ENDOTHELIUM -DEPENDENT RELAXATION OF INTERNAL MAMMARY ARTERY PRODUCED BY RESVERATROL

NOVAKOVIĆ ALEKSANDRA*, BUKARICA GOJKOVIĆ LJILJANA**, PERIĆ M***, NEŽIĆ D***, ĐUKANOVIĆ B***, LEŠIĆ A***, BUMBAŠIREVIĆ M****, and MARKOVIĆ-LIPKOVSKI JASMINA*

*School of Pharmacy, Belgrade; **School of Medicine, Belgrade;
***Institute of Cardiovascular Diseases "Dedinje", Belgrade;
****Institute of Orthopedics and Traumatology, Clinical Center of Serbia, Belgrade

Resveratrol, a polyphenol present in wine, has been thought to be responsible for cardiovascular benefits associated with moderate wine consuption. It is also present in the plant Polygonum Cuspidatum. The mechanism of cardiovascular benefits probably includes vaso-relaxation, antioxidant and anti-platelet effects of resveratrol. The mechanisms by which resveratrol causes vasodilatation are uncertain. The aim of this study was to investigate the mechanism(s) of resveratrol-induced vasorelaxation in human internal mammary artery (HIMA) with endothelium. HIMA rings were precontracted by phenylephrine. Resveratrol induced relaxation of the HIMA rings with endothelium. L-NAME, an inhibitor of NO synthase, and methylene blue, an inhibitor of guanylate cyclase, abolished relaxation of HIMA, induced by resveratrol. Highly selective blocker of ATP-sensitive K^+ channels, glibenclamide as well as a nonselective blocker of big Ca-sensitive K^+ channels, charybdotoxin did not block resveratrol-induced relaxation of HIMA. 4-Aminopyridine and margatoxin, blockers of voltage-gated K^+ (K_V) channels, abolished endothelium-dependent relaxation of HIMA, induced by resveratrol. In conclusion, we have shown that resveratrol induces relaxation of HIMA with endothelium. It seems that NO and smooth muscle K_V channels are included in this relaxation.

Key words: mammary artery, K^+ channel, NO, resveratrol, vasorelaxation

INTRODUCTION

Epidemiological studies have suggested that dietary factors, including moderate wine consumption, might reduce the risk of cardiovascular diseases (Diebolt et al., 2001). The lower incidence of coronary artery disease in Soutern French and other Mediterranean populations, despite a diet rich in saturated fat and high smoking habits (the so-called French paradox), has been attributed to prolonged and moderate red wine consumption by these populations (Orallo et

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al., 2002). It is considered that resveratrol, as a polyphenol, is present in red wine in significant amounts, and is partly responsible for the cardiovascular benefits associated with wine consumption. The mechanism of cardiovascular benefits probably includes vasorelaxation, antioxidant and anti-platelet effects of resveratrol (Wu *et al*., 2001).

The mechanisms by which resveratrol causes vasodilatation are uncertain. Today is known, that resveratrol-induced vasorelaxation may either be endothelium-dependent (attenuated by L-NAME) or endothelium-independent (Naderali *et al*., 2001). 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 (Jager and Nguyen-Duong, 1999; Andriambeloson *et al*., 1999). Recently, it has been shown that some endothelial and peripherial effects of resveratrol are mediated by activation of Ca^{2+} activated and voltage-gated K^+ (K_V) channels (Wu, 2003, Granados-Soto *et al*., 2002).

HIMA is the vessel of choice for coronary artery bypass grafting (CABG). Spasm of arterial graft conduit may occur during harvesting or immediately following CABG (Rosenfeldt *et al*., 1999). The mechanism of graft spasm is not yet elucidated although physical (mechanical manipulation, temperature changes), and humoral factors (circulating vasoconstricting substances) have been evoked (He *et al*., 1995). We have shown that potassium channel openers, pinacidil, levcromakalim and rilmakalim, are potent antivasoconstrictors and vasodilatating agents on the HIMA and that they can be considered as potential drugs in the prevention of the bypass graft spasm (Gojkovic Bukarica *et al*., 1997, Novakovic *et al*., 2003). Resveratrol is present in the plant Polygonum Cuspidatum. Its effects on coronary bypass grafts have not been studied yet. Since some authors (Andriambeloson *et al*., 1999; Chen *et al*., 1996; Naderali *et al*., 2000) have studied the effects of resveratrol in animals, the authors performed the study on human arteries, which are important both in cardiac and orthopedic surgery. Therefore, the aim of this study was to investigate the effect of resveratrol on HIMA, with endothelium, used as coronary bypass grafts, and to define the contribution of different K^+ channel subtypes in resveratrol action on this blood vessel.

MATERIAL AND METHODS

The HIMA (n = 49) segments were collected from male patients (n = 49) undergoing CABG suffering from coronary artery disease. Only arteries without macroscopic evidence of atherosclerosis were used. All patients gave their formal consent for excision of the remaining tissue. The experimental protocol was approved by The Ethical Committee of Institute for cardiovascular diseases "Dedinje". Research has been carried out in the accordance with Declaration of Helsinki (2000) of the World Medical Association. The vessels were excised within 10 min of clamping the blood flow and placed in cold (4ºC) Krebs-Ringer-bicarbonate solution. After excision, the vessels were immediately transported to the laboratory.

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Assessment of vascular function

The HIMA segments were dissected free from connective tissue. They were cut into rings (3 mm) and were mounted between two stainless-steel triangles in an organ bath containing 10 ml Krebs-Ringer-bicarbonate solution (37°C, pH of 7.4), aerated with 95% O_2 and 5% CO_2. One of the triangles has been attached to a displacement unit allowing fine adjustments 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 have been 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 (Gojkovic Bukarica *et al*., 1997). The resting tension was 2 g (Schoeffter *et al*., 1988). Vascular rings were allowed a further 30 min to equilibrate before being contracted with phenylephrine.

Concentration-response curves were obtained by the cumulative addition of resveratrol (1-100 M) to ring segments contracted to a stable plateau by adding phenylephrine (10 M). Increasing concentrations of resveratrol have been 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 different K^+-channel blockers and a 20 min equilibration period; 2) contraction to phenylephrine and the concentration-response curve to resveratrol.

We have examined the effects of resveratrol onto the rings with intact endothelium. To assess the endothelial integrity of the preparation we have used acetylcholine (20 M). Endothelium was considered to be intact when this drug produced a strong vasorelaxation ($>80\%$) of precontracted vascular rings. Failure of arteries to relax to acetylcholine was considered to indicate a state of endothelial denudation.

Data analysis

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 the maximum response (EC_{50}) was determined graphically for each curve by linear interpolation.

The results are expressed as the means ± standard error of the mean (SEM); *n* refers to the number of experiments. Statistical difference between means was determined by Student's *t*-test, and a *P* value <0.05 was considered statistically significant.

Drugs

The following drugs were used: *trans*-resveratrol, phenylephrine, acetylcholine, glibenclamide, charybdotoxin, 4-amynopiridine (4-AP), and margatoxin (Sigma Chemical Co, St Louis, MO, USA). Resveratrol was dissolved in 70% v/v ethanol with further dilution in distilled water before use. Working

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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 L and the concentrations given were the calculated final concentrations of the bath solution.

RESULTS

Effects of resveratrol on precontracted HIMA

Figure 1 shows the effect of resveratrol on the HIMA precontracted with phenylephrine (10 M). Resveratrol (1-100 M) induced a concentration-dependent relaxation of HIMA rings with endothelium (EC_{50} values of 34.7 ± 0.11 M, maximal response 86 ± 2 %, n = 49), (Fig. 1).
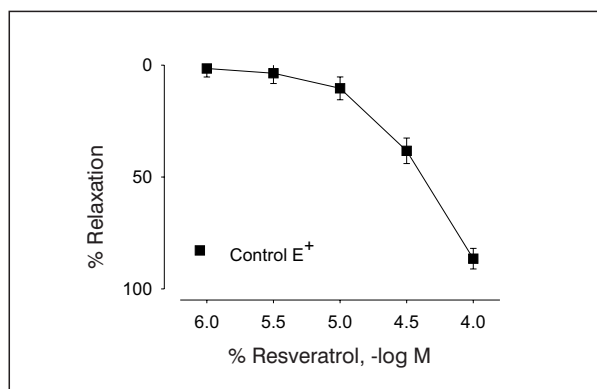

Figure 1. Concentration-response curve for resveratrol in the human internal mammary artery. Rings were precontracted with phenylephrine (10 M). Responses are expressed as a percentage of the maximum possible relaxation, i.e., the return of tension to the pre-phenylephrine level. Each point represents the mean ± SEM (n=49)

Effects of L-NAME and methylene blue on resveratrol-induced relaxation

L-NAME (100 M) and methylene blue (10 M) abolished resveratrol-induced relaxation of HIMA (maximal responses: 83 ± 2 % in the absence vs. 2 ± 2 % in the presence of L-NAME, p<0.01, n = 7, and maximal responses: 83 ± 2 % in the absence vs. 16 ± 2 % in the presence of methylene blue, p<0.011, n = 7, respectively (Figure 2A and 2B).

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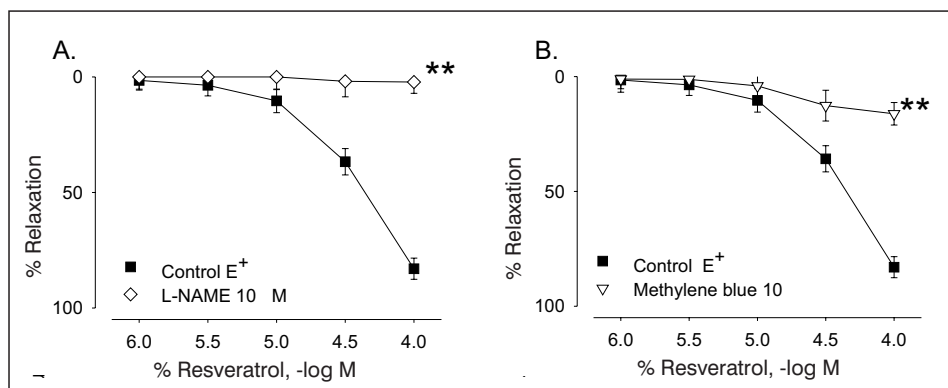of internal mammary artery produced by resveratrol

Figure 2. Antagonism of the relaxant effect of resveratrol by a L-NAME and methylene blue in the human internal mammary artery. Concentration-response curves for resveratrol in the absence and presence of L-NAME (A) and methylene blue (B). 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 the mean ± SEM (n = 7). Significance of differences: ** $P < 0.01$

Effects of potassium channel antagonists on the resveratrol-induced relaxation of HIMA with endothelium

Glibenclamide (10 M), charybdotoxin (10 nM), 4-AP (3 mM), and margatoxin (10 nM) did not affect basal tension of HIMA (n=7, each; data not shown).

Glibenclamide (10 M), a selective ATP-sensitive K^+ (K_{ATP}) channels blocker did not significantly modify the relaxation of HIMA induced by resveratrol ($EC_{50} = 36.1 \pm 0.14$ M in the absence vs. 38.9 ± 0.11 M in the presence of glibenclamide, $P > 0.05$; maximal response 86 ± 2 % in the absence vs. 83 ± 5 % in the presence of glibenclamide, $P > 0.05$, n = 7) (Fig. 3A).

Charybdotoxin (10 nM), an inhibitor of big Ca-sensitive K^+ (BK_{Ca}) channels as well as K_V channels did not affect the relaxation of HIMA produced by resveratrol ($EC_{50} = 38.5 \pm 0.17$ M in the absence vs. 43.1 ± 0.12 M in the presence of charybdotoxin, $P > 0.05$; maximal response 88 ± 2 % in the absence vs. 83 ± 2 % in the presence of charybdotoxin, $P > 0.05$, n = 7) (Fig. 3B).

4-AP (3 mM), predominant blocker of K_V channels abolished resveratrol-induced relaxation of HIMA (maximal response: 89 ± 2 % in the absence vs. 4 ± 2% in the presence of 4-AP, $P < 0.01$, n = 7) (Fig. 3C).

Margatoxin (10 nM), potent inhibitor of $K_V1.1$, $K_V1.2$, $K_V1.3$, and $K_V1.6$ channels abolished the resveratrol-induced relaxation of the HIMA (maximal responses: 83 ± 2 % in the absence vs. 4 ± 2 % in the presence of margatoxin, $p < 0.01$, n = 7) (Fig. 3D).

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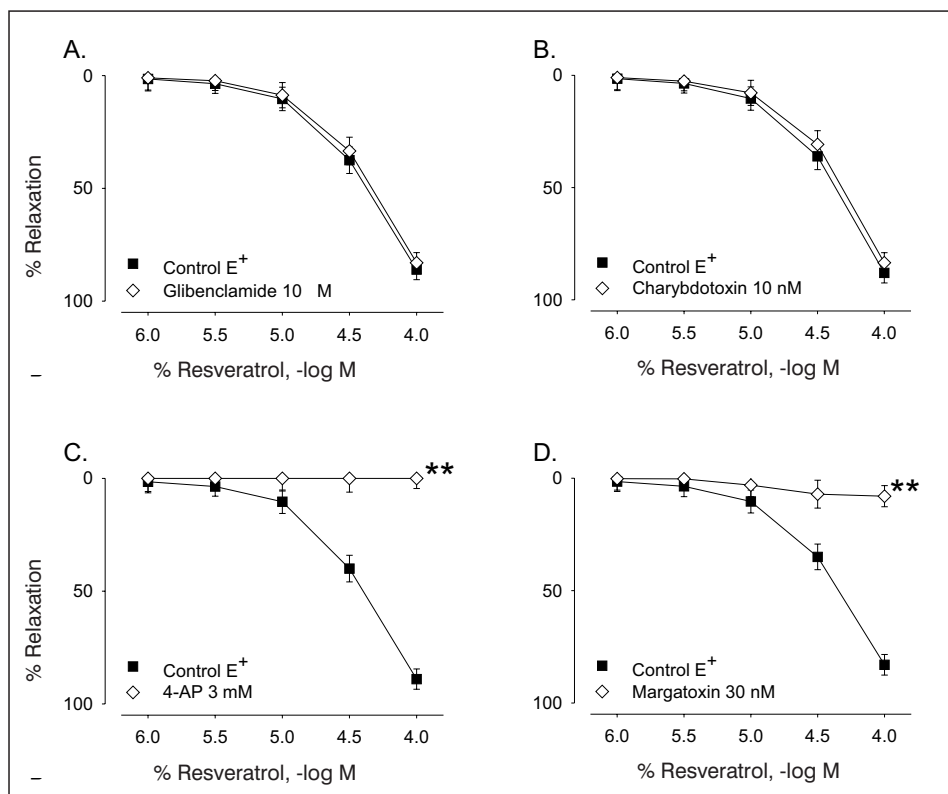of internal mammary artery produced by resveratrol

Figure 3. Antagonism of the relaxant effect of resveratrol by a K^+ channel inhibitors in the human internal mammary artery. Concentration-response curves for resveratrol in the absence and presence of: glibenclamide (A), charybdotoxin (B), 4-amynopiridine (C) and margatoxin (D). Arteries were contracted with phenylephrine (10 M). 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 the mean ± SEM (n = 7). Significance of differences: ** $P<0.01$

DISCUSSION

Several studies suggested vasorelaxant effects of polyphenolic compounds (Andriambeloson *et al*., 1997; Fitzpatrick *et al*., 1995). Resveratrol is thought to be a prime compound of the polyphenols, causing relaxation of the rat aorta, mesenteric and uterine artery of guinea pig, prior contracted by phenylephrine, noradrenaline or KCl (Chen and Pace-Asciak, 1996; Naderali *et al*., 2000; Orallo *et al*., 2001; Naderali *et al*., 2001). In the present study, we have shown that resveratrol induces concentration-dependent relaxation of HIMA with endothelium.

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Today it is known that the endothelium-dependent effect of resveratrol is apparent at low concentrations (10-30 M) and is blocked by inhibitors of NO synthase. In our study, pretreatment with L-NAME, an inhibitor NO synthesis, completely abolished endothelium-dependent relaxation of HIMA rings produced by resveratrol. This finding is in agreement with previous reports where resveratrol-induced vasodilatation was attenuated by inhibitors of NO synthesis (Chen and Pace-Asciak, 1996; Fitzpatrick *et al*., 1995; Naderali *et al*., 2001). In addition, the relaxation induced by resveratrol was blocked by methylene blue, an inhibitor of soluble guanylate cyclase (Kuriyama *et al.*, 1995, Moncada *et al*., 1997). These results correspond to those reported in the rat aorta by Orallo *et al*. (2002). According to this, it seems that endothelium-dependent vasorelaxation of HIMA caused by resveratrol could be mediated by endothelial generation and release of NO. Indeed, NO is the predominant endothelium derived dilator of the HIMA and synthesized after stimulation with vasoconstrictor phenylephrine (Archer *et al*., 2003). The mechanism underlying the NO-induced vasodilatation has been intensively investigated. Current knowledge suggests a central role for cGMP-dependent activation PKGI which can phosphorylate different membrane proteins. NO can also activate KCa channels and increase the outward potassium current. It has been shown that this action of NO can be both independent and dependent on activation PKGI (Gewalting and Kojda, 2002). Finally, cGMP-dependent inhibition of voltage-gated Ca-channels might also be involved in the mechanism of vasodilatation induced by NO. The relative contribution of each of these PKGI and K^+ channels dependent vasodilating mechanisms of NO remains to be determined (Gewalting and Kojda, 2002).

To determine whether the K^+ channels mediated relaxation of HIMA with endothelium induced by resveratrol, we used different potassium channel blockers.

To analyze the contribution of K_{ATP} channels to the endothelium-dependent resveratrol-induced relaxation of the HIMA, we used glibenclamide (10 M). Glibenclamide is known as one of the most selective blockers of K_{ATP} channels, although when used in a high concentration (>30 M), it may block some other types of K^+ channels (Sturgess *et al*., 1985, Schmid-Antomarchi *et al*., 1987, Cook and Quast, 1990). In the present study, glibenclamide did not inhibit the relaxation of HIMA induced by resveratrol. Accordingly, it seems that K_{ATP} channels are not involved in the pathway by which resveratrol produces a relaxation of the HIMA. This result is in agreement with the view that glibenclamide does not antagonize antinociceptive effect of resveratrol (Granados-Soto *et al*., 2002).

To analyze the possibility that the endothelium-dependent relaxation of the HIMA, evoked by resveratrol, is mediated via BK_{Ca} channels, charybdotoxin was tested. It has been shown that charybdotoxin blocks BK_{Ca} channels in the vascular smooth muscle (Cook and Quast, 1990). However, charybdotoxin is not a specific blocker of BK_{Ca} channels, it also inhibits intermediate conductance Ca^{2+}-activated K^+ channels and K_V channels (1.2 and 1.3 channels) (Feld *et al*., 2002; Suarez-Kurtz *et al*., 1999). In each case, channel inhibition occurs with similar potency in the low nanomolar range (*Kd* $\sim$ 0.3 and 10 nM) (Wallner *et al*., 1999). The concentration of charybdotoxin used in our study was sufficient to

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block BK_{Ca} channels, but did not alter relaxation of the HIMA induced by resveratrol. Accordingly, it seems that charybdotoxin-sensitive channels are not involved in the mechanism of resveratrol-induced relaxation of the HIMA. This finding is in contrast to the finding of Wu *et al*. (2003) who demonstrated that resveratrol opens BK_{Ca} channels in the vascular endothelial cell.

To analyze the contribution of K_V channels to the resveratrol-induced relaxation in the HIMA, we used 4-AP. This compound is the most widely used blocker in the identification of potassium channel types. With very few exceptions, 4-AP have been shown to be without effect on BK_{Ca} (Ritchie, 1987). Using low millimolar concentration, 4-AP achieved some selectivity for K_V channels (Beech and Bolton, 1989). This feature complies with the results given by our experiments i.e. 4-AP (3 mM) antagonized resveratrol-induced relaxation of HIMA rings with comparable potency. Thus, our finding supports a relevant participation of K_V channels in the relaxation of HIMA produced by resveratrol. Consistent with this idea is the result obtained by Granados-Soto *et al*. (2002) that suggested that activation of K_V channels participated in the peripheral nociceptive effect of resveratrol.

We used margatoxin in order to test which subtype of K_V channels was included in resveratrol-induced relaxation of HIMA. This peptide is highly selective inhibitor of the K_V1 channels, especially 1.1, 1.2, 1.3, and 1.6 subtypes, but displays no affinity for the mammalian BK_{Ca} channel (Garcia-Calvo *et al*., 1993, Cheong *et al*., 2001). $K_V1.2$ and $K_V1.3$ were identified in vascular smooth muscle cell of rat mesenteric artery; $K_V1.1$ and $K_V1.6$ were detected in smooth muscle cell of rat aorta and rat pulmonary artery but not in smooth muscle cell of mesenteric artery (Xu *et al*., 1999; Archer *et al*., 1998). This reflects tissue differences in expression of K_V1 channels subtypes. In our study, th applied margatoxin (10 nM) was sufficient to block $K_V1.1$, $K_V1.2$, $K_V1.3$, and $K_V1.6$ channels, by abolishing resveratrol-induced relaxation, suggests that those channels might be included in the mechanism of resveratrol-induced endothelium-dependent vasodilatation of HIMA (Garcia-Calvo *et al*., 1993, Cheong *et al*., 2001). However, the fact that charybdotoxin, a potent blocker of $K_V1.2$ and $K_V1.3$ did not reproduce the effect of margatoxin suggests that $K_V1.1$ and/or $K_V1.6$ may be the relevant target. This observation needs further evaluation.

In conclusion, we have shown that resveratrol can induce relaxation of HIMA with endothelium. It seems that NO and 4-AP- and margatoxin-sensitive voltage-gated K^+ channels located in the smooth muscle of HIMA are included in this relaxation. Further investigations have been necessary to explain the nature of interaction between NO and K^+ channels in HIMA.

Address for correspondence:
Ass. Prof.: Dr. Ljiljana Gojković-Bukarica
Department of Clinical Pharmacology, Pharmacology and Toxicology,
School of Medicine, P.O.Box 840,
University of Belgrade, Belgrade 11129,
Serbia and Montenegro;
E-mail: bukarica@rcub.bg.ac.yu

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ENDOTEL-ZAVISNA RELAKSACIJA UNUTRAŠNJE TORAKALNE ARTERIJE PROUZROKOVANA REZVERATROLOM

NOVAKOVIĆ ALEKSANDRA, GOJKOVIĆ-BUKARICA LJILJANA, PERIĆ M, NEŽIĆ D, DJUKANOVIĆ B, LEŠIĆ A, BUMBAŠIREVIĆ M i MARKOVIĆ-LIPKOVSKI JASMINA

SADRŽAJ

Smatra se da rezveratrol kao jedna polifenolna komponenta prisutna u značajnim količinama u crnom vinu, smanjuje rizik od razvoja ateroskleroze i koronarne bolesti. U mehanizam kardioprotektivnog delovanja verovatno su uključeni antioksidativno, antitrombocitno i vazodilatatorno delovanje rezveratrola. Mehanizam vazodilatacije još uvek nije poznat, pa je cilj ovog rada bio da se ispitaju efekti i mehanizam vazorelaksantnog delovanja rezveratrola na humanoj unutrašnjoj torakalnoj arteriji sa endotelom. Unutrašnja torakalna arterija je prekontrahovana fenilefrinom. Rezveratrol je koncentracijski-zavisno relaksirao unutrašnju torakalnu arteriju čoveka. L-NAME, inhibitor NO sintaze, i metilensko plavo, inhibitor solubilne gvanilat ciklaze, su antagonizovali relaksaciju unutrašnje torakalne

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arterije sa intaktnim endotelom, prouzrokovanu rezveratrolom. Visoko selektivni blokator ATP-senzitivnih K$^+$ kanala, glibenklamid, kao i neselektivni blokator velikih Ca-senzitivnih K$^+$ kanala, karibdotoksin nisu antagonizovali rezveratrolom indukovanu relaksaciju unutrašnje torakalne arterije. 4-Aminopiridin i margatoksin, blokatori voltažnih K$^+$ kanala su antagonizovali relaksaciju prouzrokovanu rezveratrolom.

Na osnovu ovih činjenica se može zaključiti da je endotel-zavisna relaksacija unutrašnje torakalne arterije čoveka, prouzrokovana rezveratrolom, verovatno posredovana NO. Izgleda, da su 4-aminopiriin- i margatoksin-senzitivni K-kanali smešteni u membrani vaskularnih glatko-mišićnih ćelija humane unutrašnje torakalne arterije, uključeni u mehanizam endotel-zavisne relaksacije prouzrokovane rezveratrolom.