Available online at www.sciencedirect.com
SCIENCE DIRECT
Vascular Pharmacology 44 (2006) 231–237
Vascular Pharmacology
www.elsevier.com/locate/vph

Resveratrol decreases calcium sensitivity of vascular smooth muscle and enhances cytosolic calcium increase in endothelium

Mesut Buluc, Emine Demirel-Yilmaz *

Department of Pharmacology and Clinical Pharmacology, Ankara University, School of Medicine, Sihhiye, Ankara 06100, Turkey

Received 21 December 2004; received in revised form 29 March 2005; accepted 30 December 2005

Abstract

Resveratrol causes endothelium dependent and independent relaxation of vascular smooth muscle. This study investigated the mechanisms behind the effect of resveratrol on vascular tone. Resveratrol (0.1 mM) inhibited KCl-stimulated contractions in endothelium-denuded rat aorta and this inhibition was not reversed by tetraethylammonium (TEA) (5 mM), glyburide (3 μM), ouabain (0.1 mM), thapsigargin (1 μM), or indomethacin (10 μM). KCl (90 mM) increased the intracellular free calcium concentration ($[Ca^{2+}]_i$) in the isolated smooth muscle cells from the rat aorta and resveratrol (0.1 mM) did not inhibit the KCl-stimulated $[Ca^{2+}]_i$ increase. The $CaCl_2$ (0.1–100 μM) stimulated contractions were inhibited by resveratrol (0.1 mM) in the Triton X-100 skinned smooth muscle of the aorta. In heart valve endothelium, resveratrol (0.1 mM) augmented the acetylcholine (10 μM) stimulated $[Ca^{2+}]_i$ increase. Resveratrol-induced augmentation of the acetylcholine-stimulated $[Ca^{2+}]_i$ elevation was reversed by glyburide (3 μM), but not by TEA (5 mM). The present study indicated that resveratrol affected vascular smooth muscle and endothelium in different ways. Resveratrol decreased the Ca^{2+} sensitivity but did not affect the KCl-stimulated $[Ca^{2+}]_i$ increase in the vascular smooth muscle. In the endothelial cells, resveratrol enhanced the agonist-stimulated $[Ca^{2+}]_i$ increase that might trigger nitric oxide synthesis from endothelial cells.

Keywords: Resveratrol; Vascular smooth muscle; Endothelium; $[Ca^{2+}]_i$; Ca^{2+} sensitivity

1. Introduction

Moderate wine consumption has been linked to a lower incidence of cardiovascular disease; this was described as the "French Paradox" (German, 2000). Natural compounds have been invoked to explain the beneficial effects of moderate wine consumption on coronary heart disease (Hertog et al., 1993). Resveratrol, a natural phytoalexin produced in grapes, is present in wine together with other polyphenols (Soleas et al., 1997). Resveratrol has been found to have several pharmacological effects, including anti-inflammatory, anti-platelet, anti-oxidant, anti-fungal, and anti-cancer properties (Fremont, 2000). Moreover, it is able to cause relaxation of vascular smooth muscle. It had been reported that resveratrol induced relaxation in several vascular beds of the rat, pig, guinea pig and sheep by acting in endothelium dependent and independent ways (Chen and Pace-Asciak, 1996; El-Mowafy, 2002; Jager and Nguyen-Duong, 1999; Naderali et al., 2000). In addition, our previous study showed that resveratrol potentiated acetylcholine-stimulated endothelium-dependent relaxation and inhibited the vessel contractions stimulated by some contractile agents in the rat thoracic aorta (Buluc and Demirel-Yilmaz, 2002). However, the mechanism of the resveratrol effects on blood vessel tone has not been investigated. Therefore, the aim of the present study was to identify the mechanisms behind the effect of resveratrol on vascular smooth muscle cells and endothelium.

2. Methods and materials

2.1. Preparation of tissue and measurement of contractility

Wistar albino rats, weighing 150–250 g, were anesthetized with an intraperitoneal injection of sodium pentothal 35 mg/kg. The thoracic aorta was removed and freed of fat and connective tissue. After being cut into 2–3 mm rings, the endothelium was removed with cotton thread. All rings were suspended in an organ bath containing oxygenated (5% CO_2 in 95% O_2) and

* Corresponding author. Tel./fax: +90 312 310 6268.
E-mail address: dyilmaz@medicine.ankara.edu.tr (E. Demirel-Yilmaz).

1537-1891/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.vph.2005.12.003

warmed (37 °C) Krebs solution. The solution contained (in mM) 112 NaCl, 5 KCl, 1.2 NaH_2PO_4, 11.5 dextrose, 0.5 $MgCl_2$, 2.5 $CaCl_2$, and 25 $NaHCO_3$ (pH 7.4). Isometric contractions of the aorta rings were measured using force-displacement transducers (Grass FT.03), and were recorded on a Grass polygraph (model 79D) under 2 g of initial tension. The rings were allowed to equilibrate for 45 min before the initiation of the study. During the resting periods, the bath solution was replaced every 10 min. The absence of endothelium was verified before the experiment by acetylcholine (1 μM) stimulated relaxation in precontracted (phenylephrine 0.1 μM) rings. After a 45 min resting period, cumulative concentration response curves to KCl were determined in all preparations by the stepwise addition of the drug to the bathing medium, which were then washed with Krebs solution and left for 45 min. Then resveratrol was added to the medium for 15 min and the response to KCl was determined again. In another series of experiments, second KCl responses were recorded in the presence of resveratrol plus some other drugs. Contraction was plotted as a percentage of the maximum contraction of the KCl in the first concentration response curve for each ring.

2.2. Isolation of vascular smooth muscle cells

Smooth muscle cells were isolated from a 2–3 cm segment of thoracic aorta. After the endothelium and adventisia were removed, the tissue was cut into 1 mm strips and placed in calcium free physiological salt solution (PSS) containing (in mM) 120 NaCl, 5.9 KCl, 11.5 dextrose, 10 HEPES, 1.4 $MgCl_2$, and 0.2 EGTA, at pH 7.4. The strips were then incubated at 37 °C in calcium free PSS containing 8 mg/ml of papain, 0.5 mg/ml of dithiothreitol, and 1 mg/ml of bovine serum albumin for 30 min with gentle shaking in a water bath. After that the strips were incubated in calcium free PSS containing 2 mg/ml of collagenase, 1 mg/ml of elastase, and 1 mg/ml of trypsin inhibitor for 30 min under the same conditions. At the end of the incubation period, tissues were placed in fresh calcium free PSS and gently triturated by a fire-polished Pasteur pipette. Undispersed pieces of tissue were removed by nylon mesh filtration (mesh opening 100 μM) and the solution containing single cells was centrifuged at 450 rev min^{-1} for 5 min. The supernatant was discarded and smooth muscle cells were suspended in normal PSS (0.2 mM EGTA replaced with 1 mM $CaCl_2$) containing 1 μM membrane-permeant fura 2 acetoxymethyl ester (Fura 2-AM; 1 mM stock in dimethyl sulfoxide (DMSO)). Smooth muscle cells were placed on glass coverslips and loaded for 60 min at room temperature in the dark. After the incubation period, cells were transferred into PSS and then a tissue chamber and placed on the stage of an inverted microscope (Leica DM IRB).

2.3. Isolation of the heart valve

Isolation and intracellular free calcium concentration ($[Ca^{2+}]_i$) measurement in intact endothelial cells from the heart valve were described previously (Laskey et al., 1994). The rat heart was rapidly excised and placed in PSS. The apex of the ventricle and the right and left atria were removed. The mitral valve was dissected free and kept in PSS for fluorescent dye loading. The valve preparation was loaded with 2 μM Fura 2-AM in PSS for 60 min in the dark at room temperature. Then tissue was transferred to PSS and pinned in a specially designed tissue chamber. The chamber was placed on the stage of the inverted microscope (Leica DM IRB).

2.4. Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ of isolated vascular smooth muscle cells from rat aorta and heart valve endothelium was measured using a microscope-based fluorimeter (Photon Technology International (PTI), Ratio Master). The cells were excited alternately at 340- and 380-nm wavelengths of ultraviolet light. The emitting fluorescence was collected by photomultiplier tube (PTI, 184) at 510 nm. The fluorescence signal was recorded as digital data using a computer program designed by PTI (Felix). The autofluorescence of unloaded cells and tissue was minimal, and background signals were obtained from a region of the chamber away from valve and smooth muscle cells. Pairs of fluorescence signal ratios at 340- and 380-nm excitation wavelengths were collected every 1 s and then the background was subtracted. The ratio of the two intensities at 340- and 380-nm excitation (F_{340}/F_{380}) was reported as a relative measure of the $[Ca^{2+}]_i$. No calibration was attempted because of the uncertainty of the conventional calibration method in living cells. Cells were stimulated by injection of drugs in the tissue chamber using a standard pipette. Cell responses were recorded at room temperature in the absence and presence of resveratrol (0.1 mM) using separate preparations.

2.5. Skinning of vascular tissue

A skinning procedure described by Van Heijst et al. (1999) was modified and adapted to our system. The rat thoracic aorta was rapidly removed and placed in Krebs solution. After removing the fat and connective tissue, the thoracic aorta was cut into 2–3 mm rings and denuded of endothelial cells. The aorta rings were mounted in organ baths containing Krebs buffer maintained at 37 °C continuously bubbled with 95% O_2–5% CO_2 at pH 7.4. The absence of endothelium was verified before the experiment by acetylcholine (1 μM) relaxation in precontracted (phenylephrine 0.1 μM) rings. After a 45 min resting period, response to KCl (40 mM) was determined in all preparations by the addition of the drug to the bathing medium, which were then washed with Krebs solution and left for 45 min. Then the aorta rings were incubated in a relaxing solution containing (in mM) 10 MOPS, 1 EGTA, 5 $MgCl_2$, and 160 KCl, at pH 7.0 for 15 min. The skinning of the smooth muscle preparations was achieved by incubation with 1% Triton X-100 in a relaxing solution at 22 °C for 30 min. Triton X-100 is a detergent and forms small channels of consistent size that facilitate ion permeation through the cell membrane. After the skinning, the solution was changed to relaxing solution and then the rings were incubated in a contracting solution containing (in mM) 10 MOPS, 1 EGTA, 2 $MgCl_2$, 160 KCl, 3 Mg-ATP, and 10 creatine phosphate (pH 7.0) for 30 min. Cumulative concentration response curves to $CaCl_2$ (0.1–100 μM) were

recorded by stepwise addition of the drug to the bathing medium. Free Ca^{2+} concentration in the medium was calculated with a computer program (Fabiato, 1988). The contractile responses were obtained in the absence and presence of resveratrol in different rings. Contraction was plotted as a percentage of the contraction of the KCl (40 mM) for each ring.

2.6. Chemicals

Analytic grades of reagents for all solutions were obtained from Sigma (St. Louis, MO, USA). Fura 2-AM was from Molecular Probes (Eugene, OR, USA). Resveratrol was a kind gift from Pharmascience (Montreal, Canada). Thapsigargin was provided by Alomone Labs (Jerusalem, Israel).

2.7. Statistics

Values are expressed as Mean±SEM. Statistic analysis was performed with the Student's *t*-test. In the skinning experiments, the repeated-measures ANOVA was used to test the contraction differences among $CaCl_2$ measurements and to compare the two groups in terms of contraction level. In the $[Ca^{2+}]_i$ measurements, differences among the groups for response of acetylcholine were evaluated by Kruskal–Wallis variance analysis. When the P value from the Kruskal–Wallis test is statistically significant, multiple comparison test was used to know which groups differ from which others. Values were considered significantly different when $p<0.05$.

3. Results

3.1. Resveratrol inhibits KCl-stimulated contractions

In our previous study, KCl-stimulated contractions of the rat thoracic aorta were attenuated by resveratrol in high concentration (0.1 mM) and there was no significant change in contractions when the denuded aortic rings were subjected to lower concentrations of drug (1 and 10 μM) (Buluc and Demirel-Yilmaz, 2002). The mechanism of resveratrol-induced inhibition was investigated using blockers of some possible target proteins in the present study. In the presence of resveratrol (0.1 mM), the KCl-induced maximum contraction of the endothelium denuded aortic rings was significantly inhibited (37.13±3.72% of the control) ($p<0.01$) (Fig. 1A).

The inhibitory effect of resveratrol on KCl-stimulated contractions was not reversed by tetraethylammonium (TEA) (5 mM), glyburide (3 μM), ouabain (0.1 mM), thapsigargin (1 μM), or indomethacin (10 μM) which are blockers of the calcium dependent K channels (K_{Ca}) and ATP sensitive K channels (K_{ATP}), and inhibitors of the Na-K-ATPase, Ca-ATPase and cyclooxygenase, respectively (Fig. 1A, $n=5–12$).

3.2. Resveratrol has no effect on KCl-stimulated $[Ca^{2+}]_i$ increases in vascular smooth muscle cells

Isolated vascular smooth muscle cells from the rat aorta responded to the application of 90 mM KCl with a rise in $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ was determined as a ratio elevation for all experiments at the peak of the spike recorded from each cell group. The effect of resveratrol on the KCl-stimulated $[Ca^{2+}]_i$ increase was examined by incubation of another group of cells with the drug for 10 min before KCl stimulation. The mean peak increase in the ratio stimulated by KCl was not significantly different: in the absence and presence of resveratrol (0.1 mM), Δ ratio was 0.406±0.079 and 0.509±0.084, respectively (Fig. 1B, $n=12$).

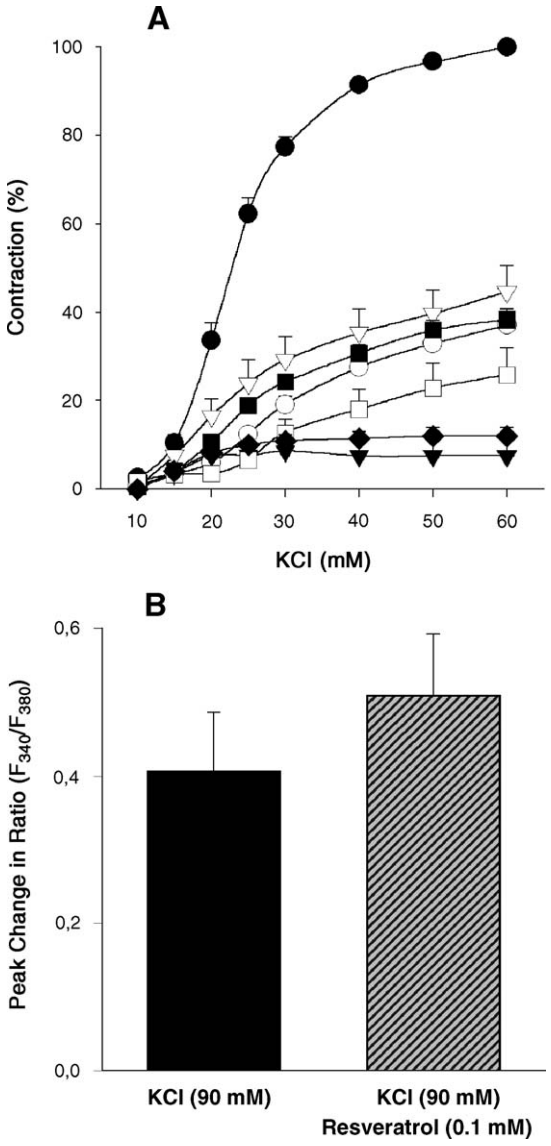

Fig. 1. Effect of resveratrol on KCl-stimulated contractions and $[Ca^{2+}]_i$ elevation in vascular smooth muscle. (A) The resveratrol-induced inhibition of KCl-stimulated contractions was not reversed by TEA, glyburide, ouabain, thapsigargin and indomethacin in endothelium-denuded rat aorta. (●) Control, in the presence of (○) resveratrol (0.1 mM), and resveratrol plus (▼) TEA (5 mM), (▽) glyburide (3 μM), (■) ouabain (0.1 mM), (♦) thapsigargin (1 μM), (□) indomethacin (10 μM) ($n=5–12$). (B) Resveratrol did not significantly affect the KCl-stimulated $[Ca^{2+}]_i$ increase in the isolated vascular smooth muscle cells. Histograms show the mean values of Δ increase in ratio stimulated by KCl (90 mM) without and with resveratrol (0.1 mM) ($n=12$). The values are given as mean±SEM.

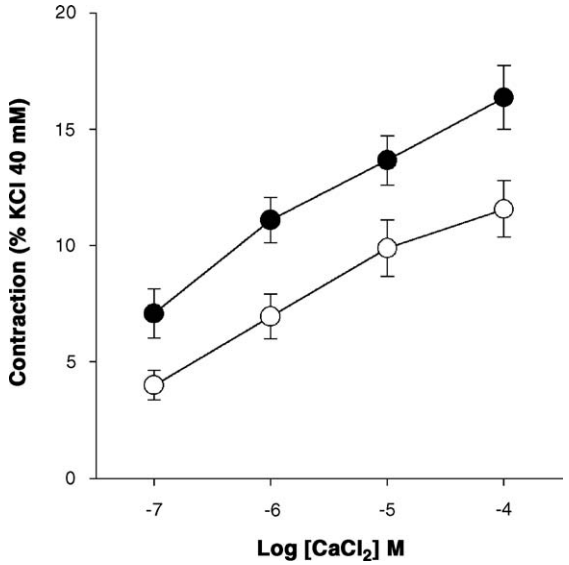

Fig. 2. Inhibitory effect of resveratrol on $CaCl_2$-stimulated contractions in skinned rat aorta. Contractions induced by $CaCl_2$ (0.1–100 μM) are significantly inhibited by resveratrol (0.1 mM) ($p<0.05$). (●) Control, (○) in the presence of resveratrol. Values are expressed as mean±SEM ($n=15–20$).

3.3. Ca^{2+} sensitivity of vascular smooth muscles is decreased by resveratrol

We postulated that the distal step of excitation–contraction coupling in vascular smooth muscle cells was influenced by resveratrol and investigated the Ca^{2+} sensitivity of smooth muscle. In Triton X-100 skinned smooth muscle from the rat thoracic aorta, $CaCl_2$ (0.1–100 μM) stimulated contractions. These contractions were significantly decreased by resveratrol (0.1 mM) (16.36±1.37 and 11.58±1.22, % maximum contraction in the absence and presence of resveratrol, respectively, $n=15–20$) ($p<0.05$) (Fig. 2).

3.4. Resveratrol enhances acetylcholine-stimulated $[Ca^{2+}]_i$ increases in heart valve endothelium

It has been previously reported that endothelium-dependent inhibition of vessel tone by resveratrol or red wine polyphenols occurs due to increased production/release of nitric oxide (NO) from endothelial cells (Chen and Pace-Asciak, 1996; Fitzpatrick et al., 1993; Naderali et al., 2000). NO is synthesized from the L-arginine by the NO-synthase (NOS) (Stuehr, 1997). The endothelial isoform of NOS (eNOS) is activated by $[Ca^{2+}]_i$ increase (Long and Stone, 1985; Rubanyi and Vanhoutte, 1988; Schmidt et al., 1992). In the present study, the effect of resveratrol on the endothelial $[Ca^{2+}]_i$ increase was researched in isolated valve endothelium from the rat heart, which contained intact endothelial cells on both surfaces. In the valve endothelium, acetylcholine (10 μM) stimulated a mean peak increase in ratio (Δ ratio=0.050±0.008, $n=6$). In the presence of resveratrol (0.1 mM), the $[Ca^{2+}]_i$ increase stimulated by acetylcholine was significantly enhanced (Δ ratio=0.103±0.016, $n=10$) ($p<0.05$) (Fig. 3A).

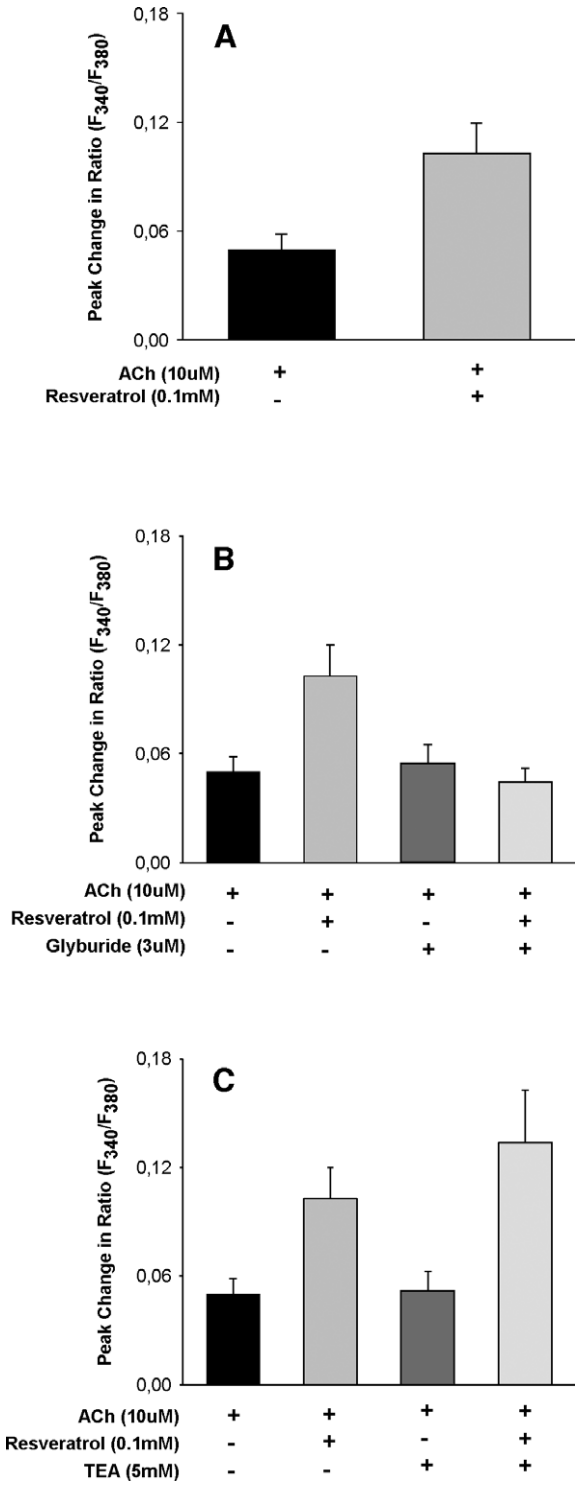

Fig. 3. Resveratrol-induced enhancement of acetylcholine-stimulated $[Ca^{2+}]_i$ elevation in the heart valve endothelium. (A) The acetylcholine-stimulated increase in the ratio was significantly enhanced by resveratrol ($p<0.05$). (B) Glyburide significantly blocked resveratrol-induced potentiation ($p<0.05$). (C) TEA did not significantly change the resveratrol-induced potentiation of the acetylcholine-stimulated increase in the ratio. Histograms show that the mean values of Δ increase in ratio (A) stimulated by acetylcholine (10 μM) alone and after incubation with resveratrol (0.1 mM) or (B) resveratrol (0.1 mM) plus glyburide (3 μM) and (C) resveratrol (0.1 mM) plus TEA (5 mM). The values are given as mean±SEM ($n=6–10$).

3.5. Resveratrol-induced enhancement of $[Ca^{2+}]_i$ increase is dependent on K_{ATP} channels activation

In the endothelial cells, $[Ca^{2+}]_i$ increase is mainly regulated by membrane potential, which is controlled by potassium channels activity. To investigate whether the resveratrol-induced enhancement of the acetylcholine-stimulated $[Ca^{2+}]_i$ increase was mediated by potassium channels activation, potassium channel blockers were used. The resveratrol-induced enhancement of the acetylcholine-stimulated $[Ca^{2+}]_i$ increase was blocked by glyburide (3 μM), which is a K_{ATP} channel blocker, but glyburide alone did not affect acetylcholine response (0.045 ± 0.007 and 0.055 ± 0.01, Δ ratio in the presence of resveratrol plus glyburide and only glyburide, respectively, $n = 6$–7; Fig. 3B). On the other hand, acetylcholine-stimulated $[Ca^{2+}]_i$ increase and resveratrol-induced augmentation were not significantly affected by TEA (5 mM), which is a K_{Ca} channels blocker (0.134 ± 0.029 and 0.052 ± 0.01, Δ ratio in the presence of resveratrol plus TEA and only TEA, respectively, $n = 6$; Fig. 3C).

4. Discussion

The present study indicated that the natural phytoallexin resveratrol decreases vascular tone by influencing two main types of cell in the vessel wall, i.e. endothelial cells and smooth muscle cells. Resveratrol decreases the calcium sensitivity of myofilaments in vascular smooth muscle and enhances agonist-stimulated $[Ca^{2+}]_i$ increase in endothelium.

Several studies have shown that vascular relaxation was achieved with resveratrol in several vascular beds. Resveratrol-induced vessel relaxation was documented in the rat aorta, porcine coronary arteries, guinea-pig mesenteric and uterine arteries and sheep coronary arteries (Chen and Pace-Asciak, 1996; El-Mowafy, 2002; Jager and Nguyen-Duong, 1999; Naderali et al., 2000). These studies indicated that resveratrol exerts both direct and indirect vasodilator effects on the blood vessel by endothelium-independent (non-NO-mediated) and endothelium-dependent (NO-mediated) mechanisms, respectively.

No possible mechanism of resveratrol-induced endothelium-independent (non-NO-mediated) relaxation of vascular smooth muscle has yet been completely explained. Our previous study showed that the vessel contractions stimulated by KCl were inhibited by resveratrol in endothelium denuded rat aorta (Buluc and Demirel-Yilmaz, 2002). In the present study, we examined the mechanism underlying the endothelium-independent effect of resveratrol on vascular smooth muscle and tested the modulation of possible target molecules that cause attenuation of smooth muscle tone. In endothelium denuded aortic rings, resveratrol-induced inhibition of the KCl-stimulated contractions was not reversed by TEA, glyburide, ouabain, thapsigargin or indomethacin, which are blockers of the K_{Ca} and K_{ATP} channels, and inhibitors of the Na-K-ATPase, Ca-ATPase and cyclooxygenase, respectively. None of these cellular processes played a part in the endothelium-independent attenuation of vascular smooth muscle contraction by resveratrol.

KCl-induced depolarization of the smooth muscle cell membrane activates voltage gated Ca^{2+} channels, influx of Ca^{2+} and then $[Ca^{2+}]_i$ increase (Bolton, 1979; Somlyo and Somlyo, 1968; Van Breemen et al., 1986). Resveratrol-induced inhibition of Ca^{2+} influx has been shown in thrombin-stimulated platelets (Dobrydneva et al., 1999). We hypothesized that resveratrol would inhibit KCl-stimulated $[Ca^{2+}]_i$ increase and examined $[Ca^{2+}]_i$ in the vascular smooth muscle cells isolated from the rat aorta. However, resveratrol did not decrease the KCl-stimulated $[Ca^{2+}]_i$ increase in the isolated vascular smooth muscle cells in the present study. Thus it is postulated that the $[Ca^{2+}]_i$ increase was not influenced by resveratrol in the vascular smooth muscle cells from rat aorta and the distal step of excitation–contraction coupling was responsible for the reduction of the contraction.

Although the contraction of the smooth muscle cell is primarily regulated by the $[Ca^{2+}]_i$ increase, different levels of tension may occur at the same $[Ca^{2+}]_i$ in muscle cells. It has been shown that $[Ca^{2+}]_i$ and force are not invariably tightly coupled (Somlyo and Somlyo, 1994); this phenomenon is called "Ca^{2+} sensitivity" (Karaki et al., 1988; Morgan and Morgan, 1984; Rembold and Murphy, 1988). Furthermore, it has been shown that KCl-induced Ca^{2+} sensitization was obtained in the vascular smooth muscle (Mita et al., 2002; Sakurada et al., 2003). In our study, the Ca^{2+} sensitivity was examined using skinned vascular smooth muscle preparations. We defined the effect of exogenously added $CaCl_2$ on the contraction using isolated Triton X-100-skinned smooth muscle from the rat thoracic aorta. Resveratrol inhibited $CaCl_2$ stimulated contraction in skinned vascular smooth muscle in the present study. All these results indicated that attenuation of vascular smooth muscle contraction and endothelium-independent relaxation induced by resveratrol depend on reduced Ca^{2+} sensitivity of muscles fibers. On the other hand, it has been reported that Ca^{2+} sensitivity of vascular smooth muscle was inhibited by cGMP/cGMP-dependent protein kinase (Sauzeau et al., 2000) and resveratrol increased cGMP by activation of particulate guanylyl cyclase (El-Mowafy, 2002). Thus, resveratrol-induced cGMP increase may be responsible for the reduction of Ca^{2+} sensitivity. However, further studies are needed to identify the exact target molecule of resveratrol in the vascular smooth muscle cell.

On the other hand, endothelium-dependent (NO mediated) vascular smooth muscle relaxation was also induced by resveratrol (Chen and Pace-Asciak, 1996; Naderali et al., 2000). NO is the most popular factor derived from vascular endothelium. Furchgott and Zawadzki (1980) first showed that endothelial cells play a pivotal role in relaxation stimulated by acetylcholine in the rabbit aorta due to the release of a factor called EDRF, and it has since been demonstrated that NO accounts for the most of biological properties of EDRF (Ignarro et al., 1987; Palmer et al., 1987). NO is a signaling molecule in blood vessels, where a continuous formation from endothelial cells acts on the underlying smooth muscle to maintain vasodilatation and blood flow (Moncada et al., 1991). Our previous study demonstrated that resveratrol potentiated acetylcholine-stimulated endothelium-dependent relaxation but not sodium nitroprusside-stimulated endothelium-independent relaxation

(Buluc and Demirel-Yilmaz, 2002). In that study, it was postulated that endothelium-dependent inhibition of vessel tone by resveratrol likely occurs due to increased production and/or release of NO from the endothelial layer of the vessel wall. NO is generated from the L-arginine–citrulline pathway by the enzyme nitric oxide synthase (NOS) (Stuehr, 1997). The endothelial isoform of NOS is constitutive and regulated in a $[Ca^{2+}]_i$ dependent manner (Long and Stone, 1985; Rubanyi and Vanhoutte, 1988; Schmidt et al., 1992). In our study we defined the effect of resveratrol on $[Ca^{2+}]_i$ in endothelial cells, using isolated heart valve endothelium. The acetylcholine-stimulated $[Ca^{2+}]_i$ increase was enhanced by resveratrol. Therefore, we hypothesize that resveratrol-induced potentiation of the $[Ca^{2+}]_i$ increase would modulate NOS activation and then NO release from endothelial cells. Our conclusion is supported by other research, which also reported that resveratrol including red wine extracts increased $[Ca^{2+}]_i$ in bovine aortic endothelial cells and caused NO release (Martin et al., 2002).

Agonist-stimulated release of NO from endothelial cells is reduced in high K^+ solution and is not inhibited by Ca^{2+} channel blockers, which suggests that voltage-dependent channels are not involved in agonist-stimulated NO secretion in vascular endothelium (Jayakody et al., 1987; Takeda et al., 1987). Although the ion channels that allow the influx of Ca^{2+} into the endothelial cells are voltage independent, E_m nonetheless plays an important role in regulating Ca^{2+} entry (Adams et al., 1989; Busse et al., 1988). E_m determines the electrochemical gradient ($E_m - E_{Ca}$) that provides the driving force for Ca^{2+} influx. It is important therefore to consider the ion channels that modulate the E_m of the vascular endothelial cells, as they can influence NO release by regulating Ca^{2+} flux (Demirel et al., 1994; Schilling, 1989). Therefore, K^+ channels play the most important role in the regulation of the endothelial membrane potential and Ca^{2+} entry. In this study, resveratrol-induced potentiation of the acetylcholine-stimulated $[Ca^{2+}]_i$ increase in valve endothelium was completely reversed by the K_{ATP} channels blocker glyburide, whereas the K_{Ca} channels blocker TEA did not affect this augmentation. This result suggested that resveratrol-induced potentiation of the $[Ca^{2+}]_i$ increase in endothelial cells was associated with the activation of glyburide-sensitive K^+ channels. However, K_{Ca} channels activity induced by resveratrol in the endothelial cell line (HUV-EC-C) was reported (Li et al., 2000). This diverse effect of resveratrol is probably the result of different endothelial cell preparations.

The results of this study indicate that in the vascular wall resveratrol affects endothelial and vascular smooth muscle cells in different ways. Endothelium-dependent attenuation of vessel tone by resveratrol was due to an increase in $[Ca^{2+}]_i$ in endothelial cells. Direct inhibition of the vascular smooth muscle contraction via a decrease in the Ca^{2+} sensitivity of the contractile apparatus is the endothelium-independent part of resveratrol action.

Acknowledgements

This study was supported by a research grant (2001-08-09-070) from the Ankara University Research Foundation. The authors are very grateful to Pharmascience for the generous gift of resveratrol and Alomone Labs for free sample of thapsigargin. We are also grateful to Dr. Atilla Elhan for the statistical analyses and Russell Fraser for the editing of the English.

References

Adams, D.J., Barakeh, S., Laskey, R., van Breemen, C., 1989. Ion channels and regulation of intracellular calcium in vascular endothelial cells. FASEB J. 3, 2389–2400.

Bolton, T.B., 1979. Mechanisms of action of transmitters and other substances on smooth muscle. Physiol. Rev. 59, 606–718.

Buluc, M., Demirel-Yilmaz, E., 2002. Possible mechanism for depression of smooth muscle tone by resveratrol. In: Varro, A., Vegh, A. (Eds.), Adv. Rec. Cardiovas. Res. Monduzzi Editore, Bologna Italy, pp. 55–59.

Busse, R., Fichtner, H., Lückhoff, A., Kohlhardt, M., 1988. Hyperpolarization increased free calcium on acetylcholine stimulated endothelial cells. Am. J. Physiol. 255, H965–H969.

Chen, C.K., Pace-Asciak, C.R., 1996. Vasorelaxing activity of resveratrol and quercetin in isolated rat aorta. Gen. Pharmacol. 27, 363–366.

Demirel, E., Rusko, J., Laskey, L.E., Adams, D.J., Van Breemen, C., 1994. TEA inhibits ACh-induced EDRF release: endothelial Ca^{2+} dependent K^+ channels contribute to vascular tone. Am. J. Pharmacol. 36, H1135–H1141.

Dobrydneva, Y., Williams, R.L., Backmore, P.F., 1999. Trans-resveratrol inhibits calcium influx in thrombin-stimulated human platelet. Br. J. Pharmacol. 128, 149–157.

El-Mowafy, A.M., 2002. Resveratrol activates membrane-bound guanylyl cyclase in coronary arterial smooth muscle: a novel signaling mechanism in support of coronary protection. Biochem. Biophys. Res. Commun. 291, 1218–1224.

Fabiato, A., 1988. Computer programs for calculating total from specified free or free from specified total ionic concentrations in aqueous solutions containing multiple metals and ligands. Methods Enzymol. 157, 378–417.

Fitzpatrick, D.F., Hirschfield, S.L., Coffey, R.G., 1993. Endothelium-dependent vasorelaxing activity of wine and other grape products. Am. J. Physiol. 265, H774–H778.

Fremont, L., 2000. Biological effects of resveratrol. Life Sci. 66, 663–673.

Furchgott, R.F., Zawadzki, J.V., 1980. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288, 373–376.

German, J.B., 2000. The health benefits of wine. Annu. Rev. Nutr. 20, 561–593.

Hertog, M.G.L., Feskens, E.J.M., Hollman, P.C.H., Katan, M.B., Kromhout, D., 1993. Dietary antioxidant flavonoids and risk of coronary heart disease. The Zutphen Elderly Study. Lancet 342, 1007–1011.

Ignarro, L.J., Buga, G.M., Wood, K.S., Byrns, R.E., Chaudhuri, G., 1987. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc. Natl. Acad. Sci. U. S. A. 84, 9265–9269.

Jager, U., Nguyen-Duong, H., 1999. Relaxant effect of trans-resveratrol on isolated porcine coronary arteries. Arzneim.-Forsch. 49, 207–211.

Jayakody, R.L., Kappagoda, S., Senaratne, M.P., Sreeharan, N., 1987. Absence of effect of calcium antagonists on endothelium dependent relaxation in rabbit aorta. Br. J. Pharmacol. 91, 155–164.

Karaki, H., Sato, K., Ozaki, H., 1988. Different effects of norepinephrine and KCl on the cytosolic Ca^{2+}-tension relationship in vascular smooth muscle of rat aorta. Eur. J. Pharmacol. 151, 325–328.

Laskey, R.E., Adams, D.J., van Breemen, C., 1994. Cytosolic $[Ca^{2+}]_i$ measurements in endothelium of rabbit cardiac valves using imaging fluorescence microscopy. Am. J. Physiol. 266, H2130–H2135.

Li, H.F., Chen, S.A., Wu, S.N., 2000. Evidence for the stimulatory effect of resveratrol on Ca^{2+}-activated K^+ current in vascular endothelial cells. Cardiovas. Res. 45, 1035–1045.

Long, C.J., Stone, T.W., 1985. The release of endothelium-derived relaxant factor is calcium dependent. Blood Vessels 22, 205–208.

Martin, S., Andriambeloson, E., Takeda, K., Andriantsitohaina, R., 2002. Red wine polyphenols increase calcium in bovine aortic endothelial cells: a basis to elucidate signaling pathways leading to nitric oxide production. Br. J. Pharmacol. 135, 1579–1587.

Mita, M., Yanagihara, H., Hishinuma, S., Saito, M., Walsh, M.P., 2002. Membrane depolarization-induced contraction of rat caudal arterial smooth muscle involves Rho-associated kinase. Biochem. J. 364, 431–440.

Moncada, S., Palmer, R.J.M., Higgs, E.A., 1991. Nitric oxide: physiology pathophysiology and pharmacology. Pharmacol. Rev. 43, 109–142.

Morgan, J.P., Morgan, K.G., 1984. Stimulus-specific patterns of intracellular calcium levels in smooth muscle of ferret portal vein. J. Physiol. 351, 155–167.

Naderali, E.K., Doyle, P.J., Williams, G., 2000. Resveratrol induces vasorelaxation of mesenteric and uterine arteries from female guinea pigs. Clin. Sci. 98, 537–543.

Palmer, R.M.J., Ferridge, A.G., Moncada, S., 1987. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 327, 524–526.

Rembold, C.M., Murphy, R.A., 1988. Myoplasmic $[Ca^{2+}]_i$ determines myosin phosphorylation in agonist-stimulated swine arterial smooth muscle. Circ. Res. 63, 593–603.

Rubanyi, G.M., Vanhoutte, P.M., 1988. Calcium and activation of the release of endothelium-derived relaxing factor. Ann. N. Y. Acad. Sci. 522, 226–233.

Sakurada, S., Takuwa, W., Sugimoto, N., Wang, Y., Seto, M., Sasaki, Y., Takuwa, Y., 2003. Ca^{2+}-dependent activation of Rho and Rho kinase in membrane depolarization-induced and receptor stimulation-induced vascular smooth muscle contraction. Circ. Res. 93, 548–556.

Sauzeau, V., Le Jeune, H., Cario-Toumaniantz, C., Smolenski, A., Lohmann, S.M., Bertoglio, J., Chardin, P., Pacaud, P., Loirand, G., 2000. Cyclic GMP-dependent protein kinase signaling pathway inhibits RhoA-induced Ca^{2+} sensitization of contraction in vascular smooth muscle. J. Biol. Chem. 275, 21722–21729.

Schilling, W.P., 1989. Effect of membrane potential on cytosolic calcium of bovine aortic endothelial cells. Am. J. Physiol. 257, H778–H784.

Schmidt, H.H.H.W., Pollock, J.S., Nakane, M., Försterman, U., Murad, F., 1992. Ca^{2+}/calmodulin-regulated nitric oxide synthases. Cell Calcium 13, 427–434.

Soleas, G.J., Diamandis, E., Goldberg, D.M., 1997. Resveratrol: a molecule whose time has come? And gone? Clin. Biochem. 30, 91–113.

Somlyo, A.V., Somlyo, A.P., 1968. Electromechanical and pharmacomechanical coupling in vascular smooth muscle. J. Pharmacol. Exp. Ther. 159, 129–145.

Somlyo, A.V., Somlyo, A.P., 1994. Signal transduction and regulation in smooth muscle. Nature 372, 231–236.

Stuehr, D.J., 1997. Structure-function aspects in the nitric oxide synthases. Annu. Rev. Pharmacol. Toxicol. 37, 339–359.

Takeda, K., Schini, V., Stoeckel, H., 1987. Voltage-activated potassium but not calcium currents in cultured bovine aortic endothelial cells. Pflugers Arch. 410, 385–393.

Van Breemen, C., Cauvin, C., Johns, A., Leijten, P., Yamamoto, H., 1986. Ca^{2+} regulation of vascular smooth muscle. Fed. Proc. 45, 2746–2751.

Van Heijst, B.G.V., De Wit, E., vander Heide, U.A., Blange, T., Jongsma, H.J., de Beer, E.L., 1999. The effect of length on the sensitivity to phenylephrine and calcium in intact and skinned vascular smooth muscle. J. Muscle Res. Cell Motil. 20, 11–18.