vitisin C was determined by IR spectrum, UV spectrum, fast-atom bombardment MS and from $^1$H- and $^{13}$C-NMR spectra (see Figure 1) [4]. The purity of vitisin C was determined to be 100 % by TLC and from $^1$H- and $^{13}$C-NMR spectra.

**Aortic preparation and mounting**

All animal experiments were carried out in accordance with the Guidelines for Animal Experimentation, Hirosaki University. Rabbit aortic preparation was performed as described previously [11]. Male white rabbits (2.0–2.5 kg) were purchased from Clea Japan Inc. The rabbits were anaesthetized with sodium pentobarbital (30 mg/kg) and then the thoracic cavity was opened. The thoracic aorta was removed carefully, cleaned of adhering fat and connective tissue, and cut into rings (2–3 mm length). Each ring was mounted suitably between two platinum stirrups in an isolated tissue bath filled with a Krebs–Henseleit solution [composition in mM: NaCl, 120; KCl, 4.7; CaCl$_2$, 1.3; MgSO$_4$, 1.2; NaHCO$_3$, 25.0; KH$_2$PO$_4$, 1.2; d-(+)-glucose, 11.7] maintained at 37$^\circ$C and bubbled continuously with a 95 % O$_2$/5 % CO$_2$ mixture. The resting tension was adjusted to 2 g. Tension was measured with an isometric force transducer. In some experiments, the endothelium of some segments was removed by gently rubbing the intima surface of the aorta with a glass rod. Endothelium integrity or lack of endothelium-dependent responses of segments was confirmed by monitoring carbachol (3 µM)-mediated vasodilation before further experimentation.

After an equilibration period of 1 h, the vessels were twice contracted with phenylephrine (1 µM) in order to test their contractile capacity. Then the tissue was washed three times over a 1 h period, and the concentration–response curve determination was repeated. After ascertaining the presence or absence of endothelium, rings of aorta were contracted by phenylephrine (1 µM) and, when a steady tension level was reached, vitisin C was added cumulatively. Cumulative additions were made as soon as the response to the preceding addition levelled off. The relaxation evoked by each concentration of vitisin C is indicated by expressing the percentage contraction compared with that induced by 1 µM phenylephrine alone (100 %). To measure the response of the aortic ring in the presence of atropine (1 µM) or l-NAME (300 µM),...
each drug was added 10 min before the addition of phenylephrine (1 µM).

**Measurement of cGMP**

Rabbit aortic strips were incubated at 37°C in gassed (95 % O₂ and 5 % CO₂) Krebs–Henseleit solution. When the contractile responses of the strips to phenylephrine (1 µM) had reached a plateau, strips were exposed to 3-isobutyl-1-methylxanthine (1 µM), a phosphodiesterase inhibitor. After 5 min, strips were exposed either to carbachol (1 µM) or to vitisin C (10 µM) in the absence or in the presence of l-NAME (300 µM; added 5 min before exposure to vitisin C). The reaction was stopped by rapidly exchanging Krebs–Henseleit solution in the bath for ice-cold 10 % (w/v) trichloroacetic acid. Following homogenization and centrifugation, the supernatant was collected for cGMP determination using an enzymic assay developed in our laboratory [12]. Protein content was determined by the Bradford method [12a].

**Cell culture**

Segments (6–7 cm in length) were cut from freshly excised rabbit aortas and placed in ice-cold physiological salt solution (composition in mM: NaCl, 118.3; KCl, 4.7; CaCl₂, 2.5; MgSO₄, 1.2; KH₂PO₄, 1.2; NaHCO₃, 25.0; EDTA, 0.026; glucose, 11.1). The segments were used within 3 h. Under sterile conditions, the aortas were cleaned of connective tissue, and washed in 10 ml of Dulbecco’s modified Earle’s medium to remove adhering blood elements. Endothelial cells were obtained by lightly scraping the intimal surface of four aortas with a sterile scalpel blade (no. 10). The suspension, composed of single and small clumps of cells, was then transferred to a dish (30 mm). After a 24 h incubation at 37°C in an incubator (5 % CO₂), the culture medium was replaced with fresh medium in order to remove any non-adhering cells. The cells were subcultured once they reached confluence.

**45Ca²⁺ uptake**

45Ca²⁺ uptake measurements were performed by the method of Ohkura et al. [13]. Cells were suspended in PBS (pH 7.4) including trypsin, and then washed with PBS to remove trypsin. Cells were centrifuged at 120 g and cell pellets were suspended in Dulbecco’s modified Earle’s medium. The cell suspension was added to each well of a 24-well flat-bottom culture plate (Corning). After 2 days, almost all of the cells had adhered to the bottom of the plate, having reached confluence. The adherent cells were washed twice with 1 ml of BSS solution (10 mM Hepes buffer, adjusted to pH 7.4, containing 146 mM NaCl, 4 mM KCl, 0.2 mM CaCl₂, 0.2 mM MgCl₂, 10 mM glucose and 0.1 % BSA) at 37°C. To initiate 45Ca²⁺ uptake, the preincubation solution was replaced by 200 µl of Hepes buffer containing 0.44 µCi of 45Ca²⁺ with or without vitisin C (3 µM), carbachol (1 µM) or A23187 (10 nM). A low Ca²⁺ concentration (0.5 mM) was used to increase the incorporation of radioactive 45Ca²⁺ into the cells, because Ca²⁺ and 45Ca²⁺ were introduced into the cells proportionally, as described previously [13]. At the indicated times, the incubation was terminated by aspirating the 45Ca²⁺ uptake solution and washing each well three times with 0.5 ml of ice-cold Hepes buffer containing 3 mM LaCl₃. The cells were solubilized with 200 µl of 0.2 % (w/v) SDS, and an aliquot of the SDS lysate was measured for 45Ca²⁺ radioactivity. Cell protein per well was determined by the Bradford method [12a]. In some experiments, we investigated the changes in 45Ca²⁺ influx into endothelial cells induced by vitisin C, carbachol, A23187 and BayK8644 in the presence of nifedipine (10 µM) or SK&F96365 (10 µM), which were used as a 20 min pretreatment.

**Statistical analysis**

Group comparisons were performed by ANOVA with the Student–Newman–Keuls post hoc correction procedure or with Student’s t test. Values are presented as means ± S.E.M.; P < 0.05 was considered to indicate statistical significance.

**RESULTS**

**Effect of vitisin C on rabbit thoracic aorta**

The contractile response of endothelium-intact rabbit aorta to phenylephrine (1 µM) was dose-dependently inhibited not only by carbachol (0.3–3 µM; Figure 2a), but also by vitisin C (1–10 µM; Figure 2b). This effect was also evoked by KCl (30 mM) and 5-hydroxytryptamine (serotonin; 1 µM) (results not shown). In endothelium-denuded rabbit aorta, neither carbachol nor vitisin C up to 10 µM relaxed the phenylephrine-induced contraction (Figures 2c and 2d). In the endothelium-intact aorta, the inhibition by vitisin C of the contraction induced by phenylephrine was abolished by pretreatment with l-NAME (300 µM), a potent inhibitor of NO synthase, but not with atropine (1 µM), a non-selective muscarinic cholinoreceptor antagonist (Figure 3).
Figure 2  Effects of carbachol (0.3–3 µM; a, c) and vitisin C (1–10 µM; b, d) on the phenylephrine (1 µM)-induced contractile response
(a, b) Endothelium-intact rabbit thoracic aorta; (c, d) endothelium-denuded rabbit thoracic aorta.

Figure 3  Dose–response curves for vitisin C-induced relaxation of phenylephrine-precontracted rabbit aortic rings in the presence (○) or absence (●) of (a) atropine (1 µM) or (b) L-NAME (300 µM)
Values are means ± S.E.M. of six experiments.

Effect of vitisin C on cGMP level
As shown in Figure 4, the cGMP level was 9.5 ± 0.7 pmol/mg of protein in endothelium-intact rabbit thoracic aorta precontracted with phenylephrine (1 µM). Both carbachol (1 µM) and vitisin C (10 µM) produced a significant increase in the cGMP level, to 19.3 ± 6.0 and 20.8 ± 3.5 pmol/mg of protein respectively. The increase in cGMP level induced by vitisin C was not observed in strips pretreated with 300 µM L-NAME (7.7 ± 1.0 pmol/mg of protein). The cGMP level in endothelium-denuded strips precontracted with phenylephrine (1 µM) was 11.3 ± 3.6 pmol/mg of protein in the presence of vitisin C (10 µM).

Ca²⁺ influx into rabbit aortic endothelial cells induced by vitisin C
We investigated Ca²⁺ influx into the endothelial cells prepared from rabbit thoracic aorta in the presence of vitisin C, and compared it with that induced by carbachol and A23187. The EC₅₀ values for the relaxation of phenylephrine (1 µM)-precontracted endothelium-intact rabbit aorta were determined as 3 µM for vitisin C, 1 µM for carbachol and 10 nM for A23187 (results not shown). All of these compounds time-dependently increased ⁴⁵Ca²⁺ influx into the endothelial cells, reaching plateau at 2 min after addition of ⁴⁵Ca²⁺ (Figure 5). ⁴⁵Ca²⁺ influx into endothelial cells over 2 min was 140.6 ± 5.9 %
Vasorelaxation effects of vitisin C

Figure 4  cGMP levels in rabbit aortic strips precontracted with phenylephrine (1 µM)
cGMP levels were measured in endothelium-intact aortic strips in the absence (control) or in the presence of carbachol (1 µM), vitisin C (10 µM) or vitisin C + L-NAME (300 µM), and in endothelium-denuded strips in the presence of vitisin C (10 µM). Values are means ± S.E.M. of five experiments. Significant differences:
*P < 0.05 compared with control; **P < 0.01 compared with cGMP level in the presence of vitisin C and L-NAME.

Figure 5  Time course of changes in 45Ca2+ uptake in the presence of vitisin C (open bars), carbachol (closed bars) and A23187 (grey bars)
Values are means ± S.E.M. of six experiments. Significant differences: *P < 0.05 compared with 45Ca2+ uptake at 0.5 min.

Figure 6  Effects of SK&F96365 on 45Ca2+ uptake induced by carbachol, A23187 and vitisin C
Shown is the 45Ca2+ uptake induced by carbachol (1 µM), A23187 (10 nM) and vitisin C (3 µM) in the absence (open bars) or presence (closed bars) of SK&F96365 (10 µM) during 2 min in cultured rabbit thoracic endothelial cells. Values are means ± S.E.M. of six experiments. Significant differences: *P < 0.05 compared with 45Ca2+ uptake in the absence of SK&F96365.

Figure 7  Effects of nifedipine on 45Ca2+ uptake induced by carbachol, A23187, vitisin C and BayK8644
Shown is the 45Ca2+ uptake induced by carbachol (1 µM), A23187 (10 nM), vitisin C (3 µM) and BayK8644 (100 nM) in the absence (open bars) or presence (closed bars) of nifedipine (10 µM) during 2 min in cultured rabbit thoracic endothelial cells. Values are means ± S.E.M. of six experiments. Significant differences: *P < 0.05 compared with 45Ca2+ uptake in the absence of nifedipine.

of the baseline value for vitisin C, 163.3 ± 18.1 % for carbachol and 119.7 ± 6.6 % for A23187.

In the presence of SK&F96365 (10 µM), a potent blocker of receptor-operated Ca2+ channels, 45Ca2+ influx induced by carbachol was significantly inhibited (control, 196.23 ± 21.8 %; SK&F96365, 138.4 ± 26.9 %; P < 0.05), whereas the 45Ca2+ influx induced by vitisin C (control, 152.4 ± 29.4 %; SK&F96365, 145.1 ± 11.1 %) and A23187 (control, 133.6 ± 16.4 %; SK&F96365, 158.1 ± 12.0 %) were not affected (Figure 6). Nifedipine (10 µM), a blocker of dihydropyridine-sensitive Ca2+ channels, inhibited 45Ca2+ influx induced by vitisin C (control, 136.4 ± 6.3 %; nifedipine, 100.5 ± 7.1 %; P < 0.05) and carbachol (control, 164.3 ± 19.9 %; nifedipine, 117.2 ± 5.4 %), but not by A23187 (control, 130.5 ± 14.8 %; nifedipine, 125.8 ± 15.4 %) (Figure 7). BayK8644 (100 nM), an agonist of dihydropyridine-sensitive Ca2+ channels, was also antagonized by nifedipine (control, 167.1 ± 18.4 %; nifedipine, 122.2 ± 9.8 %; P < 0.05).

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The ability of vitisin C to transfer Ca$^{2+}$ was compared with that of A23187. A23187 significantly transferred Ca$^{2+}$ from an aqueous phase buffered at pH 7.5 into a bulk organic phase (control, 30 ± 4.7 ng; A23187, 46.3 ± 6.7 ng; P < 0.05) (Figure 8). The amount of Ca$^{2+}$ transported approached half of the molar amount of A23187 added. On the other hand, vitisin C (control, 12.6 ± 2.5 ng; vitisin C, 14.7 ± 2.2 ng) was unable to transfer Ca$^{2+}$ ion into a bulk organic phase when the pH of the aqueous medium was pH 7.5.

**DISCUSSION**

In the present study, we demonstrated that vitisin C inhibited the contractile responses of rabbit aorta in an endothelium-dependent manner by increasing the production of NO. Further, we revealed that endothelium-dependent vasorelaxation induced by vitisin C was caused by the influx of Ca$^{2+}$ ions via nifedipine-sensitive cation channels.

Vitisin C is an oxidative tetramer of resveratrol. It is known that low doses of resveratrol cause the concentration-dependent relaxation of noradrenaline-precontracted arteries from rats, indicating that the endothelium-dependent component of relaxation is induced by resveratrol, which activates endothelial NO synthesis [9]. Thus we expected vitisin C to relax the rabbit thoracic aorta because of its structural similarity to resveratrol. Accordingly, vitisin C dose-dependently inhibited the contraction of rabbit aorta induced by phenylephrine. The vasorelaxation effect of vitisin C was inhibited by L-NAME, but not by atropine. In endothelium-denuded rabbit aorta, vitisin C was ineffective in attenuating phenylephrine-induced contraction. Moreover, vitisin C (10 µM) did not increase the cGMP level in endothelium-denuded aorta precontracted with phenylephrine (1 µM), but did produce a significant increase in the cGMP level in endothelium-intact rabbit aorta precontracted with phenylephrine (1 µM), and this increase was abolished in the presence of L-NAME. From these results, we concluded that vitisin C has endothelium-dependent vasodilatory effects in the rabbit aorta that are mediated by NO generation.

It is well established that an increase in the Ca$^{2+}$ concentration within the endothelial cell is the critical intracellular step for the activation of NO synthase, leading to the production of NO and subsequent endothelium-dependent vasorelaxation. In the present study, we confirmed that vitisin C, like carbachol and A23187, significantly enhanced $^{45}$Ca$^{2+}$ influx into endothelial cells derived from rabbit aorta. The $^{45}$Ca$^{2+}$ influx induced by vitisin C, but not that induced by A23187, was inhibited significantly in the presence of nifedipine, a blocker of L-type Ca$^{2+}$ channels. SK&F96365, which blocks receptor-operated Ca$^{2+}$ channels, did not inhibit the induction of $^{45}$Ca$^{2+}$ influx by vitisin C. It is inferred from these results that vitisin C facilitates Ca$^{2+}$ influx into endothelial cells via nifedipine-sensitive Ca$^{2+}$ channels or as a Ca$^{2+}$ ionophore.

Generally, Ca$^{2+}$ ionophores contain carboxy groups and are assumed to directly facilitate the transport of Ca$^{2+}$ across the plasma membrane by forming a complex with Ca$^{2+}$. It is known that Ca$^{2+}$ ionophores, for example A23187 and ionomycin, primarily form a 1:1 complex with Ca$^{2+}$ and transport Ca$^{2+}$ across the target cell membrane [14]. However, vitisin C has a polyphenol structure without a carboxy group. We demonstrated that vitisin C does not have any ability to produce a complex with Ca$^{2+}$ (Figure 8). These results suggest that vitisin C does not act as a Ca$^{2+}$ ionophore.

Since all of the available evidence suggests the absence of voltage-dependent L-type Ca$^{2+}$ channels from endothelial cells, non-specific cation channels that are permeable to Ca$^{2+}$ have been reported to mediate Ca$^{2+}$ influx into these cells [15]. Indeed, we have not been able to demonstrate the existence of L-type Ca$^{2+}$ channels by reverse transcription–PCR methods using primer pairs that targeted rabbit cardiac, brain, smooth muscle and epithelial L-type Ca$^{2+}$ channels (K. Seya, K.-I. Furukawa and S. Motomura, unpublished work). Rahmani et al. [16] demonstrated that nifedipine-sensitive Ca$^{2+}$ channels exist in both endothelium and smooth muscle cells of Sprague–Dawley rats. However, it is unclear whether this nifedipine-sensitive Ca$^{2+}$ channel on the plasma membrane of endothelial cells derived from rabbit thoracic aorta is a voltage-dependent Ca$^{2+}$ channel or a non-selective cation channel. The precise mechanism by which vitisin C induces endothelial NO-dependent vasorelaxation awaits further investigation.
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