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Mechanisms of endothelium-dependent vasorelaxation induced by procyanidin B2 in venous bypass graft

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ABSTRACT

Cardioprotective abilities of procyanidins, might, at least in part, attribute to their vasodilator properties. The present study was undertaken to assess the vasorelaxant effect of procyanidin B2 on isolated human saphenous vein (HSV) and its underlying mechanisms. Procyanidin B2 relaxed phenylephrine-induced contraction of HSV rings in concentration-dependent manner. The relaxation was dependent on the presence of endothelium and was strongly affected by L-NAME, hydroxocobalamin or ODQ, the inhibitors of NO/cGMP pathway. Indomethacin significantly affected only the relaxation produced by the highest concentrations of procyanidin B2. Apamin and TRAM-34 combination, in the presence of L-NAME and indomethacin, did not additionally decreased procyanidin B2-induced relaxation. In the presence of K^+ channel blockers, relaxation induced by procyanidin B2 was partially attenuated by 4-aminopyridine, significantly inhibited by glibenclamide and almost abolished by iberiotoxin. Procyanidin B2 also relaxed the contractions induced by phenylephrine or caffeine in Ca^{2+}-free solution. Finally, nifedipine slightly, while thapsigargin strongly antagonized HSV relaxation. Our results indicate that procyanidin B2 induces endothelium-dependent relaxation of HSV, which results primarily from stimulation of NO production, as well K^+ channels opening, especially BK_{Ca}, and partially K_{ATP} and K_V. Regulation of the intracellular Ca^{2+} release and inhibition of Ca^{2+} influx probably contribute to procyanidin B2-induced relaxation.

1. Introduction

Recently there has been an upsurge of interest in the therapeutic potentials of medicinal plants as an inexhaustible source of polyphenols. Numerous epidemiological studies have suggested an inverse correlation between polyphenol-enriched diet consumption and risks of cardiovascular diseases.[1] Polyphenols are mainly present in fruits like apple, apricot, blackberry and grapes and derived products like fruit juices or jams; also in tea, cocoa, red wine and cereals.[2] In plant polyphenols, the most active fractions have been found in procyanidins which are oligomers and polymers of the flavan-3-ols, particularly (−)-epicatechin.[3] Some of their health-promoting properties, like free radical scavenging activity[4] and inhibition of low-density lipoprotein oxidation[5] keep them very interesting subject for investigation as promising agents for protection against cardiovascular diseases. It has also been shown that procyanidins cardioprotective abilities might, at least in part, attribute to their vasodilator properties. Recent *in vitro* reports have shown that many procyanidin-rich extracts from medicinal herbs traditionally used to treat hypertension, anemia or heart failure, elicit endothelium-dependent vasorelaxation that is mediated via increased nitric oxide (NO) production and subsequent guanylate

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cyclase (GC) activation.[6] Even more, Aldini et al.[7] suggested that prostacyclin, besides NO, is also involved in the human internal mammary artery (HIMA) relaxation induced by a grape-seed procyanidin-rich extract. In addition, it has been suggested that endothelium-dependent vasorelaxation of rat aorta, induced by procyanidins from different sources (apple, Crategus), results from both hyperpolarization via multiple K^+ channels opening and activation of NO/cGMP pathway.[8,9] Moreover, procyanidin-rich products are also proved to have *in vivo* blood pressure lowering effect in several experimental models of hypertension.[6,8] The diverse vasorelaxation mechanisms reported so far may be caused by multiple active procyanidins present in extracts of different origin. In order to evaluate the particular compound which mediates beneficial vascular effects of some sources with the highest content of procyanidin dimers (grape seeds, apples, cacao beans, or pine bark extract),[2] we have focused to shed more light on vasorelaxing properties of procyanidin B2, composed of two (−)-epicatechin molecules. Accordingly, the aim of the present study was to investigate vasorelaxant effect of procyanidin B2 on human saphenous vein (HSV) and its underlying mechanisms, such as roles of NO, different K^+ channels, and Ca^{2+}.

2. Materials and methods

2.1. Tissue preparation

HSV grafts were supplied from 54 randomized male patients undergoing coronary artery revascularization. All the patients were Caucasian (mean age ± standard deviation of mean (S.D.M.); 58 ± 5) and they were informed in detail about the aims of investigation and had given their consent for the excision of remaining tissue. The use of the excess vessels was approved by the Ethics Committee of Institute for Cardiovascular Diseases "Dedinje" and conforms to the principles outlined in the Declaration of Helsinki. After excision, the vessels segments were immediately placed in cold (4 °C) Krebs–Ringer bicarbonate solution (118.3 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4 and 11.1 mM glucose), and transported to the laboratory for further study.

The grafts were removed free of connective tissue and fat before cutting into 3 mm long rings. In some experiments the endothelium was removed mechanically by rubbing the lumen with a stainless steel wire. The rings were mounted between two stainless steel triangles, one of which was fixed to the bottom of organ bath, while the other was connected to a force-displacement transducer (LETICA, Spain), in a 10 ml organ bath filled with aerated (95% O_2–5% CO_2) Krebs–Ringer bicarbonate solution at 37 °C and equilibrated for 60 min under a resting tension of 2 g. A computer-assisted data acquisition system (AD Instruments, Power Lab/4SP) recorded the changes in isometric tension during the experiments.

2.2. Experimental protocol

2.2.1. Role of endothelium in the relaxation induced by procyanidin B2

After equilibration, the rings were pre-contracted with phenylephrine (10 μM) or high K^+ solution (80 mM) to reach stable and sustained contraction (appropriate time is approximately 15 min), followed by exposing the rings to increasing concentration of procyanidin B2 (0.001–3 μM) to obtain concentration–response curves. The relaxation response to each dose was allowed to develop for 15–20 min, until a stable baseline was obtained. To assess whether endothelium mediates procyanidin B2-induced relaxation, concentration–relaxation curves to procyanidin B2 were constructed in both endothelium intact and endothelium denuded rings. Endothelium viability was assessed by rings exposure to 1 μM acetylcholine.

To determine which endothelial mediator(s) was/were related to the vasodilator effect of procyanidin B2, the endothelium-intact HSV rings were pre-incubated with specified inhibitors of endothelium-derived relaxing factors for 20 min before phenylephrine-induced contraction and construction of the second concentration–response curves to procyanidin B2. Curves obtained without any pretreatment were taken as control.

2.2.2. Role of K^+ channels in procyanidin B2-induced relaxation

To test the involvement of different K^+ channels in the vasorelaxing action of procyanidin B2, selective K^+ channel blockers were added to rings, approximately 20 min before the phenylephrine-induced contraction, and construction of second concentration–response curves to procyanidin B2. In some experiments effects of certain K^+ channels blockers were tested after the inhibition of endothelium-derived relaxing factors synthesis.

2.2.3. Role of Ca^{2+} in procyanidin B2-induced relaxation

To study whether the inhibition of extracellular Ca^{2+} influx was involved in procyanidin B2-induced relaxation, some rings were pretreated with nifedipine, specific L-type Ca^{2+} channel blocker, for 20 min before contraction with phenylephrine and addition of increasing concentrations of procyanidin B2. Finally, rings with no added blocking drug were taken as control.

In the next set of experiments, to clarify whether the procyanidin B2 interferes with Ca^{2+} release from intracellular stores, rings were equilibrated in a Ca^{2+}-free Krebs–Ringer bicarbonate solution (containing EGTA) and then stimulated with phenylephrine or caffeine (25 mM) to induce a stable contraction. This is followed by construction of cumulative concentration–response curves to procyanidin B2.

Finally, the possible role of sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) in the procyanidin B2-induced relaxation was tested by incubation the rings, in the normal Krebs–Ringer bicarbonate solution, with thapsigargin alone, or in the presence of L-NAME and indomethacin, 40 min prior to pre-contraction with phenylephrine. The cumulative concentration–response curves to procyanidin B2 were then constructed and compared with control.

2.3. Drugs

The following drugs were used: procyanidin B2, phenylephrine hydrochloride, acetylcholine iodide, Nω-Nitro-L-arginine methyl ester (L-NAME), 1H-[1,2,4]Oxadiazolo [4,3-a]quinoxalin-1-one (ODQ), hydroxocobalamin, indomethacin, apamin, TRAM-34 (1-[(2-chlorophenyl) diphenylmethyl]-1H-pyrazole), iberiotoxin, glibenclamide, 4-aminopyridine (4-AP), nifedipine, thapsigargin and caffeine (Sigma–Aldrich Inc., St. Louis, MO, USA). Procyanidin B2 was dissolved in distilled water on the day of use. Glibenclamide, TRAM-34, ODQ and indomethacin were dissolved in dimethyl sulfoxide. Thapsigargin and nifedipine were dissolved in absolute ethanol. Other chemicals were dissolved in distilled water. The drugs were added directly to the bath and the concentrations are expressed as final molar concentrations in the bath solution.

2.4. Treatment of data and statistics

The relaxation response to each dose was expressed as the percentage of relaxation of vasoconstrictor-induced tone. The cumulative concentration–relaxation curves were analyzed using a

non-linear least squares fit of individual experimental data and then the concentration of procyanidin B2 producing 50% of the maximum response (EC_{50}) was calculated and presented as pD_2 (pD_2 = -log EC_{50}). Data are expressed as the mean ± standard deviation (S.D.) and *n* indicates the number of experiments. Statistical comparisons of the cumulative responses of relaxation under different treatments were performed by two-way analysis of variance (ANOVA) with repeated measures, followed by a post-hoc Bonferroni test to detect the individual differences. The comparisons of EC_{50} and the maximal responses of two different groups were performed by paired Student's *t*-test or unpaired *t*-test when appropriate. All calculations were done using the SPSS statistical software (version 10.0; International Business Machines Corp, Armonk, NY).

3. Results

3.1. Relaxing effect of procyanidin B2 on phenylephrine- or high K^+-contracted HSV rings

In endothelium intact HSV pre-contracted with phenylephrine, procyanidin B2 (0.001−3 μM) produced a concentration-dependent vasorelaxation with pD_2 value of 6.86 ± 0.06 and the maximal response of 89.7 ± 6.2% (n = 10). Endothelial removal did not affect the contractile response to phenylephrine, but the relaxant response to procyanidin B2 was significantly diminished in denuded preparations (maximal response 23.0 ± 4.8%; n = 10) ($P < 0.01$) (Fig. 1A).

In endothelium intact HSV pre-contracted with high K^+ solution (80 mM), procyanidin B2 (0.001−3 μM) produced a concentration-dependent vasorelaxation with pD_2 value of 6.73 ± 0.06 and the maximal response of 73.7 ± 6.5% (n = 8) (Fig. 2).

The time-matched control did not affect phenylephrine- or high K^+-induced vessel tone (data not shown).

3.2. Role of endothelium-derived vasoactive factors on the procyanidin B2-induced relaxation

Pre-treatment of endothelium intact HSV rings with a competitive endothelial NO synthase (NOS) inhibitor, L-NAME (100 μM), a NO scavenger, hydroxocobalamin (3 μM), or with an inhibitor of soluble GC, ODQ (10 μM), markedly inhibited the vasorelaxant effect of procyanidin B2 (Table 1, Fig. 1B). Indomethacin (10 μM), a cyclooxygenase (COX) inhibitor, significantly affected only the relaxation produced by the highest concentrations of procyanidin B2 (Table 1, Fig. 1C). Moreover, in the presence of L-NAME and indomethacin, adding combination of apamin (50 nM) and TRAM-34 (10 μM), selective blockers of small and intermediate conductance Ca^{2+}-activated K^+ (K_{Ca}) channels (SK_{Ca} and IK_{Ca}), respectively, caused further reduction of procyanidin B2-induced relaxation, but did not reach statistical significance (Table 1, Fig. 1D).

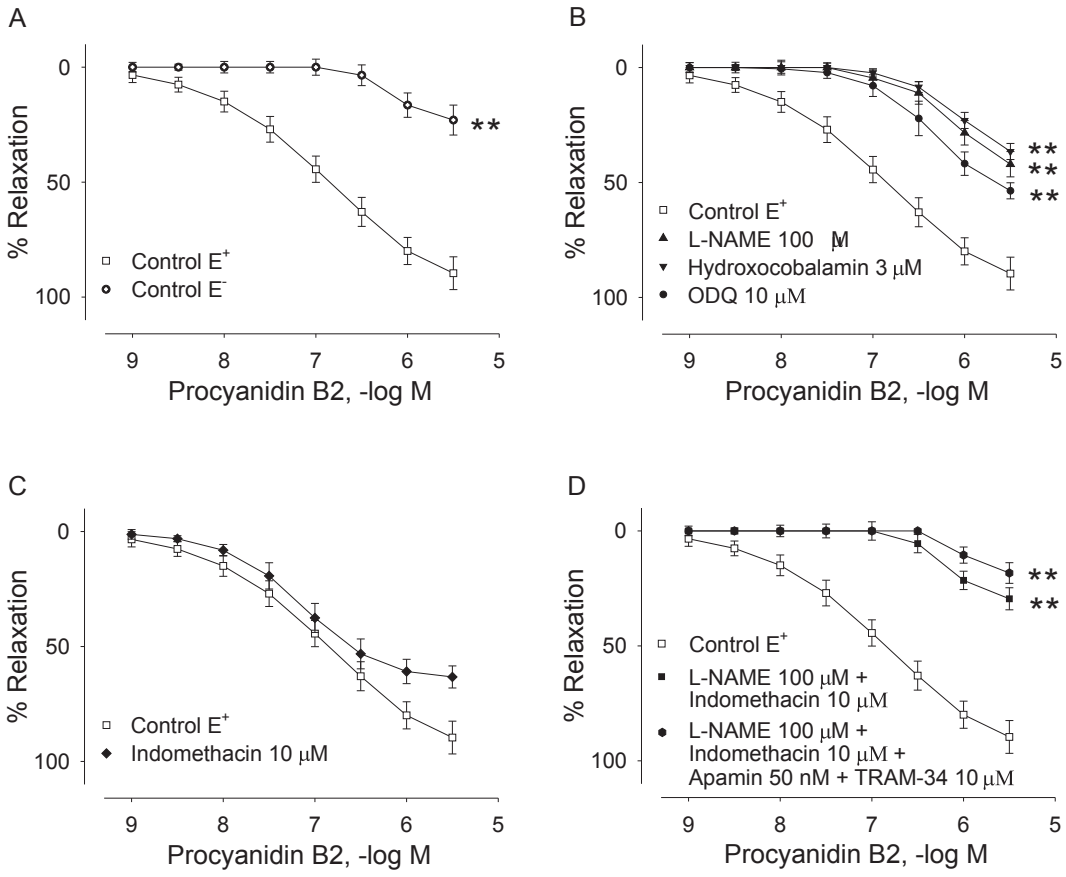

Fig. 1. Role of endothelium-derived vasoactive factors on the procyanidin B2-induced relaxation of human saphenous vein pre-contracted by phenylephrine (10 μM). Concentration-response curves to procyanidin B2 in the presence (control E^+) and absence (control E^-) of endothelium (A) and effects of pre-incubation with L-NAME (100 μM) (B), hydroxocobalamin (3 μM) (B), ODQ (10 μM) (B), indomethacin (10 μM) (C), combination of indomethacin (10 μM) and L-NAME (100 μM) with or without apamin 50 nM and TRAM-34 10 μM combination (D) on the procyanidin B2-induced relaxation in endothelium-intact human saphenous vein. Responses are expressed as a percentage of the maximum possible relaxation i.e., the return of tension to the level before contraction with phenylephrine. Each point represents the mean ± S.D. (n = 4–10). $^{**}P < 0.01$ vs. control E^+ (two-way analysis of variance).

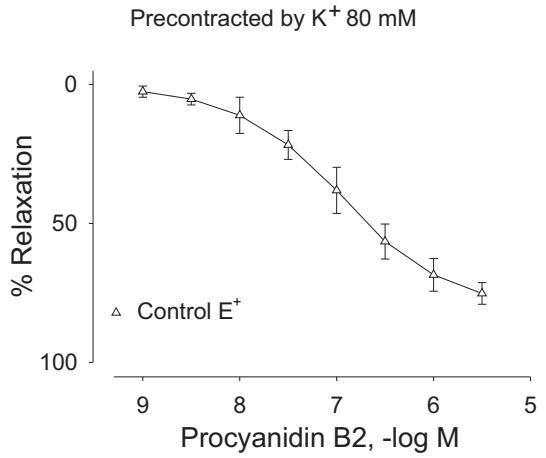

Fig. 2. Concentration-response curve to procyanidin B2 of human saphenous vein pre-contracted by high K^+ (80 mM). Responses are expressed as a percentage of the maximum possible relaxation i.e., the return of tension to the level before contraction with high K^+. Each point represents the mean ± S.D. (n = 8).

Table 1
Effects of various blockers on the procyanidin B2-induced relaxation of HSV.

	n	pD_2	E_{max} (%)
Control E^+	6	6.89 ± 0.02	89.6 ± 5.8
L-NAME 100 μM	6	nc	42.05 ± 4.8**
Control E^+	6	6.90 ± 0.03	91.3 ± 6.1
Hydroxocobalamin 3 μM	6	nc	36.5 ± 3.5**
Control E^+	5	6.91 ± 0.02	94.6 ± 5.3
ODQ 10 μM	5	5.14 ± 0.54**	53.6 ± 6.5*
Control E^+	7	6.82 ± 0.06	88.5 ± 5.9
Indomethacin 10 μM	7	6.57 ± 0.07	65.3 ± 4.8*
Control E^+	4	6.90 ± 0.03	90.6 ± 3.4
L-NAME 100 μM + Indomethacin 10 μM	4	nc	29.4 ± 4.5**
L-NAME 100 μM + Indomethacin 10 μM + Apamin 50 nM + TRAM-34 10 μM	4	nc	18.4 ± 3.5**
Control E^+	5	6.85 ± 0.04	86.5 ± 5.3
4-AP 0.5 mM	5	5.84 ± 0.06**	55.61 ± 5.5**
Control E^+	5	6.83 ± 0.05	93.0 ± 5.9
Glibenclamide 10 μM	5	nc	47.2 ± 6.5**
Control (E^-)	5	nc	23.0 ± 4.8%
Iberiotoxin 100 nM	5	nc	21.5 ± 3.2%
Control (E^+)	5	6.91 ± 0.03	88.8 ± 3.7
Iberiotoxin 100 nM	5	nc	22.6 ± 3.5**
L-NAME 100 μM Indomethacin 10 μM + Iberiotoxin 100 nM	5	nc	27.3 ± 5.5**
Control (E^+)	5	6.88 ± 0.04	89.5 ± 6.9
Nifedipine 1 μM	5	6.24 ± 0.06*	62.5 ± 5.5*
Control (E^-)	5	nc	22.5 ± 5.8%
Thapsigargin 1 μM	5	nc	20.4 ± 3.5%
Control (E^+)	5	6.91 ± 0.04	87.7 ± 4.5
Thapsigargin 1 μM	5	nc	16.4 ± 3.2**
L-NAME 100 μM Indomethacin 10 μM + Thapsigargin 1 μM	5	nc	25.3 ± 6.5**

The results are expressed as mean ± S.D. n, number of experiments; pD_2 = -log EC_{50}; E_{max}, maximal relaxation; 4-AP, 4-aminopyridine; nc, not calculated; *$P < 0.05$ vs. control, **$P < 0.01$ vs. control (paired Student's *t*-test).

Two-way ANOVA for all concentrations revealed no significant differences after incubation with indomethacin compared with control ($P > 0.05$) and apamin/TRAM-34/L-NAME/indomethacin combination compared with L-NAME plus indomethacin alone ($P > 0.05$), but demonstrated significant differences after incubation with all other inhibitors compared with control ($P < 0.01$ for all comparisons).

3.3. Effects of K^+ channel blockers on the relaxation produced by procyanidin B2

4-AP (0.5 mM), a predominant blocker of voltage-gated K^+ (K_V) channels, partially but significantly antagonized the relaxation of endothelium-intact HSV rings produced by procyanidin B2 (Table 1, Fig. 3A). On the other hand, the procyanidin B2-induced relaxation was significantly inhibited by glibenclamide (10 μM), a selective ATP-sensitive K^+ channels (K_{ATP}) inhibitor, with a strong reduction of maximal response (Table 1, Fig. 3B).

Iberiotoxin (100 nM), a highly selective blocker of large conductance K_{Ca} (BK_{Ca}), almost abolished the procyanidin B2-stimulated relaxation in endothelium-intact rings (Table 1, Fig. 4A), while did not affect the relaxation in endothelium-denuded rings (Table 1, Fig. 4B). Also, in the presence of L-NAME and indomethacin, iberiotoxin did not cause further decreased in procyanidin B2-induced relaxation (Table 1, Fig. 4C).

Two-way ANOVA for all concentrations revealed significant differences after incubation with 4-AP ($P < 0.05$), iberiotoxin ($P < 0.01$) and glibenclamide ($P < 0.01$) compared with control, but demonstrated no significant differences after pre-treatment with iberiotoxin/L-NAME/indomethacin combination compared with L-NAME plus indomethacin alone ($P > 0.05$), or after pretreatment with iberiotoxin in endothelium-denuded rings, compared with control ($P > 0.05$).

3.4. Role of Ca^{2+} in procyanidin B2-induced relaxation

Nifedipine (1 μM), a specific L-type Ca^{2+} channel blocker, slightly antagonized HSV rings relaxation produced by procyanidin B2 (Table 1, Fig. 5A).

In Ca^{2+}-free Krebs–Ringer bicarbonate solution, cumulative addition of procyanidin B2 caused concentration-dependent relaxation of HSV rings pre-contracted either by phenylephrine ($pD_2 = 5.89 \pm 0.11$; maximal response 57.8 ± 3.0%; n = 4) or caffeine ($pD_2 = 6.73 \pm 0.03$; maximal response 93.6 ± 9.1%, n = 4) (Fig. 5B and C).

Thapsigargin (1 μM), a SERCA inhibitor, strongly inhibited the response to procyanidin B2 in HSV rings pre-contracted by phenylephrine in normal Krebs–Ringer bicarbonate solution (Table 1 and Fig. 5D). In contrast, tapsigargin pretreatment neither affect the relaxation of endothelium-denuded rings (Table 1 and Fig. 5E), nor caused any further reduction of procyanidin B2-induced relaxation, in the presence of L-NAME and indomethacin (Table 1, Fig. 5F).

Two-way ANOVA for all concentrations revealed no significant differences after incubation with thapsigargin/L-NAME/indomethacin combination compared with L-NAME plus indomethacin alone ($P > 0.05$), and after pre-treatment with thapsigargin in endothelium-denuded rings ($P > 0.05$), but demonstrated significant differences after incubation with nifedipine ($P < 0.05$) and thapsigargin in endothelium-intact rings ($P < 0.01$) compared with control.

4. Discussion

The investigation reported in this paper has focused on the effects of procyanidin B2 on HSV. The present study revealed that procyanidin B2 induces the endothelium- and concentration-dependent relaxation of HSV in a way greatly related to the NO production (Fig. 6). Previous publications have indicated that several procyanidin-rich fractions from different origin induce strong endothelium-dependent relaxations of various types of blood vessels.[6,8,9] However, in the vast majority of studies, blood vessels were from animal origin and plant extracts were used, so it

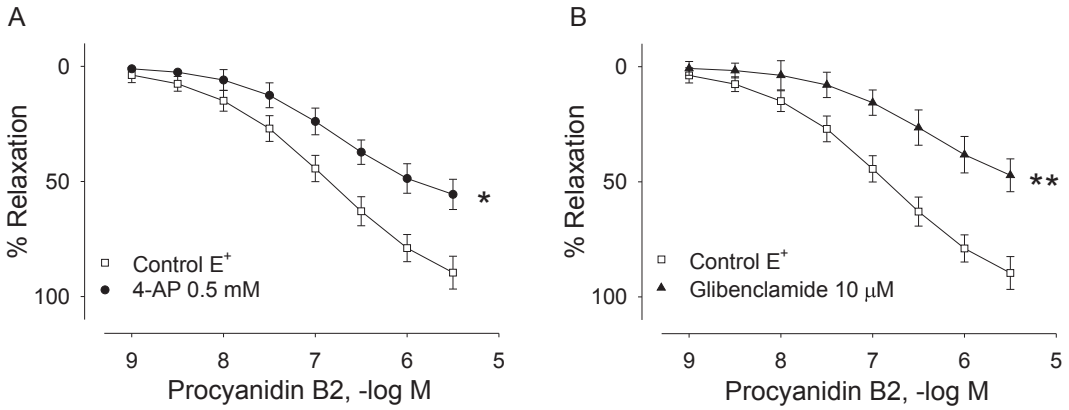

Fig. 3. Effects of K^+ channel blockers on the procyanidin B2-induced relaxation of human saphenous vein pre-contracted by phenylephrine (10 μM). Concentration-response curves to procyanidin B2 in the absence and presence of 4-aminopyridine (4-AP, 0.5 mM) (A), and glibenclamide (10 μM) (B). Responses are expressed as a percentage of the maximum possible relaxation i.e., the return of tension to the level before contraction with phenylephrine. Each point represents the mean ± S.D. (n = 5). * $P < 0.05$ or ** $P < 0.01$ vs. control (two-way analysis of variance).

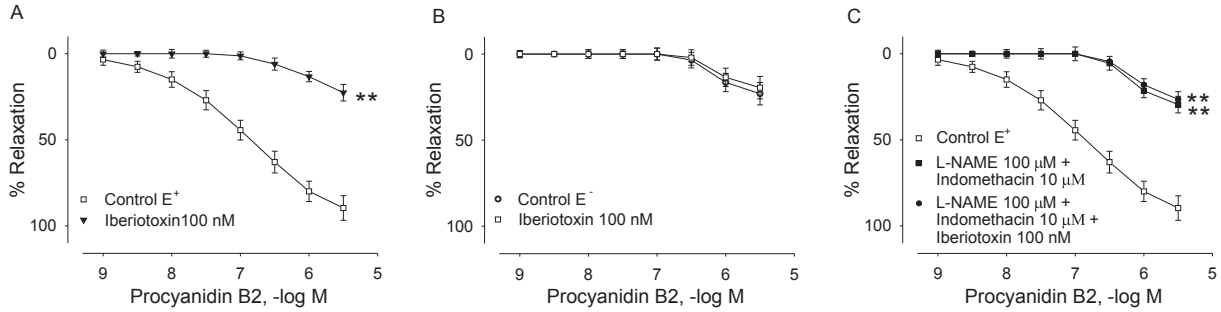

Fig. 4. Contribution of BK_{Ca} channels on the procyanidin B2-induced relaxation of human saphenous vein pre-contracted by phenylephrine (10 μM). Concentration-response curves to procyanidin B2 in the absence and presence of iberiotoxin (100 nM): (A) in endothelium-intact rings, (B) in endothelium-denuded rings, (C) in endothelium-intact rings after pre-incubation with L-NAME (100 μM)/indomethacin (10 μM) combination. Responses are expressed as a percentage of the maximum possible relaxation, i.e., the return of tension to the level before contraction with phenylephrine. Each point represents the mean ± S.D. (n = 5). ** $P < 0.01$ vs. control (two-way analysis of variance).

was not possible to evaluate the contribution of individual substances to the total vasorelaxant effect. Our study represents one of the rarely attempts to describe the vasorelaxing features of isolated procyanidin and its possible mechanisms of action. Previously, it has been proposed that activity of the procyanidins increases with polymerization degree, epicatechin content, and number of galloyl units.[10] Results from the present study support this possibility because vasorelaxing potency of procyanidin B2, as (−)-epicatechin dimer, on HSV rings (EC_{50} = 0.13 μM), was about 300 times greater than potency of (−)-epicatechin monomer reported on the same blood vessel (EC_{50} = 44.67 μM).[11] On the other hand, comparison of procyanidin B2 vasorelaxant effect on human arterial and vein graft, revealed its similar potency (EC_{50} was 0.13 μM vs. 0.10 μM, respectively).[12]

It is known that alteration in the endothelial function precedes the development of morphological atherosclerotic changes and later clinical complications.[13] Also, the endothelium represents an important target for a variety of polyphenols.[7,12] In our study, removal of functional endothelium drastically reduced the relaxant response to procyanidin B2, suggesting significant endothelial dependence of its vasorelaxant action. The importance of the endothelium was first recognized by its effect on vascular tone which is regulated through production of vasoactive factors, such as NO, prostacyclin and endothelium-derived hyperpolarizing factor (EDHF).[14] As the vasorelaxant properties of procyanidin B2 were significantly reduced in the presence of L-NAME or hydroxocobalamin, the stimulation of NO production is probably involved in procyanidin B2-induced responses in HSV. Furthermore, it has been suggested that the principal mediator responsible for human vein graft flow-mediated dilatation is NO.[15] The participation of the NO/cGMP pathway in procyanidin B2 action was further verified by the findings that relaxation was effectively decreased by treatment with ODQ. However, unlike the NO synthesis inhibition or NO scavenging, the inhibition of GC produced a lesser reduction of HSV relaxation suggesting that NO-mediated vasodilation does not only depend on soluble GC activation, but also on some other mechanisms which will be discussed in more detail.

Further, we tested involvement of other endothelial mediators, such as prostacyclin and EDHF, in the vascular effects of procyanidin B2. The fact that indomethacin had no effect on the relaxation induced by low procyanidin B2 concentrations, but only influenced maximal relaxation of HSV, suggested prostacyclin involvement in procyanidin B2 action on HSV rings primarily in high concentrations. Similar with the present results, we have previously shown significant contribution of NO to procyanidin B2-induced relaxation of HIMA, while, comparing with HSV, prostacyclin participation was more important in lower concentrations of procyanidin B2 suggesting its vessel-specific action.[12] This is line with previous findings that capacity of HIMA to produce prostacyclin in both a basal and a stimulated state is greater than that of the HSV.[16]

EDHF contribution to the regulation of blood flow and vascular resistance may be the greatest at the level of small blood vessels.[17] Particularly, K_{Ca} mediate endothelial hyperpolarization in response to humoral stimulation. SK_{Ca} and IK_{Ca} channels are mainly

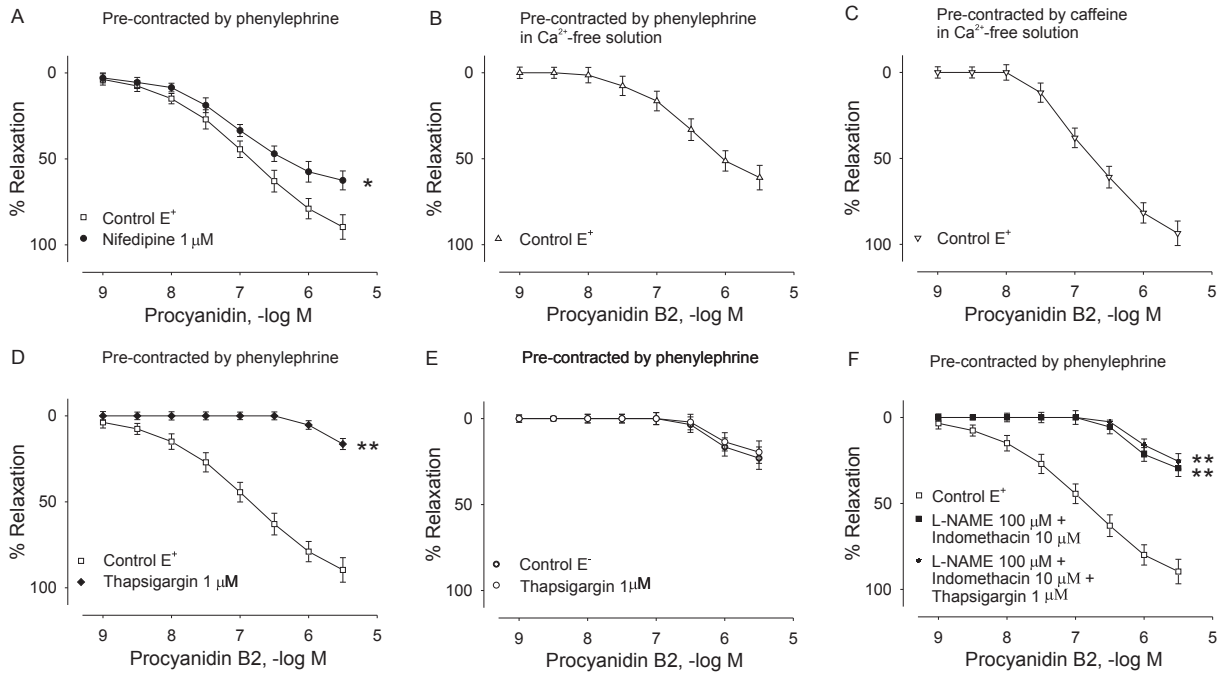

Fig. 5. Role of Ca^{2+} in the procyanidin B2-induced relaxation. Concentration-response curves to procyanidin B2 in the human saphenous vein: (A) pre-contracted by phenylephrine (10 μM) in the absence and presence of nifedipine (1 μM); (B) pre-contracted by phenylephrine (10 μM) in Ca^{2+}-free Krebs–Ringer bicarbonate solution; (C) pre-contracted by caffeine (25 mM) in Ca^{2+}-free Krebs–Ringer bicarbonate solution; (D) pre-contracted by phenylephrine (10 μM) in the absence and presence of thapsigargin (1 μM) in endothelium-intact rings; (E) pre-contracted by phenylephrine (10 μM) in the absence and presence of thapsigargin (1 μM) in endothelium-denuded rings and (F) pre-contracted by phenylephrine (10 μM) in the absence and presence of thapsigargin (1 μM) after pre-incubation with L-NAME (100 μM)/indomethacin (10 μM) combination. Responses are expressed as a percentage of the maximum possible relaxation, i.e., the return of tension to the level before contraction with phenylephrine or caffeine. Each point represents the mean ± S.D. (n = 4–5). * $P < 0.05$ or** $P < 0.01$ vs. control (two-way analysis of variance).

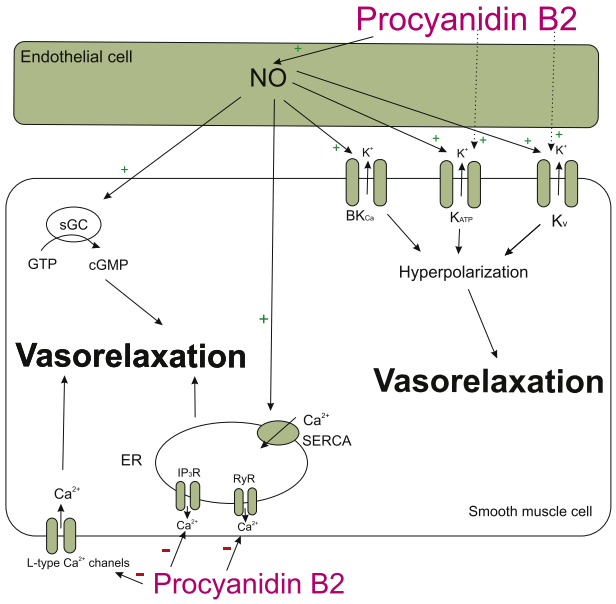

Fig. 6. Mechanism of vasorelaxant effect of procyanidin B2 on human saphenous vein (HSV). This figure emphasizes endothelium importance for procyanidin B2 action on HSV. Procyanidin B2 exerts its vasorelaxant effect mostly via stimulation of NO production followed by K^+ channel opening, especially large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel, and partially ATP-dependent K^+ (K_{ATP}) channel and voltage-dependent K^+ (K_V) channel, as well as guanylate cyclase (GC) stimulation. Additionally, procyanidin B2causes reduction of intracellular Ca^{2+} concentration through impairing both Ca^{2+} release, via NO-dependent SERCA inhibition, and Ca^{2+} influx, via voltage-dependent Ca^{2+} channels, cGMP, cyclic guanosine monophosphate; ER, endoplasmic reticulum; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; RyR, ryanodine receptor; IP_3R, receptor for inositol triphosphate.

expressed on endothelium and their inhibition, in the presence of NOS and COX inhibitors, leads to the prevention of endothelium-dependent hyperpolarization.[18] However, our findings with apamin and TRAM-34 in addition to L-NAME plus indomethacin excludes possible involvement of EDHF in the vasorelaxant effect of procyanidin B2 in HSV. Although less important then in HIMA, EDHF-mediated hyperpolarization has been detected in HSV,[19] but it is also shown that could be abolished by surgical graft preparation.[20]

In order to analyze the contribution of different K^+ channels subtypes to the procyanidin B2-induced relaxation in the HSV rings, we used agents that are known to possess a selective K^+ channel-blocking activity.

4-AP produced small but significant shift to the right in the concentration–response curves to procyanidin B2, suggesting at least partial contribution of K_V channels in the relaxation of HSV. This result is in agreement with the studies done byMatsui et al.[9] and Byun et al.,[3] who proposed that these channels are included in procyanidin-induced hyperpolarization and subsequent vasorelaxation, while in HIMA rings their significance have been shown only in high concentrations of procyanidin B2.[12]

On the other hand, similar with results obtained on HIMA, the present study suggests that procyanidin B2-induced opening of K_{ATP} channels notably contributes to its relaxant effect. Considering that these channels contribute to the regulation of coronary blood flow during hypoxia, acidosis, ischemia and ischemic preconditioning,[21] procyanidin B2 impact on K_{ATP} channels could be important. However, conflicting results reported in the literature about involvement of these channels in procyanidins action could be the consequence of different procyanidin-rich extracts use, but it is also known that expression and composition of K_{ATP} channels in

vascular smooth muscle cells vary between vascular beds, with vessel size, and among cells of individual vascular segment.[22]

Finally, as pretreatment with iberiotoxin almost abolished the concentration–response curves to procyanidin B2, the opening of BK_{Ca} channels seems to contribute to its relaxant activity to a great extent. This is in accordance with our previous results obtained with procyanidin B2 and (−)-epicatechin on isolated bypass grafts.[11,12] Considering that BK_{Ca} channels, although predominantly expressed on vascular smooth muscle cells, were detected on some endothelial cells,[23] there is a possibility that their activation by procyanidin B2 results in endothelial hyperpolarization, followed by NO production. However, in our study, procyanidin B2 was still able to induce relaxation in endothelium-intact segments pre-contracted by high K^+, when contribution of K^+ channels is abolished. Also, iberiotoxin neither produce any additional inhibition of procyanidin B2-induced relaxation in the presence of L-NAME and indomethacin, nor influenced the relaxation of endothelium-denuded rings. These results suggest the minor or no involvement of endothelial BK_{Ca} channels in stimulation of NO production, yet opening of BK_{Ca} channels on smooth muscle cells as downstream effectors of NO-dependent relaxation. These results are in accordance with the studies conducted by Hohn et al.[24] and Gruhn et al.[25] who demonstrated that among the cellular mechanisms involved in NO-induced relaxation of HSV rings is the modulations of smooth muscle cells BK_{Ca} channels. Considering that BK_{Ca} channels contra regulate the myogenic tone in a pressure-dependent fashion in HSV exposed to chronically high *in vivo* pressure,[26] it is not surprising existence of channels alteration in pathophysiological conditions, such as hypertension, diabetes and hypoxia.[27] Thus, assuming the influence of procyanidin B2 on K^+ channels, particularly on BK_{Ca} channels, it could be speculated about its positive effect on vascular function and its potential use in cardiovascular diseases.

Calcium acts as an intracellular signal that controls many cellular processes, such as contractile activity of smooth muscle cells, and flavanols impact on Ca^{2+} movement has been previously reported.[28] Our results showed that, in Ca^{2+}-free medium, procyanidin B2 completely relaxed HSV rings pre-contracted by phenylephrine and caffeine, which induced contraction through the inositol-trisphosphate and ryanodine receptors activation on the sarcoplasmic reticulum, respectively.[29,30] These findings suggest significant influence of procyanidin B2 on internal Ca^{2+} release induced by both types of receptors, which is in agreement with our previous results obtained with (−)-epicatechin.[31] However, only inositol-trisphosphate receptor system was important for procyanidin B2 relaxant effect reported on HIMA,[18] emphasizing its tissue-specific action once more.

At the intracellular level, the regulation of cytosolic Ca^{2+} homeostasis depends not only on Ca^{2+} release, but also on Ca^{2+} re-uptake by SERCA.[30] In the present study, procyanidin B2-induced relaxation of HSV endothelium-intact rings was greatly affected by thapsigargin, which imply that SERCA stimulation is very important for the pathway by which these flavanol produces the relaxation. Involvement of endothelial SERCA could be excluded based on results with thapsigargin in endothelium-denuded rings or in the presence of L-NAME/indomethacin combination. Comparing with previous results, it seems that SERCA stimulation is relatively more important in procyanidin B2-induced relaxation on HSV, than on HIMA rings.[12] Furthermore, it has been suggested that NO can enhance SERCA activity either directly, or indirectly via PKG activation.[32,33] Adachi et al.[32] have also suggested that PKG can decrease Ca^{2+} release from intracellular stores. Taking into account the important contribution of NO in procyanidin B2 action in HSV, it could be speculated that enhanced SERCA activation or decreased intracellular Ca^{2+} releasing are the steps involved in NO-dependent relaxation induced by procyanidin B2 on this blood vessel. Finally, in our study, nifedipine caused slight but significant reduction of the relaxation produced by procyanidin B2, suggesting that this flavanol probably could inhibit the influx of extracellular Ca^{2+}, similar with findings reported for (−)-epicatechin on the same blood vessels.[11]

In conclusion, we emphasize endothelium importance for procyanidin B2 action on HSV. Mechanism of relaxant effect probably involves stimulation of NO production, followed by soluble GC activation, as well K^+ channels opening, especially BK_{Ca}, and partially K_{ATP} and K_V. Additionally, reduction of intracellular Ca^{2+} concentration through impairing both Ca^{2+} influx and Ca^{2+} release, contributes to procyanidin B2-induced relaxation of HSV.

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Declaration of Competing Interest

The authors indicated no potential conflict of interest.

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