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Relaxing Effects of Phytoestrogen α-Zearalanol on Rat Thoracic Aorta Rings *in Vitro*

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Abstract

The aim of this research is to investigate the vasorelaxing effects and mechanisms involved in the phytoestrogen α-zearalanol (α-ZAL) in rat thoracic aortas rings. Intact or endothelium denuded rat thoracic aortas rings were put in individual organ chamber to observe the endothelium-dependent or independent vasorelaxing effects of α-ZAL (10^{-10}-10^{-5} M). The thoracic aortas rings were pre-contracted with phenylephrine. The relaxing effects of α-ZAL were observed and the influence of N^{ω}-nitro-L-arginine methylester (L-NAME, NOS inhibitor), methylene blue (MB, guanylate cyclase inhibitor), charybdotoxin (ChTX, Ca^{2+}-activated K^+ channel blocker), glibenclamide (ATP-sensitive K^+ channel blocker), (–) BayK8644 (L-type Ca^{2+} channel agonist) and ICI182,780 (estrogen receptor antagonist) were pre-incubated with α-ZAL, respectively, to explore the possible mechanisms involved in this vasorelaxation. Furthermore, the Phospho-eNOS expression and cGMP level in the aortas tissue were detected by Western blot and radioimmunity, respectively; the NO level in perfusate was assaied by chromatometry. Our result showed that α-ZAL (10^{-10}-10^{-5} M) induced both endothelium-dependent and -independent relaxation of rat thoracic aortas rings. The vasorelaxing effects of α-ZAL were dose-dependent whether the endothelium was intact or not. In endothelium-intact aortas rings, α-ZAL-induced vasorelaxation might be inhibited by L-NAME, MB, charybdotoxin, glibenclamide and (–) BayK8644, but not ICI182,780. (–) BayK8644 could also inhibit α-ZAL-induced vasorelaxation in endothelium-denuded aortas rings.10^{-7}-10^{-5} M α-ZAL might induce the Phospho-eNOS expression in thoracic aorta tissue, increase the NO level in perfusate and cGMP content in thoracic aorta tissue. Meanwhile, L-NAME might decrease both NO and its downstream cGMP level. Methylene blue might decrease the level of cGMP. These results suggest that α-ZAL induces a partly endothelium-dependent relaxation of rat thoracic aortas rings; the possible mechanisms involved in this rapid vasorelaxation include activation of eNOS/NO/cGMP pathway, opening of VSMCs ATP-sensitive and Ca^{2+}-activated K^+ channels through secretion of EDHF from endothelium. Furthermore, this relaxation also appears to be mediated by both direct and indirect inhibition of voltage-dependent Ca^{2+} channel of VSMCs, while it is not concerned with activation of estrogen receptor.

Key Words: α-ZAL, thoracic aortas, vasorelaxation, NO, cGMP, K^+ channel, Ca^{2+} channel

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Introduction

Phytoestrogens are naturally occurring plant-derived nonsteroidal estrogens which are present in the human diet. Their chemical structure is similar to that of the estrogen, what enables them to bind the estrogen receptor thus acting as estrogen agonists or antagonists (for reviews, see 1, 24). Interest in phytoestrogens comes from the observation that people from East Asian countries showed a lower incidence of cardiovascular diseases than people from Western countries, since Asians consume 20-50 times more soyderived food per capita than Americans do, and soybeans are concentrated sources of phytoestrogens. It has been postulated that phytoestrogens might be responsible for the health-promoting effect of soy consumption (for reviews, see 2, 4, 14, 22). Recently a newly identified phytoestrogen α-zearalanol (α-ZAL) interested us. α-ZAL is a reductive product of the Gibberella zeae etabolite zearalenone. α-ZAL and zearalenone have been used as relatively safer and more efficient food additives to facilitate growth in animal husbandry (19). Recently our group has demonstrated that α-ZAL had cardiovascular-protecting property in a manner similar to estrogen. These properties include the following efficiently reducing atherogenesis and improving lipid profile in ovariectomized cholesterol-fed rabbits (6, 7), decreasing risk of thrombosis through inhibiting tissue factor expressing in endothelial cells (26), antagonizing oxidized LDL-induced imbalance between nitric oxide and endothelin-1 in endothelial cells (27), and protecting endothelial cells from homocysteine injury (9,10), meanwhile without estrogenic tissue proliferation risk (8). As we know, the change of angiotasis may also play a role in the pathogenesis of cardiovascular diseases, but the effects of α-ZAL on angiotasis remain unknown. Here we try to challenge the hypothesis that phytoestrogen α-ZAL could be a useful tool in the prevention and/or treatment of cardiovascular diseases by its vasorelaxing effects. Therefore, the aim of this study is to evaluate the vasorelaxing effects of α-ZAL in rat thoracic aorta. Furthermore, the possible mechanisms involved in pathways such as the nitric oxide synthase/nitric oxide/cyclic guanosine monophosphate (NOS/NO/cGMP), and the roles of K^+ channels, Ca^{2+} channels and estrogen receptor were also examined.

Materials and Methods

Drugs and Chemicals

α-ZAL was purified by the Institute of Biological Sciences, Chinese University of Agriculture (P.R. China), ICI182780(7α-[9-(4,4,5,5-pentafluoropentylsulphinyl)nonyl]oestra-1,3,5,(10)-triene-3, 17beta-diol) was purchased from Tocris Cookson Inc. (Ellisville, MO, USA)), Phospho-endothelial NOS (p-eNOS, Ser1177) antibody was purchased from Santa Cruz (Santa Cruz, CA, USA), the protein content BCA kit was purchased from Pierce (Rockford, IL,USA), cGMP assay kit was purchased from Atomic Energy Research Institute (Beijing, P.R. China). All other drugs and chemicals were purchased from Sigma (St. Louis, MO, USA).

For α-ZAL, 17β-estradiol (E_2) and (–) BayK8644, ethanol was used as solvent and the final concentration of ethanol in each bath was always < 0.1%. All other drugs and chemicals were dissolved in distilled water. Acetylcholine (ACh) was freshly prepared and diluted by warm modified Krebs-Henseleit Solution (KHS). The final organ bath concentrations of 0.1% ethanol used had no significant effect on the results obtained when compared to the corresponding control.

KHS was used to make up the final working solutions. KHS had a composition of the followings: NaCl 0.118 M, KCl 4.75×10^{-3} M, $MgSO_4$ 1.19×10^{-3} M, $CaCl_2$ 2.54×10^{-3} M, $NaHCO_3$ 2.5×10^{-2} M, KH_2PO_4 1.19×10^{-3} M and glucose 1.11×10^{-2} M, pH7.3-7.4.

Rat Thoracic Aorta Rings Preparation

Adult female healthy Wistar rats (12 weeks old, weighing 250-300 g) were purchased from the Animal Center of Capital Medical University (Beijing, People's Republic of China). All animals were housed in a temperature (22 ± 1°C)-controlled room with free access to standard rat chow and tap water and received humane care in accordance with the animal care provisions. The use of animals for this study had been approved by the Committee on the Use of Live Animals in Teaching and Research at the Capital Medical University following guidelines as recommended by the Helsinki Declarations for use of experimental animals. The rats were sacrificed by decapitation. The thoracic aorta was removed and placed in cold KHS at 4°C. Excess fat and connective tissue were removed and the aorta was cut into 3-4 mm rings. The rings were then immediately mounted on two stainless steel hooks under a resting tension of 2.0 g in 10 ml KHS-filled organ baths. One of these hooks was anchored and the other was attached to a force transducer for tension measurement. The preparations were allowed to equilibrate for 60 min in oxygenated condition (95% O_2: 5% CO_2) at 37°C. KHS was changed every 15 min. Basal tension of 2.0 g was maintained continuously throughout the equilibration period.

In experiments requiring disruption of en-

dothelium, rings were gently rubbed of the entire inner lumen with a stainless steel wire. For the corresponding control, perfusion was carried out using normal KHS. In functional studies, the removal of endothelium was verified by examining ACh (10^{-6} M) vasorelaxation in phenylephrine (10^{-6} M)-precontracted aorta rings.

Experimental Protocols

After the equilibration period, rings were precontracted with phenylephrine and then relaxed with ACh. Only rings that contracted more than 2.0 g and relaxed more than 80% were regarded as viable. After the viability test, the rings were allowed to return to the basal tension by changing the KHS every 10 min before further experimentation. Phenylephrine was then added until a stable and sustained contraction was achieved. Dose (10^{-10}-10^{-5} M)–relaxation curves of α-ZAL or E_2 were obtained. To assess the role of endothelium in the effects of α-ZAL or E_2, responses were elicited in endothelium denuded phenylephrine-contracted aorta rings. The functional state of the endothelium was verified by challenge of phenylephrine-contracted rings with ACh, rings producing less than 10% relaxation to 10^{-6} M ACh were used. To assess the possible involvement of NOS/NO/cGMP mechanisms in α-ZAL-elicited response, aorta rings were preincubated with either 10^{-5} M N^{ω}-nitro-L-arginine methylester (L-NAME, nitric oxide synthase inhibitor) or 10^{-5} M methylene blue (MB, guanylate cyclase inhibitor). On the other hand, the roles of K^+ channel, Ca^{2+} channel and estrogen receptor in α-ZAL-elicited response were also studied. For these purposes, aorta rings were preincubated with either 10^{-7} M charybdotoxin (ChTX, Ca^{2+}-activated K^+ channel blocker), 10^{-5} M glibenclamide (ATP-sensitive K^+ channel blocker), 10^{-6} M (–) BayK8644 (L-type Ca^{2+} channel agonist), 10^{-6} M ICI182,780 (estrogen receptor antagonist), respectively.

Western Blot Analysis of Phospho-Endothelial NOS (p-eNOS) in Aortas Tissue

After 10 min incubation, the aortas rings were chopped and placed into a 1 ml glass-glass homogenizer containing 300 μl homogenizing buffer (NaCl 0.154 M, Tris base 0.02 M, EDTA 0.01 M, sodium vanadate 0.01 M and sodium dodecyl sulphate (SDS) 2%; pH7.4). The homogenate was centrifuged for 20 min at 10,000 g at 4°C. The supernatant was collected for Western blot analysis. The protein content was determined using the Pierce BCA kit. 30 μg protein of each sample was loaded per well. Membranes were incubated for 3 h with a polyclonal rabbit anti-human p-eNOS antibody (diluted 1:500), then incubated for 1 h with goat anti-rabbit secondary antibody (diluted 1:1,000) and loaded control glyceraldehyde phosphate dehydrogenase (GAPDH, diluted 1:10,000), both conjugated to horseradish peroxidase. P-eNOS and GAPDH were visualized with enhanced chemiluminescence and exposure to Kodak Chemiluminescence film.

NO Assay in Aorta Perfusate

The NO level in thoracic aorta perfusate was assayed by chromatometry. The product of NO was shown by NO^{2-}/NO^{3-} (15). Operations strictly follow the directions of the kit.

Radioimmunity Analysis of cGMP in Aortas Tissue

After incubation, the aortas were moved to fluid nitrogen at once. 3 ml cold 10% trichloroacetic acid was added for homogenizing. The homogenate was centrifuged for 10 min at 3,000 rpm at 4°C. The supernatant was collected and extracted with ethylether four times. After moving off trichloroacetic acid and ethylether, the samples were dried on 60°C water bath, then operated strictly according to the instruction of the cGMP assay kit. The cGMP content was expressed by pmol/g wet tissue.

Data and Statistical Analysis

Rings from the same rat were used for one treatment only. Results were expressed as the means ± S.D. and n represents the number of rings used in the experiments. Relaxation (Rmax) was expressed as percentages of the phenylephrine-induced contraction. One-way analysis of variance (ANOVA) was used to determine the significant differences between treatments (SPSS). A *P*-value less than 0.05 was considered statistically significant.

Results

Direct Relaxing Effect of α-ZAL on Precontracted Rat Thoracic Aorta Rings

α-ZAL (10^{-10}-10^{-5} M) induced a dose-dependent vasorelaxation in rat thoracic aorta rings precontracted with phenylephrine. Significant differences were seen between 10^{-10}-10^{-5} M α-ZAL treatment and ethanol control. Denudation of the endothelium might attenuate, but could not entirely eliminate α-ZAL-induced relaxation. The relaxation was significantly greater by higher concentrations of α-ZAL than lower concentrations whether the endothelium was intact or not (Fig. 1).

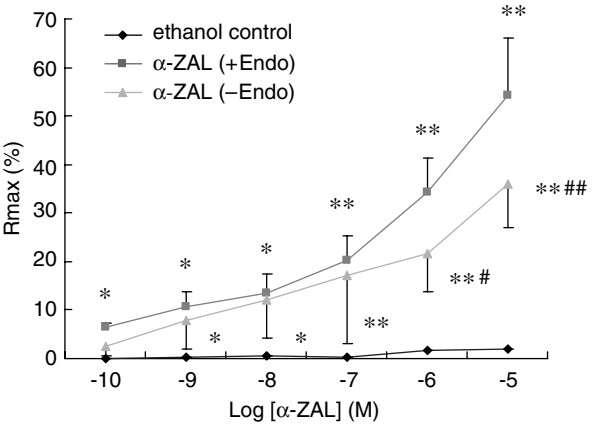

Fig. 1. Dose-dependent vasorelaxation induced by α-ZAL in phenylephrine precontracted rat thoracic aorta rings. Data are the means ± S.D. of six experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs*. ethanol control; $^{\#}P < 0.05$ *vs*. 10^{-6} M α-ZAL with endothelium-intact (+Endo), $^{\#\#}P < 0.01$ *vs*. 10^{-5} M α-ZAL (+Endo).

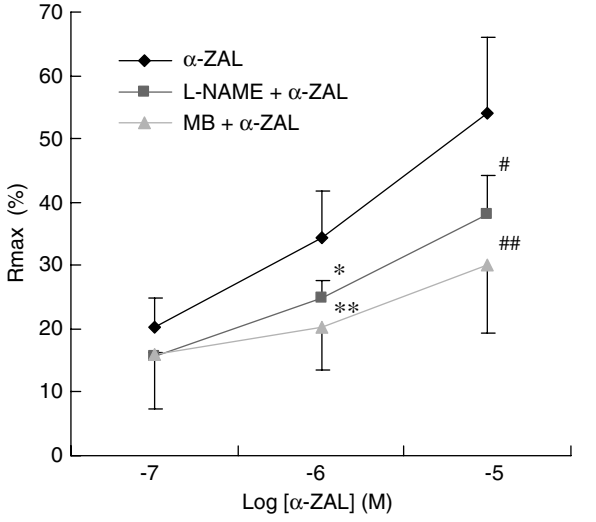

Fig. 2. Effects of L-NAME and MB on α-ZAL induced endothelium-dependent vasorelaxation in rat thoracic aorta rings. Data are the means ± S.D. of six experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ *vs*. α-ZAL (10^{-6} M); $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ *vs*. α-ZAL (10^{-5} M).

Effects of NOS /NO/cGMP Pathway on α-ZAL-Induced Vasorelaxation

To ascertain the involvement of NOS /NO/cGMP pathway in the relaxing action of α-ZAL, the effects of pretreatment with L-NAME (inhibitor of NOS) and methylene blue (MB, guanylate cyclase inhibitor) were investigated. Our results showed that both 10^{-5} M L-NAME and 10^{-5} M methylene blue could attenuate high-concentration (10^{-6} and 10^{-5} M) α-ZAL-induced vasorelaxation (Fig. 2). Furthermore, 10^{-7}-10^{-5} M

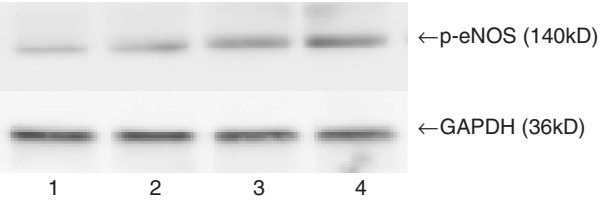

Fig. 3. Effects of 10^{-7}-10^{-5} M α-ZAL on p-eNOS expression in rat thoracic aorta tissue after 10 min of incubation. Representative Western blots from three independent experiments. Lane 1: ethanol control; lane 2: 10^{-7} M α-ZAL; lane 3: 10^{-6} M α-ZAL; lane 4: 10^{-5} M α-ZAL.

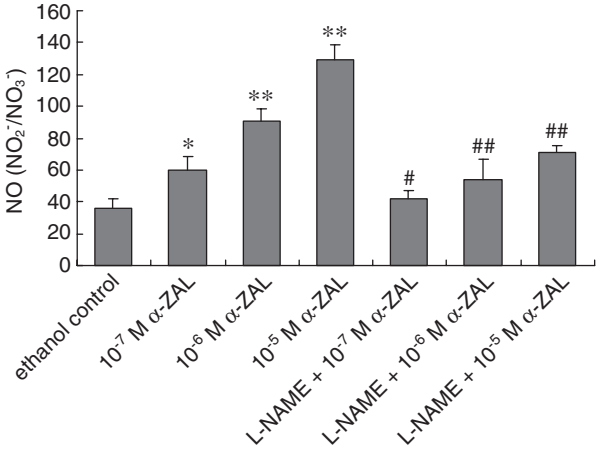

Fig. 4. Chages of NO level in rat thoracic aorta perfusate. Data are the means ± S.D. of six experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs*. ethanol control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs*. α-ZAL of same concentration.

α-ZAL might induce p-eNOS expression in rat thoracic aorta tissue (Fig. 3), increase the NO level in thoracic aorta perfusate and cGMP (product of guanylate cyclase) content in thoracic aorta tissue. Meanwhile, L-NAME, inhibitor of NOS, might decrease both NO and its downstream cGMP level (Figs. 4, 5). Methylene blue, as an inhibitor of guanylate cyclase, might decrease the level of its product cGMP (Fig. 5).

Effects of K^+ Channel Blockers on α-ZAL-Induced Vasorelaxation

To investigate the involvement of K^+ channels in the relaxant action of α-ZAL, the effects of pretreatment with charybdotoxin (ChTX), a blocker of Ca^{2+}-activated K^+ channel and glibenclamide, a blocker of ATP-sensitive K^+ channel were investigated in both endothelium-intact or denuded aorta rings. The results showed that both 10^{-7} M charybdotoxin and 10^{-5} M glibenclamide could attenuate α-ZAL-induced vasorelaxation in endothelium-intact aorta

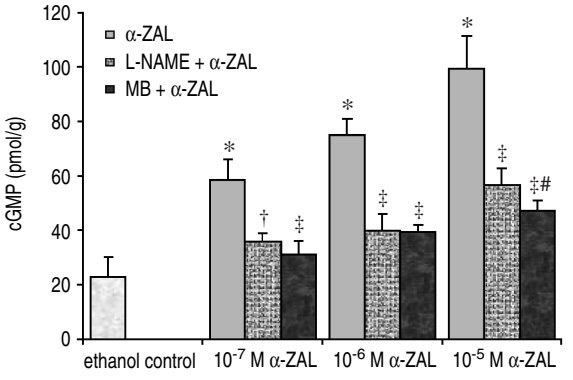

Fig. 5. Effects of α-ZAL (10^{-7}-10^{-5} M), L-NAME and MB on cGMP content of aorta tissue. Data are the means ± S.D. of six experiments. $^{*}P < 0.01$ *vs*. ethanol control; $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ *vs*. α-ZAL; $^{\#}P < 0.05$ *vs*. L-NAME + α-ZAL.

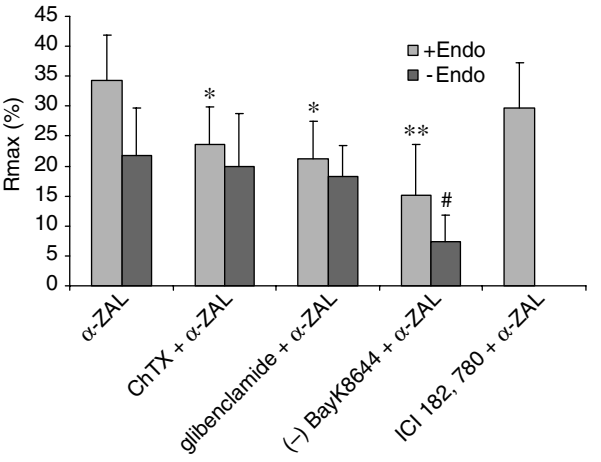

Fig. 6. Effects of ChTX, glibenclamide, (–) BayK8644 and ICI182,780 on 10^{-6} M α-ZAL –induced vasorelaxation in rat thoracic aorta rings. Data are the means ± S.D. of six experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs*. 10^{-6} M α-ZAL (endothelium-intact, +Endo); $^{\#}P < 0.01$ *vs*. 10^{-6} M α-ZAL (endothelium-denuded,-Endo).

rings. Meanwhile they had no effect on α-ZAL-induced vasorelaxation in endothelium-denuded aorta rings (Fig. 6).

Effects of Ca^{2+} Channel Agonist on α-ZAL-Induced Vasorelaxation

To investigate the involvement of Ca^{2+} channel in the relaxing action of α-ZAL, the effects of pretreatment with (–) BayK8644 (L-type Ca^{2+} channel agonist) were investigated in both endothelium-intact or denuded aorta rings. The results showed that 10^{-6} M (–) BayK8644 might attenuate ZAL-induced vasorelaxation whether the endothelium was intact or not (shown in Fig. 6).

Effects of Estrogen Receptor Antagonist on α-ZAL-Induced Vasorelaxation

Pre-incubation with the estrogen receptor antagonist ICI182,780 (10^{-6} M) did not significantly affect 10^{-6} M α-ZAL-induced vasorelaxation (Fig. 6).

Discussion

Increasing evidence has accumulated over the past few years showing that lots of phytoestrogens, including genistein, daidzein, biochanin A and reseratrol, affect vascular function in several ways (18, 23, 25). However, as far as we know, the pharmacological activtities of new phytoestrogen α-zearalanol (α-ZAL) on angiotasis had not yet been investigated. In the present study, we used female Wistar rats thoracic aorta rings to observe the vasorelaxing effect of α-ZAL and investigate the possible mechanisms involved in it.

Our results showed that α-ZAL (10^{-10}-10^{-5} M) might induce dose-dependent vasorelaxation in precontracted rat thoracic aorta rings. The relaxation was significantly greater by higher concentration of α-ZAL than lower concentration whether the endothelium was intact or not. In addition, denudation of the endothelium did not entirely eliminate α-ZAL-induced relaxation, although it was significantly attenuated. These results indicate that the partially endothelium-dependent nature of α-ZAL-induced relaxation in rat thoracic aorta rings, which is similar with 17 β -estradiol (E_2) (data not shown, also reported by Binko *et al*. (3)). The phytoestrogen plasma levels are in the nanomolar range in human consuming soy-free diets, and that the value rise to the micromolar range when soy is consumed either as an ingredient of the current diet or as an ingredient of dietary supplements (23). Conceivably, α-ZAL will elicit physiological effects on aorta rings under such situation.

Since 1980s, Furchgott and Zawadzki discovers the endothelium-derived relaxing factor (EDRF) (13), lots of vasoactive substances' vasorelaxation have been found to be mediated by EDRF, *i.e*., NO. The pathway is as follows: endothelial cells secrete NO→NO diffuses to vessel smooth muscle cells (VMSCs) by paracrine→NO activates the guanylate cyclase in VMSCs, increases the cGMP level→ activates protein kinase G (PKG)→decreases the plasma calcium level→VMSCs relax. Does the eNOS/NO/cGMP pathway also mediate the endothelium-dependent relaxation of α-ZAL? α-ZAL could induce NO production in cultured endothelial cells (27). In this study, we showed that 10^{-7}-10^{-5} M α-ZAL might

induce the p-eNOS expression in rat thoracic aorta tissue, which means activation of this enzyme, at the same time, would increase the NO level in thoracic aorta perfusate and cGMP content in thoracic aorta tissue. L-NAME, inhibitor of NOS, might decrease both NO and its downstream cGMP level with apparent attenuation of high-concentration (10^{-6} and 10^{-5} M) α-ZAL-induced vasorelaxation. Methylene blue (guanylate cyclase inhibitor) might decrease the level of cGMP, product of guanylate cyclase, with attenuation of α-ZAL-induced vasorelaxation, too. All these data implied that eNOS/NO/cGMP pathway also mediated the endothelium-dependent vasorelaxation of α-ZAL.

In addition to NO, it is possible that other vasoactive substances, such as prostacyclin and carbon monoxide also take roles in α-ZAL-induced vasorelaxation, for L-NAME could not eliminate the vasorelaxation completely.

Glibenclamide, a blocker of ATP-sensitive K^+ channels (21) might significantly reduce α-ZAL-induced relaxation in endothelium intact aorta rings (Fig. 4), which suggests that the opening of ATP-sensitive K^+ channels might be involved in this vasorelaxation. Charybdotoxin, which blocks Ca^{2+}-activated K^+ channel when used at appropriate concentrations (17), also significantly reduced α-ZAL-induced relaxation in endothelium-intact aorta rings. The opening of K^+ channels may result in the hyperpolarization of cell membrane (5). Meanwhile, neither glibenclamide nor charybdotoxin has effect on α-ZAL-induced relaxation in endothelium-denuded aorta rings, which implies that α-ZAL has no direct effect on VSMCs K^+ channels. Therefore, it is possible that the action of α-ZAL may be in part due to the release of endothelium-derived hyperpolarizing factors (EDHF) which causes hyperpolarization of the underlying VSMCs by a mechanism that involves both ATP-sensitive and Ca^{2+}-activated K^+ channels. Wide definition of EDHF also includes NO and prostacyclin (5). These results suggest that secretion of NO and other EDHF by endothelial cells, then the opening of both ATP-sensitive and Ca^{2+}-activated K^+ channels of VSMCs, hyperpolarization of the cell membrane, and eventually the block of voltage-dependent Ca^{2+} channel, may contribute to the vasorelaxation induced by α-ZAL in aorta rings. Of cause, the exact effects of α-ZAL on ion channels need to be identified by patch clamp experiments further.

α-ZAL-induced vasorelaxation occurred within minutes of treatment in rat aortic rings, which suggests that this vasorelaxation does not result from genomic effects. It has been shown that estrogen-induced relaxation is not mediated by its receptors in aortic rings and is too rapid for the classic genomic mechanism of steroid hormone action (3). These rapid effects have been attributed to the inhibition of voltage-dependent Ca^{2+} channels in VSMCs (11, 16, 20). Our results imply that the rapid vasorelaxation of α-ZAL is not mediated by estrogen receptor, for ICI182,780 (estrogen receptor antagonist) had no effect on this relaxation. Meanwhile, (–) BayK8644, agonist of L-type Ca^{2+} channel—one kind of voltage-dependent Ca^{2+} channels, might inhibit the vasorelaxation of α-ZAL both in endothelium-intact or denuded thoracic aorta rings. These results do suggest that this vasorelaxing effect is probably achieved by inhibition of voltage-dependent Ca^{2+} channel in VSMCs. It is possible that α-ZAL may have both direct and indirect inhibitions to VSMCs Ca^{2+} channel, the latter maybe through secretion of EDHF from endothelium.

In conclusion, we have demonstrated that phytoestrogen α-ZAL might induce both endothelium-dependent and -independent relaxation in rat thoracic aorta rings. α-ZAL-induced vasorelaxation is dose-dependent whether the endothelium is intact or not. The possible mêmechanisms involved in this rapid vasorelaxation include activation of eNOS /NO/cGMP pathway, opening of VSMCs ATP-sensitive and Ca^{2+}-activated K^+ channels through secretion of EDHF from endothelium. Furthermore, this relaxation also appears to be mediated by both direct and indirect inhibition of voltage-dependent Ca^{2+} channel of VSMCs, while it is not concerned with activation of estrogen receptor. Our findings illustrate a novel sight for protective effects of α-ZAL on cardiovascular diseases.

Acknowledgments

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