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A Role of Ion Channels in the Endothelium-Independent Relaxation of Rat Mesenteric Artery Induced by Resveratrol

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Abstract. Recently it has been suggested that resveratrol relaxes different isolated arteries. The present study addressed the question whether different ion channels are involved in the endothelium-independent mechanism of vasodilatation induced by resveratrol. For that purpose, we tested the action of resveratrol on the rat mesenteric artery without endothelium. Resveratrol induced concentration-dependent relaxation of rat mesenteric artery. Among the K^+-channel blockers, 4-amynopiridine (4-AP) moderately antagonized the resveratrol-induced relaxation, while glibenclamide, tetraethylammonium chloride, charybdotoxin, margatoxin, and barium chloride did not inhibit resveratrol-induced vasorelaxation. In rings, precontracted with 100 mM K^+, the relaxant responses to resveratrol were highly significantly shifted to the right compared to those obtained in rings precontracted with phenylephrine, but resveratrol-induced maximal relaxation was only slightly affected. In order to minimize the influence of K^+ channels and voltage-gated Ca^{2+} channels (VGCCs) in vascular smooth muscle, the third contraction was made by 100 mM K^+ in the presence of nifedipine. The relaxant response to resveratrol was abolished. Thus, the mechanism of vasorelaxation induced by resveratrol probably involves activation of 4-AP-sensitive K^+ channels. Its ability to completely relax the mesenteric artery precontracted with K^+-rich solution suggests that K^+ channel–independent mechanism(s) are involved in its vasorelaxant effect. It seems that interaction with VGCCs plays a part in this K^+ channel–independent effect of resveratrol.

Keywords: resveratrol, rat mesenteric artery, ion channel, vasorelaxation, K^+ channel

Introduction

Resveratrol (3,5,4'-trihydroxistilbene) was first isolated from the roots of white hellebore (*Veratrum grandiflorum*) in 1940, and later, in 1963, from the roots of *Polygonum cuspidatum*, a plant used in traditional Japanese medicine. It seems that resveratrol might be partly responsible for the cardiovascular benefits associated with red wine consumption (1 – 3). Recently, it has been suggested that resveratrol relaxes different isolated arteries, has potent antiinflammatory and antioxidant effects, and can also protect isolated hearts from ischemia-reperfusion injury (4 – 9). Despite the increasing interest in the effects of resveratrol on the cardiovascular system, there is some inconsistency in the mechanisms involved in its vasodilatatory effect that are not completely defined.

The resveratrol-induced vasorelaxation may either be endothelium-dependent (attenuated by L-NAME) or endothelium-independent (6, 7, 10). The mechanism of endothelium-independent vasorelaxation induced by

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resveratrol is uncertain. Resveratrol might become incorporated into the smooth muscle membrane, where it could either couple with a membrane receptor or interact directly with membrane ion channels, thus inducing endothelium-independent vasorelaxation (11, 12). Also, in different experimental models, resveratrol can activate adenylate cyclase and membrane-bound guanylyl cyclase and inhibit cGMP-specific phosphodiesterse-5 (13 – 15). Previously, we have shown that resveratrol produces relaxation of the isolated human internal mammary artery and rat aorta by activation of margatoxin-sensitive voltage-dependent K^+ (K_V) channels located in the smooth muscle (7, 16). In contrast, Orsini et al. (17) suggested that resveratrol derivates inhibited K_V channels in the F-11 neuroblastoma cells. Nagaoka et al. (18) reported that activation of big Ca^{2+}-activated K^+ (BK_{Ca}) channels in the smooth muscle of porcine retinal arterioles contributed to endothelium-independent relaxation caused by resveratrol. This discrepancy in the results obtained on the different experimental models strongly indicates tissue and species selectivity of resveratrol.

Therefore, the present study has addressed the question of whether different ion channels are involved in the endothelium-independent mechanism of vasodilatation induced by resveratrol. For that purpose, we tested the action of resveratrol on the rat mesenteric artery without endothelium.

Materials and Methods

Vascular rings were prepared from the mesenteric arteries of male Wistar rats, body weight of 250 – 300 g, essentially as described elsewhere (19). The studies reported in this work have been carried out in accordance with the European regulations on the protection of animals, the Declaration of Helsinki, and/or the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health.

Assessment of vascular function

The mesenteric arteries segments were dissected free from connective tissue. They were cut into rings (3 mm) and mounted between two stainless-steel triangles in an organ bath containing 10 ml Krebs-Ringer-bicarbonate solution (37°C, pH 7.4), aerated with 95% O_2 and 5% CO_2. One of the triangles has been attached to a displacement unit allowing fine adjustment of tension and the other was connected to an isometric transducer (K30; Hugo Sachs, Freiburg, Germany). The preparations were allowed to equilibrate for 30 min. During this period, the vessels were washed with a fresh buffer solution every 10 min.

After the equilibration period, the length-tension characteristics for each vessel were determined as described previously (20). The resting tension was 2 g (21). Vascular rings were allowed a further 30 min to equilibrate before being contracted with phenylephrine (1 μM).

Concentration–response curves were obtained by the cumulative addition of resveratrol (1 – 100 μM) to ring segments contracted to a stable plateau by adding phenylephrine. Increasing concentrations of resveratrol were added only after the previous concentration had produced an equilibrium response or after 20 min if no response was obtained. Therefore, the following protocol was used: 1) contraction to phenylephrine and concentration-response curve to resveratrol followed by three washes, addition of the different K^+ blockers, and a 20-min equilibration period; 2) contraction to phenylephrine and the concentration-response curve to resveratrol was determined.

In a separate series of experiments, vascular rings were first contracted with 1 μM phenylephrine to obtain the control contraction. The second contraction was produced by 100 mM K^+ (to annul the effect of K^+-channel activation) and then the increasing concentration of resveratrol was added. In a final set of experiments, the third contractions was produced by 100 mM K^+ in the presence of 1 μM nifedipine (to block the effect of both K^+- and Ca^{2+}-channel activation). Contractions produced by phenylephrine and 100 mM K^+ and those produced by 100 mM K^+ + nifedipine were of comparable amplitude. K^+-rich Krebs-Ringer bicarbonate solution was prepared by equimolar replacement of 100 mM NaCl with 100 mM KCl.

We have examined the effects of resveratrol onto the mesenteric arteries rings without endothelium. Endothelium was removed mechanically by rubbing with a steel wire. To assess the endothelial integrity of the preparation, we used acetylcholine (20 μM). Failure of arteries to relax to acetylcholine was considered to indicate a state of endothelial denudation (22).

Treatment of data and statistics

Relaxation produced by each concentration of resveratrol was measured and expressed as a percentage of the maximum possible relaxation (i.e., relaxation back to the baseline tension). The concentration of resveratrol producing 50% of its own response (EC_{50}) was determined for each curve by using a non-linear least square fitting procedure of individual experimental data and presented as pD_2 ($pD_2 = -\log EC_{50}$).

The results are expressed as the means ± S.E.M.; n refers to the number of experiments. All calculations

were done by using the computer program Graph Pad Prism (Graph Pad Software, Inc., San Diego, CA USA).

Drugs

The following drugs were used: *trans*-resveratrol, phenylephrine, acetylcholine, glibenclamide, charybdotoxin, 4-amynopiridine (4-AP), tetraethylammonium chloride (TEA), margatoxin, barium chloride ($BaCl_2$), potassium chloride (KCl), and nifedipine (Sigma-Aldrich, Inc., St. Louis, MO, USA). Resveratrol was dissolved in 70% v/v ethanol with further dilution in distilled water before use. Working concentrations of ethanol in the bath were <0.01% (v/v). Glibenclamide was dissolved in polyethylene glycol. Previous experiments showed that the solvents used had no effects on preparations at the concentrations applied. All drugs were added directly to the bath in a volume of 50 μL and the concentrations given are the calculated final concentrations in the bath solution.

Results

Effects of resveratrol on precontracted mesenteric artery

Resveratrol (1 – 100 μM) induced a concentration-dependent relaxation of rings without endothelium with pD_2 values of 4.99 ± 0.11 (maximal response 100 ± 5%, n = 10; Fig. 1).

Effects of potassium channel antagonists on the resveratrol-induced relaxation of mesenteric artery

Glibenclamide (10 μM, n = 10), a selective ATP-sensitive K^+ (K_{ATP}) channels inhibitor did not significantly modify the relaxation of mesenteric artery induced by resveratrol ($pD_2 = 4.99 \pm 0.5$ in the absence *vs* 4.89 ± 0.6 in the presence of glibenclamide, $P>0.05$; maximal response 100 ± 3% in the absence *vs* 93 ± 5% in the presence of glibenclamide, $P>0.05$) (data not shown).

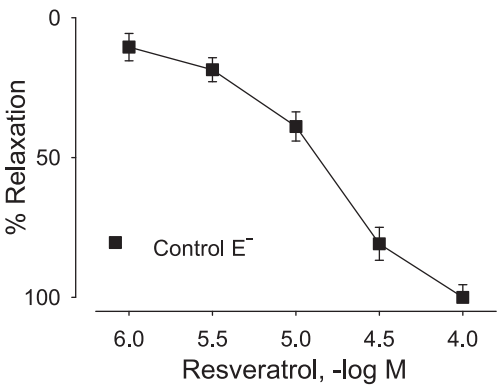

Fig. 1. Concentration–response curve for resveratrol in the rat mesenteric artery. Rings were precontracted with phenylephrine (1 μM). Endothelium was removed mechanically by rubbing with a steel wire. Responses are expressed as a percentage of the maximum possible relaxation, i.e., the return of tension to the pre-phenylephrine level. Each point represents a mean ± S.E.M. (n = 10).

TEA, a non-selective inhibitor of K^+ channels used in concentrations of 6 mM (n = 10) did not significantly modify the relaxation of the mesenteric artery induced by resveratrol ($pD_2 = 4.98 \pm 0.2$ in the absence *vs* 4.95 ± 0.1 in the presence of TEA, $P>0.05$; maximal response 100 ± 5% in the absence *vs* 100 ± 6% in the presence of TEA, $P>0.05$) (data not shown).

Charybdotoxin (10 nM, n = 10), a potent inhibitor of Ca-sensitive K^+ channels did not affect the relaxation of mesenteric artery produced by resveratrol ($pD_2 = 4.97 \pm 0.3$ in the absence *vs* 4.81 ± 0.2 in the presence of charybdotoxin, $P>0.05$; maximal response 100 ± 5% in the absence *vs* 100 ± 4% in the presence of charybdotoxin, $P>0.05$) (data not shown).

$BaCl_2$ (3 mM, n = 10), an inward rectifier K^+ (Kir)-channel antagonist, did not affect the relaxation of mesenteric artery produced by resveratrol ($pD_2 = 4.86 \pm 0.2$ in the absence *vs* 4.90 ± 0.2 in the presence of barium chloride, $P>0.05$; maximal response 100 ± 5% in the absence *vs* 100 ± 4% in the presence of barium chloride, $P>0.05$) (data not shown).

4-AP (1 mM, n = 10), a predominant blocker of K_V channels, partly inhibited resveratrol-induced relaxation of mesenteric artery ($pD_2 = 4.98 \pm 0.1$ in the absence *vs* 4.52 ± 0.3 in the presence of 4-AP, $P<0.05$; maximal response: 100 ± 2% in the absence *vs* 75 ± 2% in the presence of 4-AP, $P<0.05$) (Fig. 2A).

Margatoxin (10 nM, n = 10), a potent inhibitor of K_V1 channels, did not significantly modify the resveratrol-induced relaxation of the mesenteric artery ($pD_2 = 4.97 \pm 0.3$ in the absence *vs* 4.95 ± 0.2 in the presence of margatoxin, $P>0.05$; maximal response 100 ± 5% in the absence *vs* 97 ± 4% in the presence of margatoxin, $P>0.05$) (data not shown).

Effects of resveratrol in the presence of high K^+ and nifedipine

In the rat mesenteric artery precontracted with 100 mM K^+, relaxant response to resveratrol (1 – 100 μM) was significantly ($P<0.05$) shifted to the right (pD_2 values of 4.50 ± 0.11; maximal response 79 ± 1%, n = 10) compared to those obtained in the mesenteric artery precontracted with phenylephrine (pD_2 values of 5.00 ± 0.12; maximal response 100 ± 1%, n = 10) (Figs. 2B and 3). The difference between maximal responses was significant ($P<0.05$).

In order to minimize the influence of K^+ channels and voltage-gated Ca^{2+} channels (VGCCs) in vascular smooth muscle, the third contraction was made by 100 mM K^+ in

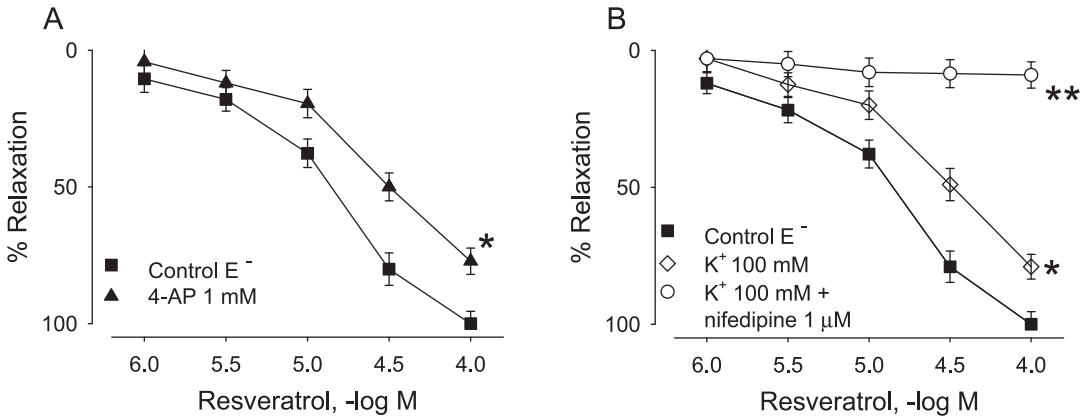

Fig. 2. Effects of resveratrol in the presence of 4-AP, high K^+, and nifedipine. A: Concentration–response curves for resveratrol were obtained in preparations precontracted with phenylephrine (1 μM) in the absence or presence of 1 mM 4-aminopyridine (4-AP). B: Concentration–response curves for resveratrol were obtained in preparations precontracted with phenylephrine (1 μM), K^+-rich (100 mM K^+) Krebs-Ringer bicarbonate solution, or K^+-rich Krebs-Ringer bicarbonate solution with addition of nifedipine (1 μM). Endothelium was removed mechanically by rubbing with a steel wire. Responses are expressed as a percentage of the maximum possible relaxation, i.e., the return of tension to the pre-phenylephrine level. Each point represents a mean ± S.E.M. (n = 10). Significance of differences: **P*<0.05, ***P*<0.01.

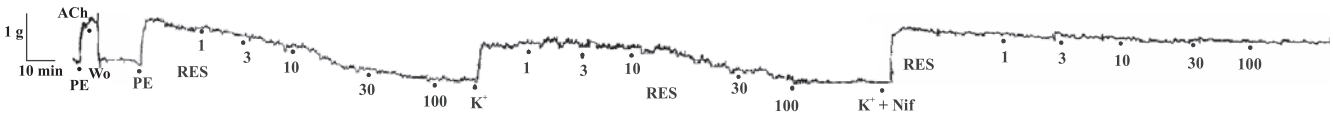

Fig. 3. Representative experiment showing relaxing activity of resveratrol (RES, μM) in rat mesenteric artery precontracted with phenylephrine (PE, 1 μM), K^+-rich (K^+, 100 mM) Krebs-Ringer bicarbonate solution, or K^+-rich Krebs-Ringer bicarbonate solution with addition of nifedipine (K^+ + Nif, 1 μM). Endothelium was removed mechanically by rubbing with a steel wire. To assess the endothelial integrity of the preparation, we have used acetylcholine (ACh, 20 μM). Failure of arteries to relax to acetylcholine was considered to indicate a state of endothelial denudation. Wo = wash preparation.

the presence of 1 μM nifedipine. Relaxant response to resveratrol (1 – 100 μM) was abolished (maximal response 9 ± 3%, n = 10) compared to those obtained in the mesenteric artery precontracted with phenylephrine (maximal response 100 ± 1%, n = 10, P<0.01; Figs. 2B and 3).

Glibenclamide (10 μM), TEA (6 mM), charybdotoxin (10 nM), $BaCl_2$ (3 mM), 4-AP (1 mM), margatoxin (10 nM), and nifedipine (1 μM) did not affect the basal tension of mesenteric artery nor the contraction induced by phenylephrine or high K^+ (data not shown, n = 5 – 7).

Discussion

Several studies suggested vasorelaxant effects of polyphenolic compounds (11, 23). Resveratrol is thought to be a prime compound of the polyphenols, causing the relaxation of human internal mammary artery, rat aorta, mesenteric and uterine artery of guinea pig, and porcine coronary artery, in which contraction had been induced by phenylephrine, noradrenaline, or KCl (5 – 7, 16, 19, 24 – 26). The mechanism of endothelium-dependent vasorelaxation is well defined by Chen and Pace-Asciak (24) who showed that resveratrol exerts an indirect vasodilatator effect on the rat aorta by a nitric oxide-mediated mechanism. However, the mechanism of endothelium-independent vasorelaxation is not completely understood.

We used different potassium channels inhibitors to determine whether the K^+ channels mediated endothelium-independent relaxation of rat mesenteric artery induced by resveratrol.

To analyze the contribution of K_{ATP} channels to the endothelium-independent resveratrol-induced relaxation of the rat mesenteric artery, glibenclamide was used (10 μM). Glibenclamide is known as one of the most selective blockers of K_{ATP} channels, although when used in a high concentration (>30 μM), it may block some other types of K^+ channels (27 – 29). In the present study, glibenclamide did not inhibit the relaxation of rat mesenteric artery induced by resveratrol. Accordingly, it seems that K_{ATP} channels are not involved in the pathway by which resveratrol produces a relaxation of the rat mesenteric artery. This result is in agreement with

the observation that glibenclamide did not antagonize the antinociceptive and vasorelaxant effect induced by resveratrol (7, 18, 30). In contrast, Wang et al. (31) have shown that resveratrol can induce negative chronotropic action and negative inotropic action by activation of K_{ATP} channels and BK_{Ca} channels in the isolated guinea-pig atrium.

To analyze the possibility that the endothelium-independent relaxation of the rat mesenteric artery, evoked by resveratrol, is mediated via BK_{Ca} channels, TEA, and charybdotoxin were tested. TEA was found to selectively block the BK_{Ca} channels in concentrations lower than 1 mM (K_d = 0.29 mM) (32), while in higher concentrations, up to 10 mM, it could block other K^+ channels as well. In our study, TEA (6 mM) at the concentration clearly sufficient to block BK_{Ca} channels did not affect the relaxation of rat mesenteric artery induced by resveratrol. Wu et al. (33) and Nagaoka et al. (18) in their studies conducted on the vascular endothelial cells and isolated porcine retinal arterioles did obtain activation of BK_{Ca} channels by resveratrol. The differences could come from tissue selectivity of the action of resveratrol. To further test the hypothesis that BK_{Ca} channels are involved in resveratrol-induced relaxation of the rat mesenteric artery, charybdotoxin was used. Originally described as a selective inhibitor of BK_{Ca} channels (34), charybdotoxin was later found to inhibit small conductance Ca^{2+}-activated K^+ and K_V channels (35). In each case, channel inhibition occurs with similar potency in the low nanomolar range (K_d approx. 0.3) (32). The concentration of charybdotoxin used in our study (10 nM) was sufficient to block BK_{Ca} channels, but did not alter relaxation of the rat mesenteric artery induced by resveratrol. It seems that charybdotoxin-sensitive channels are not involved in the mechanism of resveratrol-induced relaxation of the rat mesenteric artery. These results do not support participation of BK_{Ca} channels in the relaxation of rat mesenteric artery produced by resveratrol.

According to the lack of effect of $BaCl_2$, an inhibitor of Kir channels, it seems that those channels are probably not involved in the resveratrol-induced relaxation of rat mesenteric artery.

Two major types of K_V channel currents have been recorded in vascular smooth muscle cells, that is, the delayed rectifier outward current (IK) and transient outward K^+ current (IA). IK is sensitive to TEA and/or 4-AP, depending on the cell type studied. The whole cell patch-clamp study revealed that 4-AP–sensitive IK was the predominant K^+ conductance in smooth muscle cells from rat mesenteric arteries. The pharmacological properties of IK in rat mesenteric artery are in line with IK in other types of vascular smooth muscle cells with respect to their relative high sensitivity to 4-AP and low sensitivity to TEA (36). Using in a low milimolar concentration, 4-AP achieved some selectivity for K_V channels (37). This feature complies with the results given by our experiments, i.e., 4-AP (1 mM) partly inhibited the resveratrol-induced relaxation of rat mesenteric artery with comparable potency. Thus, our finding supports a relevant participation of K_V channels in relaxation of rat mesenteric artery produced by resveratrol. Consistent with this idea is result obtained by Granados-Soto et al. (30) who suggested that activation of K_V channels participates in the peripheral nociceptive effect of resveratrol. In contrast, Nagaoka et al. (18) have shown that resveratrol did not activate K_V channels in the isolated porcine retinal arterioles that further support its species and tissue selectivity.

A potent and selective blocker of Shaker-type (K_V1) voltage-gated K^+ channels, margatoxin, was used to determine the role of these channels in the resveratrol-induced relaxation of rat mesenteric artery. We used margatoxin in order to test which subtype of K_V channels was included in resveratrol-induced relaxation of mesenteric artery. This peptide is highly selective inhibitor of the $K_V1.1$, $K_V1.2$, and especially $K_V1.3$ channels, but displays no affinity for the mammalian BK_{Ca} channel (35, 38, 39). $K_V1.1$ and $K_V1.6$ channels were detected in smooth muscle cell of rat aorta and rat pulmonary artery, but not in smooth muscle cells of mesenteric artery (36, 40). In prior experiments, we found that margatoxin abolished resveratrol-induced relaxation of human internal mammary artery and rat aorta (7, 16). However, in the present study, margatoxin used in a concentration (10 nM) sufficient to block K_V1 channels did not change the resveratrol-induced relaxation, suggesting that K_V1 channels are not involved in the mechanism of resveratrol-induced endothelium-independent vasodilatation of rat mesenteric artery.

Our data suggest the involvement of 4-AP–sensitive K^+ channels in the relaxant effect of resveratrol, but according to relative resistance of this effect to 4-AP, it seems that resveratrol exerts endothelium-independent relaxation of the mesenteric artery by acting on multiple sites. The results of our experiments performed in K^+-rich solution also confirm the involvement of K^+ channels in the resveratrol-induced endothelium-independent relaxation of the mesenteric artery. However, the ability of resveratrol (100 μM) to almost completely (79%) relax mesenteric artery precontracted with K^+-rich solution support the notion that resveratrol, in rat mesenteric artery, acts at least in part, through a K^+ channel–independent mechanism(s). A similar K^+ channel–independent effect of resveratrol (over 30 μM)

in K^+-rich solution was obtained in human ITA and rat aorta (7, 11, 16).

Our results that nifedipine completely inhibited the K^+ channel–independent effect of resveratrol supported the thesis that VGCCs are involved in this action. This is in agreement with data obtained by other authors (41, 42). They have shown that resveratrol produced inhibition VGCCs in isolated ventricular myocytes and vascular smooth muscle cells.

In conclusion, we showed that the mechanism of action of resveratrol on vascular smooth muscle of rat mesenteric artery probably involves activation of 4-AP–sensitive K^+ channels. Its ability to almost completely relax the rat mesenteric artery precontracted with K^+-rich solution suggests that K^+ channel–independent mechanism(s) are involved in its vasorelaxant effect. It seems that interaction with VGCCs play a part in this K^+ channel–independent effect of resveratrol. However, we need additional electrophysiological experiments to further support this finding.

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