VASORELAXANT RESPONSES INDUCED BY RED WINE POLYPHENOLS: THE ROLE OF NITRIC OXIDE

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Summary

It was hypothesized that the cardioprotective effect of natural polyphenols results from their ability to protect low-density lipoprotein from oxidation, to prevent platelet aggregation and leukocyte adhesion, and to promote relaxation of vascular smooth muscle. The aim of the present study was to investigate the mechanism of vasorelaxant responses of carotid and femoral arteries induced by red wine polyphenolic compounds (Provinols™). Rings of rat carotid and femoral artery with functional endothelium were set up in a myograph for isometric recording and precontracted with phenylephrine (10^{-5} M). Provinols™ in cumulative doses (10^{-9} to 10^{-3} mg/ml) elicited endothelium- and dose-dependent relaxation of the carotid and femoral arteries with maximal relaxation of 61% and 56%, respectively, at the concentration of 10^{-5} mg/ml. The relaxant responses to Provinols™ were associated with the increase of NO synthase activity in the vascular tissues after administration of cumulative doses of Provinols™ (10^{-9} to 10^{-3} mg/ml) in the presence of Ca^{2+}. The relaxant responses induced by Provinols™ were abolished by Ca^{2+}-entry blocker, verapamil (10^{-6} M) in both arteries. In conclusion, Provinols™ elicited endothelium-dependent relaxation of rat carotid and femoral arteries by the Ca^{2+}-induced increase of NO synthase activity.

Key Words: red wine polyphenols, vasorelaxation, carotid artery, femoral artery, nitric oxide synthase, verapamil

Introduction

Many epidemiological studies have shown that regular flavonoid intake in grape juice, red wine and in some other beverages is associated with reduced risk of cardiovascular diseases. Previously it has been reported that reduction of the rate of atherosclerosis and coronary heart disease caused by daily intake of flavonoids was based mainly on the possibility of flavonoids to inhibit acute thrombus formation and to relax vascular wall as well (1,2). Polyphenolic compounds have the ability to relax precontracted smooth muscle of the arteries with intact endothelium, moreover, some of them are able to relax endothelium-denuded arteries (2,3).
Several authors have reported that extracts from grape and wine induce endothelium-dependent relaxation via enhanced generation and/or increased biological activity of nitric oxide (NO) which lead to the elevation of cGMP level (3,4). The increase in the intracellular Ca^{2+} level succeeding to the activation of NO synthase and production of NO results in the endothelium-dependent vasorelaxation (5). The biological activity of NO can be effectively increased also by the scavengers of oxygen free radicals (6,7). The mechanism brinking about the wine extract-induced increase in the level of biologically active NO needs, however, further studies.

The aim of the present study was to analyse endothelium-dependent relaxation of carotid and femoral arteries induced by polyphenolic compounds isolated from red wine (Provinols™). Moreover, NO synthase activity in the vascular tissue homogenates was determined after Provinols™ administration in the cumulative doses in pre presence or absence of Ca^{2+}.

Methods

Chemicals and drugs: Dry powder of red wine polyphenolic compounds (Provinols™) was provided by D. Ageron (Société Francaise de Distillerie, Vallont Pont d´Arc, France). The composition of Provinol was (in mg/g of dry powder): proanthocyanidins 480, total anthocyanins 61, free anthocyanins 19, catechin 38, hydroxycinnamic acid 18, flavonols 14. All the chemicals were purchased from Sigma Chemicals Co, Germany, except for [^{3}H]-L-arginine (Amersham, United Kingdom).

Analysis of Provinols™-induced relaxation: Male wistar rats, 12 weeks old were killed by cervical dislocation. Carotid and femoral arteries were removed and carefully cleaned of adhering fat and connective tissue and then cut into rings (1.5-2 mm length). Two stainless-steel wires were passed through the lumen taking care not to damage the vessel and mounted in a Mulvany-Halpern myograph chamber capable of measuring the isometric wall tension. During the experiments, the internal diameter of the vessels was 300-400 μm. The chamber was filled with physiological salt solution (composition in mM: NaCl 118, KCl 5, $NaHCO_3$ 25, $MgSO_4.H_2O$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, EDTA 1, ascorbic acid 1.1, glucose 11) maintained at 37°C and continuously bubbled with a 95% O_2 and 5% CO_2 mixture.

After the equilibration period of 45 minutes, the rings were precontracted with phenylephrine (10^{-5} M) and tested for the presence of functional endothelium by determining the ability of acetylcholine (10^{-5}M) to induce relaxation greater than 30%.

Rings of rat carotid and femoral arteries were precontracted with phenylephrine (10^{-5}M). Provinols™ in concentration range 10^{-9} - 10^{-3} mg/ml was added when contraction reached the steady state. The source of Ca^{2+} involved in the relaxation induced by Provinols™, was analyzed by testing its effect in the presence of Ca^{2+}- entry blocker, verapamil (10^{-5}M).

Determination of NO synthase activity: NO synthase activity was determined in crude homogenates of vascular tissue by measuring the formation of [^{3}H]-L-citrulline from [^{3}H]-L-arginine (Amersham,UK) as previously described by Bredt and Snyder (8) with minor modifications (9). Briefly, 50 µl of crude homogenate (30 mg of wet tissue) was incubated in the presence of 50 mM Tris-HCl, pH 7.4, containing 1 µM L-[^{3}H]arginine (specific activity 5 GBq/mmol, about 100 000 DPM), 0.5 mg/ml calmodulin, 0.5 mM β-NADPH, 250 µM tetrahydrobiopterin, 4 µM FAD, 4 µM FMN and 1mM $CaCl_2$ in a total volume of 100 µl. In some samples incubation medium was $CaCl_2$ and calmodulin free, on the contrary, EGTA (1mM) and EDTA (1mM) was added. Provinols™ was added to the incubation medium in the concentration range 10^{-9} to 10^{-3} mg/ml. After 10-min incubation at 37^{o}C, the reaction was stopped, the samples were centrifuged and supernatants were applied to 1mL Dowex 50WX-8 columns (Na^+form). [^{3}H]-L-citrulline was eluted by 2ml of water and determined by liquid scintilation counting.

Statistical analysis: The results were expressed as mean ± sem. values were considered to differ significantly if the p value was less than 0.05. For analysis one-way anova and Bonferroni test were used.

Results

Relaxant effect of Provinols™

Provinols™ in cumulative doses (10^{-9} to 10^{-3} mg/ml) elicited endothelium- and dose-dependent relaxation of the carotid and femoral arteries with maximal relaxation of 61% and 56%, respectively, at the concentration of 10^{-5} mg/ml. Relaxation of carotid and femoral arteries induced by Provinols™ was abolished in the presence of Ca^{2+}- entry blocker, verapamil (Fig. 1A, 2A).

Nitric oxide synthase activity

In the carotid and femoral artery tissue homogenates NO synthase activity was 5.01±0.30 pkat.g^{-1} and 5.17±0.31 pkat.g^{-1} protein, respectively, before the administration of Provinols™. Administration of Provinols™ (10^{-9}-10^{-4} mg/ml) increased NO synthase activity dose-dependently. The maximal activity of NO synthase in the carotid and femoral arteries was recorded with Provinols™ at the concentration of 10^{-5} and 10^{-4} mg/ml, respectively. In the absence of Ca^{2+} NO synthase activity was decreased and the

administration of Provinols™ increased it significantly less than in the presence of Ca^{2+} in both arteries investigated (Fig. 1B, 2B).

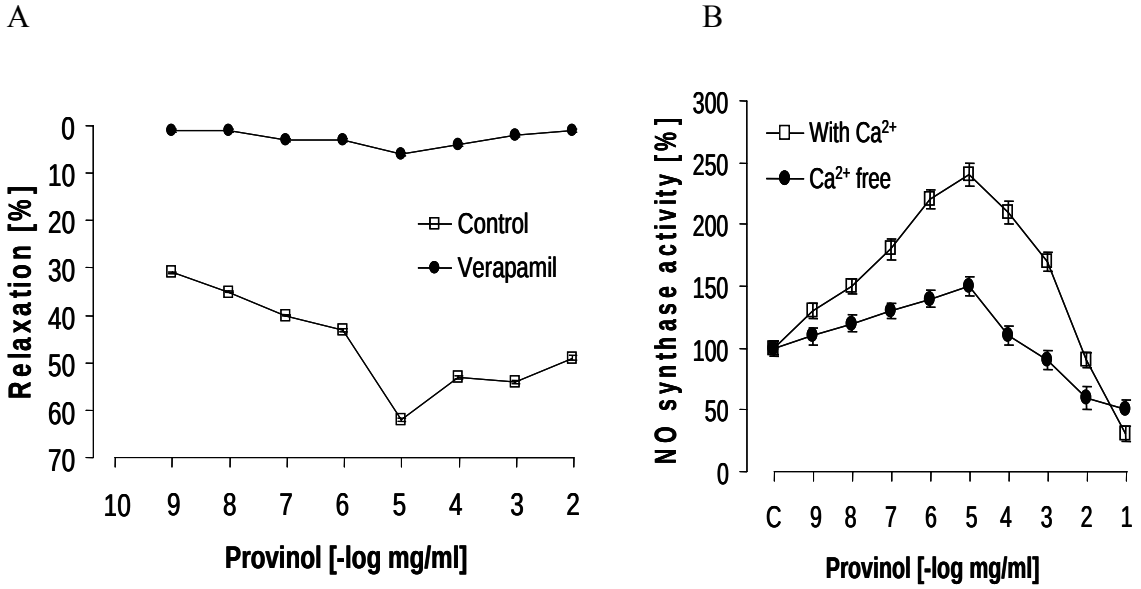

Fig. 1: Influence of the Ca2+entry blocker verapamil on the relaxation responses of carotid artery to Provinols™ administration. Responses before (□) and after (●) verapamil administration (A). NO synthase activity in the carotid artery after administration of cumulative doses of Provinols™. NO synthase activity in the presence (□) and without (●) Ca^{2+} (B).

Discussion

In the present study we have demonstrated that Provinols™ in cumulative doses (10^{-9} to 10^{-3} mg/ml) elicited endothelium- and dose-dependent relaxation of the carotid and femoral arteries with maximal relaxation of 61% and 56%, respectively, at the concentration of 10^{-5} mg/ml. The relaxant responses to Provinols™ were associated with the increase of NO synthase activity in the vascular tissues after administration of cumulative doses of Provinols™ (10^{-9} to 10^{-3} mg/ml) in the presence of Ca^{2+}. The relaxant responses induced by Provinols™ were abolished by Ca^{2+}-entry blocker, verapamil (10^{-6} M) in both arteries.

Our findings are supported by an earlier study of Fitzpatric et al. (3) who reported relaxation of intact rat aortic rings induced by red grape skin extract. This relaxation was abolished by N^G-nitro-L-arginine methyl ester and reversed by L-arginine indicating the involvement of nitric oxide in the relaxant responses. Similarly to grape skin extract, red wine polyphenolic compounds also caused a dose-dependent relaxation in rabbit aorta with intact endothelium (10). The authors documented that relaxation responses were abolished by N^G-nitro-L-arginine methyl ester and were associated with an increase in cGMP content.

Flesch et al. (4) also documented increased concentration in cGMP content in rat aortic rings after exposure to phenolic grape ingredients and red barrique wines. Since guanylate cyclase operates as an intracellular receptor for NO (11), it is possible that increased concentration of NO was responsible for the enhancement of cGMP level. This was in agreement with our finding of increased NO synthase activity after Provinols™ administration.

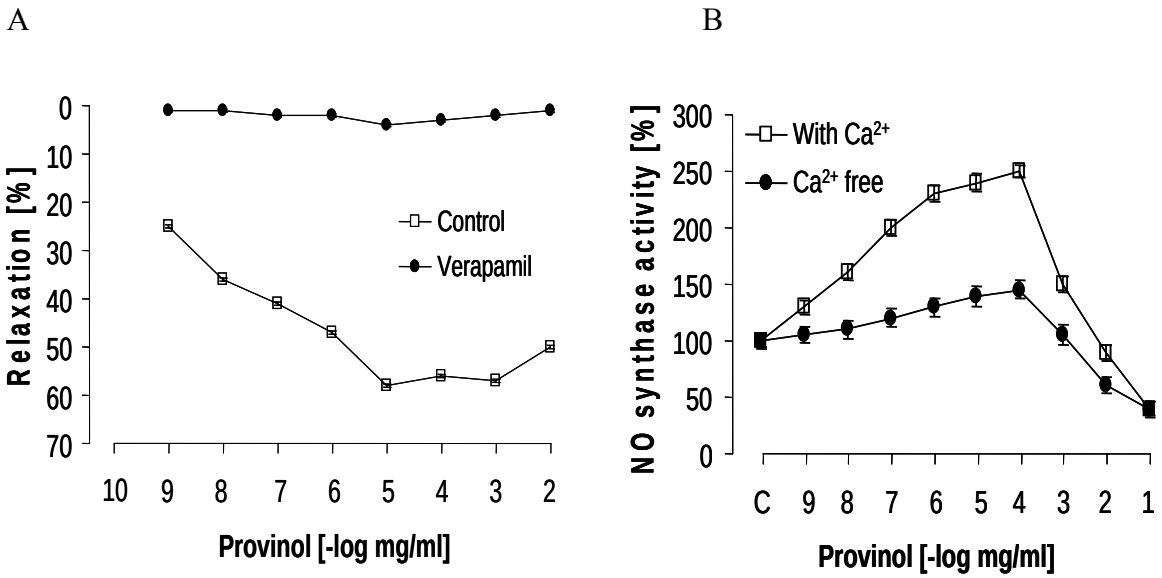

Fig. 2: Influence of the Ca2+entry blocker verapamil on the relaxation responses of femoral artery to Provinols™ administration. Responses before (□) and after (●) verapamil administration (A). NO synthase activity in the femoral artery after administration of cumulative doses of Provinols™. NO synthase activity in the presence (□) and without (●) Ca^{2+} (B).

The studies of Kang et al (12, 13) showed that the extracts of Phellinus igniarius and Sorbus commixta cortex induced relaxation of the phenylephrine-precontracted rat aorta in a dose-dependent manner, and this effect was abolished by the removal of functional endothelium. Pretreatment of the aortic tissues with N^G-nitro-L-arginine methyl ester, methylene blue, or oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) inhibited the vascular relaxations induced by Phellinus igniarius or Sorbus commixta cortex. Phellinus igniarius- and Sorbus commixta cortex- induced vascular relaxations were also markedly attenuated by the addition of verapamil or diltiazem. These results, similarly as our study, demonstrate that dilatation of vascular smooth muscle is mediated via endothelium-dependent nitric oxide-cGMP signaling pathway, with the possible involvement of L-type Ca(2+) channels.

The increase in intracellular concentration of Ca^{2+} ([Ca2^{+}]i) represents the critical step for the activation of constitutive NO synthase in the endothelial cells leading to the production of NO and the subsequent endothelium-dependent vasorelaxation (14, 15). This increase in [Ca^{2+}]i can be due to either an influx of extracellular Ca^{2+} or a release of Ca^{2+} from intracellular stores. In our study, the relaxation produced by Provinols™l was completely prevented in the presence of the Ca^{2+}- entry blocker verapamil, suggesting that the Ca^{2+} influx was crucial for the relaxation ability of the red wine polyphenolic compounds. Analogically, activity of NO synthase in the carotid and femoral arteries was increased after Provinols™ administration in the presence of Ca^{2+} while only tendency to increase NO synthase activity was seen in the Ca^{2+} free medium. These results suggested that Provinols™ increased constitutive NO synthase activity predominantly. Andriambeloson et al. (5) reported also that red wine polyphenolic compounds produced NO-dependent vasorelaxation of rat aortic rings through an extracellular Ca^{2+}-dependent mechanism. However, it cannot be excluded that a release of Ca^{2+} from intracellular stores might play a role in the endothelial NO-dependent relaxation produced by polyphenolic compounds. Indeed, after red wine polyphenolic compounds administration to the endothelial cell culture, Martin et al. (16) documented an increase of [Ca^{2+}]i from the intracellular stores, which was sensitive to the phospholipase C inhibitor.

In conclusion, our findings indicate that Provinols™ elicited endothelium-dependent relaxations of carotid and femoral arteries by stimulation of constitutive NO synthase activity which leads to the enhancement of NO production.

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