

Endothelium-Dependent Vasorelaxation Induced by Black Currant Concentrate in Rat Thoracic Aorta

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ABSTRACT—We investigated the effect of black currant (BC) concentrate on smooth muscle in rat thoracic aorta. BC concentrate dose-dependently relaxed the norepinephrine (0.1 μ M)-precontracted aorta, and the response was abolished after endothelium removal. Both oxyhemoglobin (1 μ M), a nitric oxide (NO) scavenger, and 1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one (ODQ, 0.5 μ M), an inhibitor of guanylyl cyclase (GC), inhibited the relaxing effect of BC concentrate. *N*^G-nitro-L-arginine methyl ester (L-NAME, 10 μ M), a nitric oxide synthase (NOS) inhibitor, inhibited the relaxation, and the subsequent addition of L-arginine (1 mM), a NOS substrate, reversed the inhibitory effects of L-NAME. Neither indomethacin (10 μ M), an inhibitor of cyclooxygenase, nor atropine (1 μ M), an antagonist of muscarinic receptors, modified the effect of BC concentrate. Diphenhydramine (3 μ M) and chlorpheniramine (2 μ M), selective antagonists of H₁-receptors, inhibited the relaxation, but cimetidine (0.3 mM), a selective antagonist of H₂-receptors, did not affect the relaxation. These results indicate that, in the rat aorta, BC concentrate enhances synthesis of NO, which subsequently induces the endothelium-dependent vasorelaxation via the H₁-receptors on the endothelium.

Keywords: Black currant, Endothelium, Nitric oxide, Vasorelaxation, Rat thoracic aorta

In pathological conditions such as hypertension, diabetes and atherosclerosis, endothelium-dependent vasorelaxation to different vasodilator agonists is reduced (1–3). One of the mechanisms accounting for the dysfunction of the endothelium is a decreased release of nitric oxide (NO) (4–6). Because endothelial NO plays a major role in the control of vasomotor tone and structure under physiological and pathophysiological conditions (5, 7), the development of vasodilator compounds with the ability to restore the levels of NO in the vascular system could potentially contribute to the treatment of these cardiovascular diseases.

Recently it was shown that extracts from red wines, other grape products, and various plants that contain polyphenols can induce endothelium-dependent vasorelaxation, probably via NO release or enhanced biological activity of NO, leading to an elevated accumulation of cyclic GMP in the rat aorta (8–10). Epidemiological studies (11, 12) suggested that increased consumption of vegetables and fruits with high levels of polyphenolic compounds is often associated with a low risk of degenerative diseases such as

cancer and cardiovascular disease. Berries are important sources of potential polyphenolic compounds. A mixture of anthocyanins extracted from bilberry (*Vaccinium myrtillus* L.) was reported to have biologically and pharmacologically useful properties, including vasorelaxant activity (13) and ophthalmic activity (14).

Black currants (*Ribes nigrum* L.) with high contents of polyphenolic compounds are primarily grown for industrial use in making juices, jams, and liquors. In our recent studies, we found that oral intake of black currant (BC) concentrate prepared from black currant juice had significant and beneficial effects on visual functions, reducing the dark adaptation threshold, and preventing transient refractive alteration and subjective symptoms of fatigue of the eye and low back during visual display terminal (VDT) work (15). We hypothesized that the prevention of eye and low back fatigue probably resulted from increased blood supply to these areas caused by vasorelaxation induced by BC concentrate. Therefore, the aim of the present study was to characterize the vasorelaxant effects of BC concentrate prepared from black currant juice on rat aortic rings. We found that BC concentrate induced a NO- and endothelium-dependent vasorelaxation, possibly via the histamine H₁-

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receptors on the endothelium.

MATERIALS AND METHODS

Chemicals

A powdered BC concentrate was prepared from a commercially available black currant juice by the method developed in a previous study (16). The concentrate contains 10.83% anthocyanins, consisting of 5.09% delphinidin 3-rutinoside (D3R), 1.48% delphinidin 3-glucoside (D3G), 3.76% cyanidin 3-rutinoside (C3R) and 0.50% cyanidin 3-glucoside (C3G). The total polyphenol content of BC concentrate is 19.6%, measured by Prussian blue method (17), using epicatechin as the standard.

Norepinephrine (NE), acetylcholine (ACh), N^G -nitro-L-arginine methyl ester (L-NAME), 1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one (ODQ), indomethacin, atropine, histamine, diphenhydramine, chlorpheniramine and cimetidine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Oxyhemoglobin and L-arginine were purchased from Funakoshi Co. (Tokyo) and Kanto Chemical Co. (Tokyo), respectively. Stock solutions of the drugs were prepared daily and kept on ice until used. ODQ, indomethacin, atropine, and cimetidine were dissolved in absolute ethanol to prepare 20 mM solutions, and then further dilutions were made in distilled water. Other drugs were dissolved in distilled water such that volumes of <0.025 ml were added to the organ baths. Drug concentrations are expressed as the final concentration in the organ bath.

Preparation of rat aorta rings and tension recording

All studies were performed according to the Guiding Principles for the Care and Use of Laboratory Animals of The Japanese Pharmacological Society.

Male Sprague-Dawley rats (350–450 g) were killed by cervical dislocation and then exsanguinated by carotid artery transection. The thoracic aorta was removed, carefully cleaned of adhering fat and connective tissue, and cut into rings (2–3-mm length). The rings were then mounted in a 5-ml organ bath filled with the modified Krebs solution (118.3 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl_2 , 1.2 mM MgSO_4 , 1.2 mM NaH_2PO_4 , 25.0 mM NaHCO_3 , 0.026 mM ethylenediaminetetraacetic acid calcium salt and 11.1 mM glucose, pH 7.35), maintained at $37 \pm 0.5^\circ\text{C}$ and continuously bubbled with 95% O_2 / 5% CO_2 . Tension was measured with an isometric force transducer, and resting tension was adjusted to 1.5 g. The rings were equilibrated for a period of 90 min before initiating experimental protocols, and during this period, the incubation medium was changed every 45 min.

After the equilibration period, the vessels were maximally contracted with NE (0.1 μM) to test their contractile

capacity. In some experiments the endothelium was removed by gently rubbing the intima surface with curved forceps. The presence of functional endothelium was assessed in all preparations by determining the ability of ACh (0.1 μM) to induce more than 50% relaxation of rings precontracted with NE (0.1 μM). Vessels were considered to be denuded of functional endothelium when there was no relaxation response to ACh.

Characterization of the relaxant effect of BC concentrate

After washing and returning to baseline tension, aortic rings with and without functional endothelium were precontracted to the same tension with 0.1 μM NE. When the NE-precontraction reached a steady state, increasing concentrations of BC concentrate were added cumulatively.

To characterize the involvement of NO, experiments were performed in the presence of ODQ (0.5 μM), the guanylyl cyclase (GC) inhibitor (18), added to the bath 5 min prior to the addition of NE; in another set of experiments, oxyhemoglobin (1 μM), the NO scavenger (19), was added after the relaxant effect of BC concentrate (10–30 $\mu\text{g}/\text{ml}$) was induced. Some arteries with functional endothelium were exposed to the NO synthase (NOS) inhibitor L-NAME (10 μM) (20) added to the bath 5 min before NE. When the NE-precontraction reached a steady state, BC concentrate (20 $\mu\text{g}/\text{ml}$) was added. Ten minutes later, the rings were incubated with L-arginine (1 mM) for 30 min, and then BC concentrate (20 $\mu\text{g}/\text{ml}$) was added.

To verify the participation of endothelium-derived products to the relaxant effect of BC concentrate, experiments were performed in the presence of indomethacin (10 μM), the cyclooxygenase (COX) inhibitor (21, 22), added to the bath 5 min prior to the addition of NE. To verify the participation of the muscarinic receptors and the histamine receptors to the relaxant effect of BC concentrate, atropine (1 μM), the muscarinic receptor antagonist; diphenhydramine (3 μM) and chlorpheniramine (2 μM), the histamine H_1 -receptor antagonists; or cimetidine (0.3 mM), the histamine H_2 -receptor antagonist, were added after the relaxant effect of BC concentrate (10 or 20 $\mu\text{g}/\text{ml}$).

Expression of results and statistical analyses

Data are presented as means \pm S.E.M. for the number of experiments indicated. Results from contractile experiments are expressed as percentage decreases in maximal contraction induced by NE; the point when the baseline was reached was considered 100% relaxation. Statistical analysis was performed using the Wilcoxon signed-ranks test, and $P < 0.05$ was regarded as significantly different.

RESULTS

Effect of BC concentrate on NE-contraction in endothelium-intact and denuded aorta

The vasorelaxant effect of BC concentrate was studied on NE-precontracted rat aorta (Fig. 1). In NE contracted aortic rings with functional endothelium, BC concentrate induced a concentration-dependent relaxation of vascular smooth muscles (Figs. 1A and 2). In rings without functional endothelium, BC concentrate in concentrations up to 40 $\mu\text{g/ml}$ did not produce any significant relaxant effect (Fig. 1B). These results demonstrate that in the rat aorta, BC concentrate had a vasorelaxant effect dependent on the presence of functional endothelium.

Effects of L-NAME, L-arginine, and various inhibitors on the endothelium-dependent vasorelaxation induced by BC concentrate

Pretreatment of the rings with the inhibitors did not affect the vascular tone of contraction caused by NE.

Figure 3 illustrates the effect of L-NAME and L-arginine on the endothelium-dependent vasorelaxation induced by BC concentrate. BC concentrate was used at a concentration, 20 $\mu\text{g/ml}$, that produced approximately 80% relaxation in rings with endothelium. In rings with functional endothelium, the blocking of endothelial NO synthesis by L-NAME (10 μM) inhibited the relaxation, and the subsequent addition of L-arginine (1 mM), the NOS substrate, reversed the inhibitory effects of L-NAME. The relaxation value in the presence of L-NAME was significantly de-

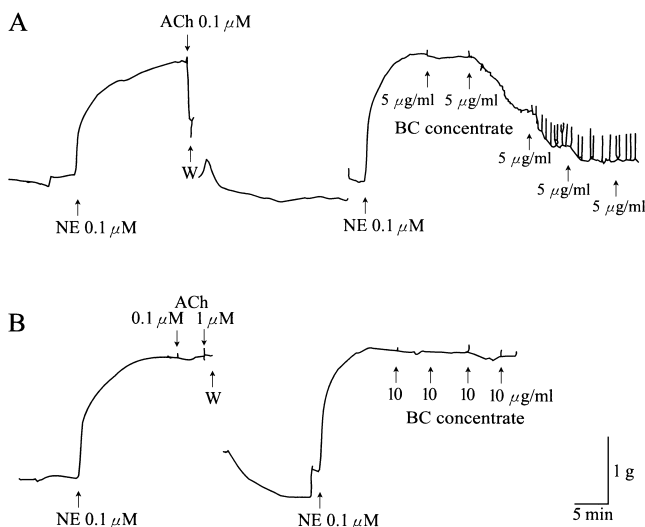


Fig. 1. Effect of black currant (BC) concentrate on NE-contraction in endothelium-intact and denuded aorta. ACh was used to determine whether the aortic ring was intact or denuded. BC concentrate was added after the exposure of the intact (A) or denuded (B) aortic ring to NE (0.1 μM).

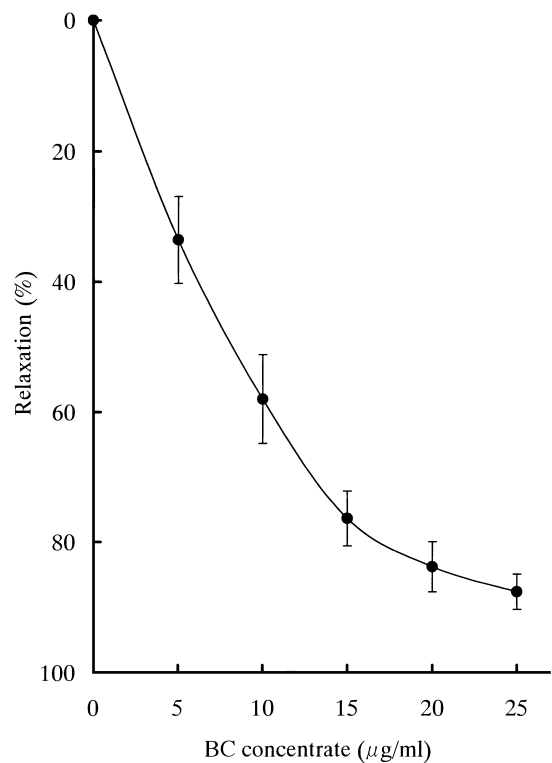


Fig. 2. Concentration response curve for black currant (BC) concentrate in NE precontracted rat thoracic aortic rings. Values are the mean of 10 experiments; vertical lines represent S.E.M.

creased from $75.4 \pm 6.5\%$ to $4.7 \pm 1.3\%$ ($n = 5$, $P < 0.001$), and the value in the presence of L-NAME plus L-arginine was significantly increased from $4.7 \pm 1.3\%$ to $19.9 \pm 1.2\%$ ($n = 5$, $P < 0.001$).

In rings with functional endothelium, ODQ (0.5 μM), the GC inhibitor, added to the bath 5 min prior to the addition of NE, inhibited the relaxation induced by BC concentrate (30 $\mu\text{g/ml}$) (Fig. 4A). In another set of experiments, after the relaxant effect of BC concentrate (10 $\mu\text{g/ml}$), the addition of oxyhemoglobin (1 μM), the NO scavenger, inhibited the relaxation (Fig. 4B). Blocking COX by indomethacin (10 μM) did not affect the BC concentrate-induced relaxation (Fig. 4C). These results indicate that the vasorelaxation induced by BC concentrate was caused by enhanced synthesis of NO in the endothelium.

Effects of the antagonists on the endothelium-dependent vasorelaxation induced by BC concentrate

The selective antagonist of the muscarinic receptors, atropine (1 μM), at a concentration at which it completely inhibited ACh-induced relaxation (0.1 μM), had no effect on the relaxant effect of BC concentrate (20 $\mu\text{g/ml}$) (Fig. 5A). The selective antagonists of the histamine H_1 -receptors, diphenhydramine (3 μM) and chlorpheniramine (2 μM), at concentrations at which they completely inhi-

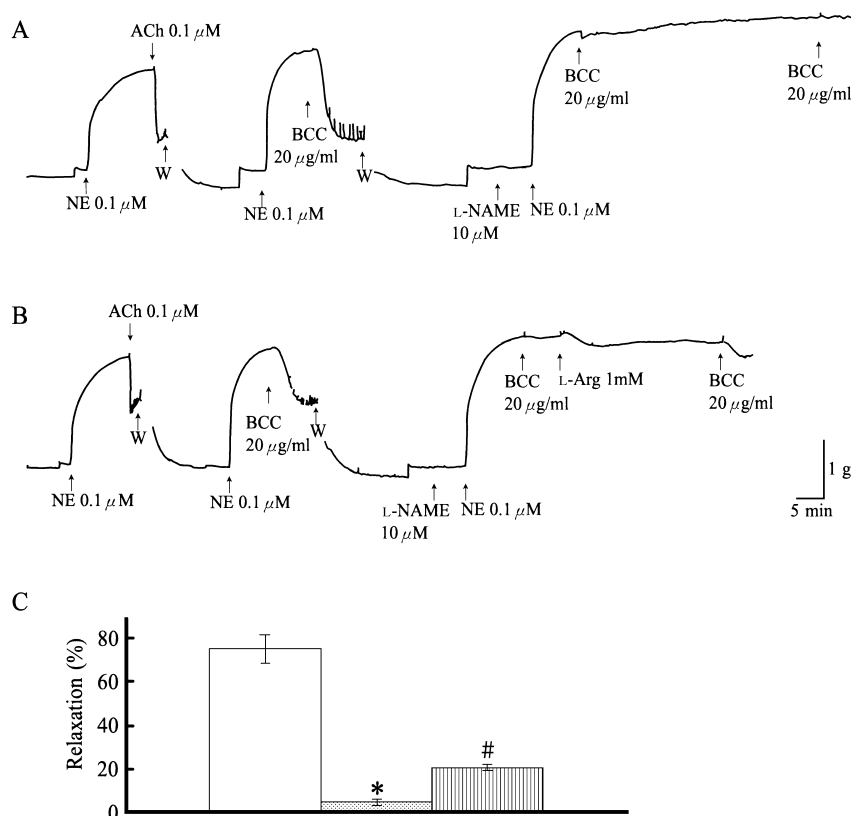


Fig. 3. Effects of L-NAME and L-arginine on the endothelium-dependent vasorelaxation induced by black currant concentrate (BCC). A: L-NAME (10 μ M) added to the bath 5 min before NE (0.1 μ M) inhibited the relaxation. B: L-NAME (10 μ M) added to the bath 5 min before NE (0.1 μ M) inhibited the relaxation; the subsequent addition of L-arginine (1 mM) reversed the inhibitory effects of L-NAME. C: Histograms show the relaxation of BC concentrate (20 μ g/ml) in the absence (empty square) or presence (dotted square) of L-NAME (10 μ M) or L-NAME (10 μ M) plus L-arginine (1 mM, square with vertical line). Values are the mean of 5 experiments; vertical lines represent S.E.M. * P <0.001, the values are significantly different from the control. # P <0.001, the values are significantly different from the response obtained in the presence of L-NAME.

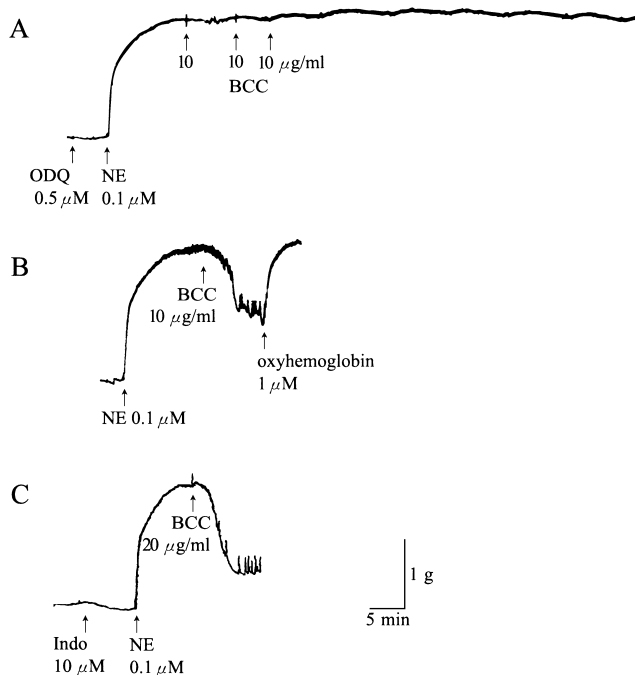


Fig. 4. Effects of ODQ (A), oxyhemoglobin (B) and indomethacin (C) on the endothelium-dependent vasorelaxation induced by black currant concentrate (BCC).

bited histamine-induced relaxation (10 μ M), inhibited the relaxant effect of BC concentrate (20 μ g/ml) (Fig. 5: B–D). In contrast, the selective antagonist of the histamine H_2 -receptors, cimetidine (0.3 mM), had no effect on the relaxant effect of BC concentrate (10 μ g/ml) (Fig. 5E).

To investigate the relaxant effect of BC concentrate by the selective antagonists of the histamine H_1 -receptors, experiments were performed in the presence of diphenhydramine (3 μ M) or chlorpheniramine (2 μ M), added to the bath 5 min prior to the addition of NE. Pretreatment of the rings with the antagonists of the histamine H_1 -receptors did not affect on vascular tone of contraction induced by NE and caused a parallel rightward shift of BC concentrate-induced relaxant response curves (Fig. 6). Mean values of relaxation induced by 5 μ g/ml and 10 μ g/ml BC concentrate were $50.6 \pm 5.5\%$ and $83.6 \pm 5.4\%$ in the control, $7.0 \pm 3.3\%$ and $38.1 \pm 8.5\%$ in the presence of diphenhydramine, and $13.5 \pm 4.9\%$ and $33.8 \pm 7.1\%$ in the presence of chlorpheniramine, respectively ($n=4$), with the differences between the control and the presence of the antagonists being statistically significant ($P<0.05$). These results suggest that the vasorelaxation induced by BC concentrate was caused via the histamine H_1 -receptors on the endothelium.

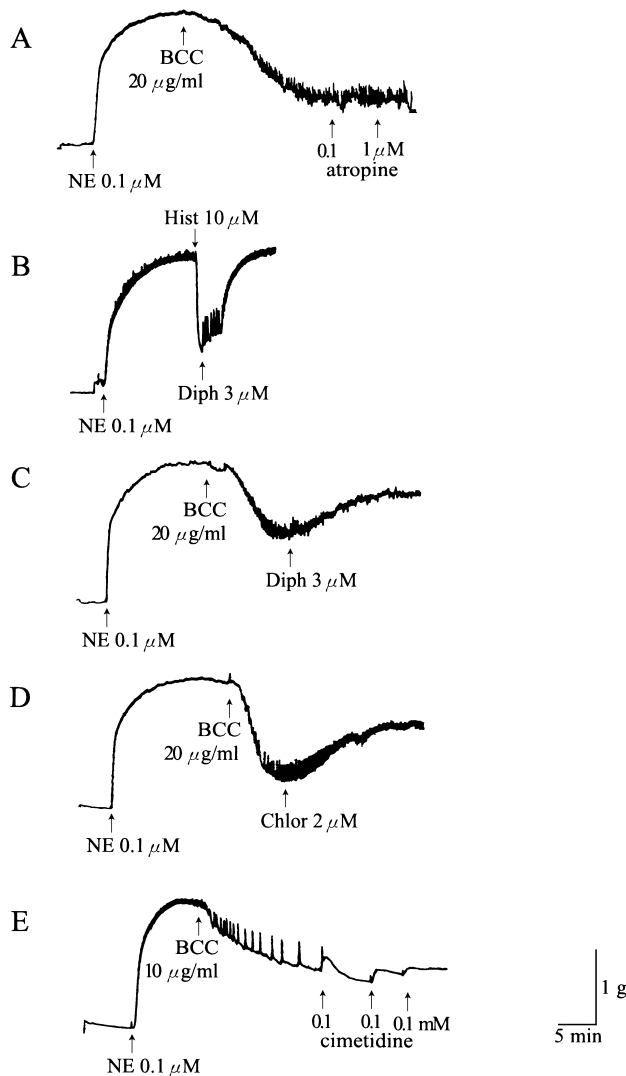


Fig. 5. Effects of the antagonists on the endothelium-dependent vasorelaxation induced by black currant concentrate (BCC). A: Effect of atropine on BCC-induced relaxation. B: Effect of diphenhydramine (Diph) on histamine (Hist)-induced relaxation. C: Effect of diphenhydramine (Diph) on BCC-induced relaxation. D: Effect of chlorpheniramine (Chlor) on BCC-induced relaxation. E: Effect of cimetidine on BCC-induced relaxation.

DISCUSSION

Results of this study clearly demonstrate that BC concentrate can induce endothelium-dependent vasorelaxation *in vitro* by a mechanism involving increased levels of NO. The relaxation is not seen in deendothelialized vascular rings. Vasorelaxation is abolished by the NO scavenger oxyhemoglobin and is inhibited by the GC inhibitor ODQ. The relaxation is not affected by the COX inhibitor indomethacin and is reduced by inhibition of NOS. Finally, the inhibitory effect by L-NAME is, in turn, reversed by the normal substrate, L-arginine.

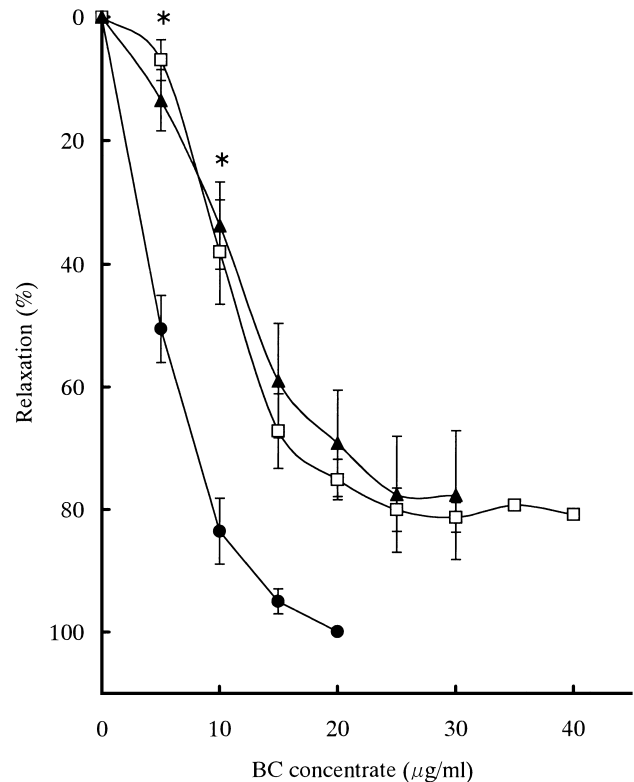


Fig. 6. Concentration response curve for black currant (BC) concentrate in NE precontracted rat thoracic aortic rings (closed circle), in the presence of diphenhydramine (3 μM, opened square) and in the presence of chlorpheniramine (2 μM, closed triangle). Values are the mean of 4 experiments; vertical lines represent S.E.M. * $P < 0.05$, the values are significantly different from the control.

Our results suggest that the vasorelaxation induced by BC concentrate could be caused via the histamine H_1 -receptors on the endothelium. The relaxation is not affected by the selective antagonist of the muscarinic receptors. Vasorelaxation is inhibited by the selective antagonists of the histamine H_1 -receptors, but not by the selective antagonists of the histamine H_2 -receptor.

In rat aorta rings precontracted by NE (0.1 μM), the relaxant effect induced by histamine (10 μM) was equivalent to the relaxant effect induced by BC concentrate (10 μg/ml), approximately 60%. In a related series of experiments using rat ileum, histamine (0.1 mM) induced the contractile response, but BC concentrate (0.3 mg/ml, three times concentration of histamine effect) did not react (data not shown). If the active components of BC concentrate were histamine or its agonists, rat ileum would show contractile response induced by 0.1 mg/ml BC concentrate. Thus, we suspect that the vasorelaxation induced by BC concentrate might be not caused by the presence of histamine or its agonists in BC concentrate.

Results from epidemiological studies suggest that a high intake of foods rich in flavonoids might reduce the risk of

cardiovascular diseases (11, 12, 23). Recently, it was reported that the flavonoids leucocyanidin (10), delphinidin (24), epicatechin 3-*O*-galate (25), and proanthocyanidins (26) have endothelial NO-induced vasorelaxant activity. However, little information is available concerning the identity of the compounds responsible for endothelial NO-induced vasorelaxant activity, and we suspect that extracts of various plants or wines that have vasorelaxant effects contain other more potent components. It was reported that delphinidin, but not other anthocyanins with closely related structures, such as malvidin and cyanidin, elicited endothelium-dependent relaxation (24). Recently, we demonstrated that orally administered D3R and C3R are directly absorbed and distributed into the blood in rats and humans (27), and C3G was reportedly absorbed in rats and humans, appearing in the blood along with its metabolites but without its aglycon (28, 29). Anthocyanins with glycosides are absorbed and appear in their intact forms in the blood, although aglycons are not detected. Thus, it would be more important to investigate the relaxant effect of glycosides than to study the effect of aglycons. Therefore, we hypothesized and investigated the relaxant effect of four anthocyanins of BC concentrate, anthocyanins with glycosides but not aglycons. We found that four kinds of anthocyanins with rutosides and glucosides present in BC concentrate did not show the relaxant effect, either alone (10 μ M) or synergistically (data not shown). Because BC concentrate contains various polyphenols in addition to anthocyanins (30, 31), we suspect the active components are categories of polyphenols—otherwise the four kinds of anthocyanins present in BC concentrate would produce the relaxant effect synergistically with the other components. More work is required before the identity of the active components and the mechanism(s) of the vasorelaxation induced by BC concentrate can be elucidated.

According to quantitative analysis with chemiluminescence, BC concentrate showed antioxidant activity by way of H₂O₂-scavenging activity (32). The free radical superoxide is known to interact with and inactivate NO (6). It is conceivable that antioxidant activity induced by BC concentrate could account for increased levels of NO in blood vessel walls as a result of decreased inactivation of NO (33). Thus, it appears that these two separate mechanisms, increased NO synthesis and decreased NO inactivation, may contribute to the beneficial effects of BC concentrate on the cardiovascular system.

Oral intake of BC concentrate can reduce the dark adaptation threshold and promote recovery from or prevent VDT work-induced transient refractive alteration and subjective symptoms of fatigue in the eye and low back in healthy subjects (15). One of the most important factors in fatigue in the low back muscles is the restriction of blood flow due to high intramuscular mechanical pressure (34).

Therefore, we extrapolate from our experiments and suggest that increased blood flow resulting from vasorelaxation induced by BC concentrate intake could help to prevent fatigue of the eye and low back.

In conclusion, our results show that BC concentrate causes endothelium-dependent vasorelaxation in vitro in rat aorta rings by increasing levels of NO production, via the histamine H₁-receptors on the endothelium. Additional research is needed to identify the active components of BC concentrate that are responsible for the observed vasorelaxation and to characterize their chemical and pharmacological properties. Our results suggest a possible beneficial effect of BC concentrate on cardiovascular function.

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