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Apigenin protects endothelium-dependent relaxation of rat aorta against oxidative stress

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Apigenin is shown to have cardiovascular effects, but the effects of apigenin on aor- tas injured by exogenous oxidants are unknown. The objective of this study was to investigate the effect of apigenin on endothelium-dependent vasorelaxation in isolated rat aortic rings exposed to superoxide anion produced by pyrogallol, and its mechanism. The male Sprague–Dawley rat thoracic aorta was rapidly dissected out and the effect of apigenin on tension of aortic rings pretreated with 500 μM pyrogallol, inducing oxidative stress injury, was measured. The activity of nitric oxide synthase (NOS), the level of nitric oxide (NO) and the inhibition of superoxide anion in aortic tissues were measured. We found that pretreatment with pyrogallol concentration-dependently decreased acetylcholine-induced endothelium-dependent vasorelaxation. Apigenin (0.5–72.0 μM) evoked a concentration-dependent relaxation in aor- tas (pD2: 5.304 ± 0.049), which was weakened by i-NAME (the maximal relaxation fell from 87.6 ± 6.7% to 37.1 ± 8.8%, P<0.01), but not by aminoguanidine and indomethacin. Apigenin markedly attenuated the inhibition of vasorelaxation induced by pyrogallol (the maximal relaxation elevated from 55.8 ± 6.6% to 69.5 ± 6.4%, and the pD2 increased from 6.559 ± 0.119 to 7.057 ± 0.145, P<0.01) and increased the inhibition of superoxide anion (from 94.6% to 74.5%), the NO level (from 77.1% to 94.4%), and the constitutive NOS activity (from 35.1% to 62.5%). These results indicate that pyrogallol decreased endothelium-dependent vasorelaxation in rat aortas via oxidative stress, which was markedly attenuated by apigenin. This may be mediated by weakening the oxidative stress and the NO reduction.

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1. Introduction

It is widely accepted that the endothelium plays important roles in maintaining normal vascular tone and blood fluidity (Gimbrone, 1995). Under normal conditions, the endothelium regulates vascular homeostasis by elaborating a variety of factors that act locally in the blood vessel wall and lumen, such as nitric oxide (NO), prostacyclin and endothelin. However, the physiological functions of endothelial cells are altered in certain cardiovascular diseases including atherosclerosis (Ludmer et al., 1986), diabetes (Rodriguez-Manas et al., 2003), and primary hypertension (Panza et al., 1995). Impairment of endothelium-dependent relaxation may be a critical initiating abnormal response in various vascular diseases (Feletou and Vanhoutte, 2006). The etiology of endothelium-dependent relaxation impairment is multi-factorial, and the underlying mechanism is not yet fully elucidated. Among these factors, overproduction of reactive oxygen species under pathophysiological conditions, alterations of endothelial nitric oxide synthase (eNOS) expression and activity, and decreased availability of NO, all have been considered responsible for the endothelial dysfunction (Feletou and Vanhoutte, 2006; Palmer et al., 1987). Intracellular oxidative stress is mainly caused by an imbalance between the activity of endogenous pro-oxidative enzymes and antioxidant enzymes (Forstermann, 2008), and thus disturb vascular cell functions and alter the release of vasodilator substances (Cai and Harrison, 2000; Rojas et al., 2006). Overall vascular function depends upon a fine balance of oxidant and antioxidant mechanisms, and clearly, factors that can modify reactive oxygen species release have the potential to protect against endothelial dysfunction.

Unfortunately, the results of long-term epidemiological trials with oral delivery of acknowledged antioxidants such as vitamin C and vitamin E have been ambiguous and do not support the use of either to reduce the risk of cardiovascular morbidity or mortality (Forstermann, 2008; Lonn et al., 2005). The prevention of reactive oxygen species formation by vasoactive agents might prove more beneficial than direct antioxidant strategies in vascular endothelial disease.

Flavonols and flavones are plant-derived polyphenolic compounds that are commonly consumed in the diet. Epidemiological studies indicating that a high dietary intake of flavonols reduces the risk of mortality due to coronary heart disease have provoked an interest in the mechanism of this cardioprotective effect (Graf et al., 2005). Apigenin, a flavone rich in the Chinese herb Flos Chrysanthemi and many other plants, has a variety of pharmacological activities, including anti-inflammatory (Gerritsen...
et al., 1995), antispasmodic (Capasso et al., 1991) and antioxidant (Cos et al., 1998). Our previous study showed that an extract of Flos Chrysanthemi attenuates the decrease of contractile function and coronary flow caused by ischaemia-reperfusion injury in isolated rat heart (Jiang et al., 2004), and causes vasodilatation in rat thoracic aorta (Jiang et al., 2005). However, the exact effects and the underlying mechanism of apigenin on normal aortas and aortas injured by exogenous oxids have not been clarified.

Therefore, the aim of the present study was to investigate whether apigenin induces vasoprotection in rat thoracic aorta with endothelium impairment induced by pyrogallol, and if so, to explore the possible mechanisms.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (4–6 months old and weighing on average 250 g) were obtained from the Experimental Animal Center of Zhejiang Academy of Medical Sciences. All procedures were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). The experiments were approved by the Ethics Committee for the Use of Experimental Animals in Zhejiang University.

2.2. Chemicals

Apigenin, pyrogallol, acetylcholine, phenylephrine, aminoguanidine, indomethacin and Nω-nitro-arginine methyl ester hydrochloride (l-NAME) were purchased from Sigma-Aldrich Inc. (Saint Louis, MO, USA). The kits for measurement of nitrite/nitrate (NO), nitric oxide synthase (NOS) activity, and inhibition of superoxide anion (O2−) were from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Indomethacin was prepared in distilled water containing 0.7% (wt./vol.) sodium carbonate; stock solution of apigenin was prepared in dimethyl sulfoxide (DMSO) and diluted in Krebs’ solution, and the final concentration of DMSO was less than 0.3%. Preliminary experiments ascertained that none of the solvents at the final concentrations used had any effect on the rat thoracic aortic rings. All other reagents were of analytical purity.

2.3. Preparation of rat thoracic aortic rings

Rats were anesthetized with chloral hydrate (400 mg/kg body weight) and killed by cervical dislocation. The descending thoracic aorta was rapidly dissected out and immersed in chilled Krebs’ solution, composed of (mM): NaCl, 118; KCl, 4.7; MgSO4·7H2O, 1.2; KH2PO4, 1.2; CaCl2, 2.5; NaHCO3, 25; and glucose, 11; and bubbled with 95% O2 +5% CO2 (pH 7.4). After the perivascular tissue was carefully removed, aortic rings approximately 4 mm in length were made into a 10% tissue homogenate in ice-cold homogenization medium containing 10 mM Tris–HCl, 0.1 mM EDTA–2Na, 10 mM sucrose, and 0.8% NaCl, pH 7.4. A supernatant was obtained from the homogenate by centrifugation (3000 rpm, 10 min, 4 °C). The NO level, the NOS activity and the inhibition of O2− were computed following the kit manual. Briefly, aortic inhibition of O2− was assayed by the modified xanthine–xanthine oxidase method (Kawczynska-Drozdz et al., 2006). The inhibition of O2− was computed following the kit manual, i.e., inhibition of O2− is the arbitrary unit (U/g protein) calculated from tissue O2− reaction system and standard O2− reaction system in the presence of O2− inhibitor. Aortic NO level was assayed by the Griess method (Zhang et al., 2007). Aortic NOS activity was assayed following the kit protocols prepared by the manufacturer. Briefly, aortic inhibition of O2− was assayed by the modified xanthine–xanthine oxidase method (Kawczynska-Drozdz et al., 2006). The inhibition of O2− was computed following the kit manual, i.e., inhibition of O2− is the arbitrary unit (U/g protein) calculated from tissue O2− reaction system and standard O2− reaction system in the presence of O2− inhibitor. Aortic NO level was assayed by the Griess method (Zhang et al., 2007). Aortic NOS activity was assayed following the kit manual. Briefly, NOS catalyzes l-arginine and O2 into NO, and NO interacts with nucleophilic substances to become a colored compound detected by a spectrophotometer at 530 nm. There are two kinds of NOS, constitutive NOS (endothelial NOS and neuronal NOS, eNOS and nNOS) that are activated by Ca²⁺,
and inducible NOS (iNOS) in which activation is Ca^{2+}-independent. To the iNOS assay tube we added an inhibitor solution which inhibits eNOS and nNOS activity via eliminating Ca^{2+}, and the results represented the iNOS activity (Llorens et al., 2005; Way et al., 1999). For total NOS assay we did not add the inhibitor solution, and the results represented the total NOS activity. The total NOS activity minus the iNOS activity gave the constitutive NOS activity (in the rat aorta the main constitutive NOS is eNOS).

2.5. Data analysis

All data are expressed as mean±S.D. The acetylcholine- or apigenin-induced maximal relaxation (E_{max}) in the aortic rings was calculated as a percentage of the contraction in response to phenylephrine (1 μM). The half maximum effective concentration (EC_{50}) was defined as the concentration of apigenin or acetylcholine that induced 50% of maximum relaxation of the contraction elicited by phenylephrine (1 μM) and was calculated from the concentration–response curve by nonlinear regression (curve fit) using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA). pD_{2} is the negative logarithm of the EC_{50}. Statistical comparisons were made using one-way ANOVA followed by Newman–Keuls test. *P<0.05, **P<0.01 compared with the control group; ***P<0.01 compared with the apigenin group.

3. Results

3.1. Effect of apigenin on relaxation in aorta precontracted by phenylephrine

Apigenin (0.5–72 μM) evoked a concentration-dependent relaxation in endothelium-intact aortic rings precontracted by phenylephrine (Fig. 1); the E_{max} reached 87.6±6.7%, and the pD_{2} was 5.304±0.049. We chose the value of pD_{2} as the experimental concentration of apigenin for subsequent experiments. Pretreatment of the endothelium-intact rings with L-NAME (100 μM) did not abolish but markedly attenuated the apigenin-induced vasorelaxation; the E_{max} fell to 37.1±8.8%. However, indomethacin (10 μM) or aminoguanidine (100 μM) did not influence the relaxation induced by apigenin (Fig. 1). In the endothelium-denuded aortic rings precontracted by phenylephrine, apigenin (0.5–72 μM) also evoked slight vasorelaxation, the E_{max} reached 28.7±7.3% (*P<0.01 vs. the endothelium-intact group, data not shown).

3.2. Effect of pyrogallol on acetylcholine-induced relaxation in aorta precontracted by phenylephrine

In the absence of pyrogallol, the E_{max} of acetylcholine-induced relaxation was 82.0%±2.7%, and the pD_{2} was 7.369±0.121 (Fig. 2). Exposure to pyrogallol (10–1000 μM) for 15 min, which produces O_{2}^{-}, resulted in a dose-dependent decrease of acetylcholine-induced relaxation (Fig. 2A). Specifically, 500 μM pyrogallol decreased the relaxation to acetylcholine compared with that in the absence of pyrogallol (P<0.01); the E_{max} decreased to 55.8±6.6%, and the pD_{2} decreased to 6.559±0.119 (Fig. 2B). No difference was seen when comparing the result of 500 μM pyrogallol to that of 1000 μM pyrogallol (P>0.05). So we selected 500 μM pyrogallol for subsequent experiments. The basal tone of rings was not altered by pyrogallol (data not shown).

3.3. Effect of apigenin on acetylcholine-induced relaxation in aorta pre-incubated with pyrogallol and precontracted by phenylephrine

Pre-incubation with apigenin attenuated the dysfunction of relaxation induced by exposure to pyrogallol (P<0.01 vs. pyrogallol group); the E_{max} increased to 69.5%±6.4% (55.8%±6.6% in pyrogallol group), and the pD_{2} increased to 7.057±0.145 (6.559±0.119 in pyrogallol group). Without exposure to pyrogallol, pre-incubation with apigenin also enhanced the acetylcholine-induced relaxation compared with that of control (P<0.01); the E_{max} increased to 91.6%±3.7% (82.0%±2.7% in control group), and the pD_{2} increased to 7.501±0.076 (P<0.05 vs. 7.369±0.121 in the control group) (Fig. 3A and B).
3.4. Effect of apigenin on O$_2^-$ inhibition, NO level and NOS activity in aorta pre-incubated with pyrogallol

Compared with the control group, the ability to inhibit O$_2^-$ was markedly decreased to 74.5% in the pyrogallol group (P<0.01). Pre-incubation with apigenin restored the inhibition of O$_2^-$ to 94.6% of that in the control group (P<0.01 vs. pyrogallol group), and no significant difference was seen when compared with the control group. Apigenin alone also increased the inhibition of O$_2^-$ to 139.9% compared with that of the control (Fig. 4A).

The NO level was markedly lower to 77.1% in the pyrogallol group compared with the control group. Pre-incubation with apigenin markedly attenuated the reduction of the NO level compared with that of the pyrogallol group, and the value was 94.4% of that in the control group. Apigenin alone also increased the inhibition of O$_2^-$ to 139.9% compared with that of the control (Fig. 4A).

Compared with the control group, exposure to pyrogallol resulted in a decrease of cNOS activity to 35.1%, accompanied by an increase of iNOS activity to 172.4% (P<0.01). Pre-incubation with apigenin attenuated these changes by enhancing the cNOS activity to 62.5% and weakening the iNOS activity to 115.4% of those in the control group (P<0.01 vs. pyrogallol group), but the cNOS activity still decreased compared with that of the controls (P<0.01). Apigenin alone also

Fig. 3. Effect of apigenin (5.0 μM) on acetylcholine (10$^{-9}$ to 10$^{-5}$ M)-induced vaso-relaxation (A) and pD2 values (B) in endothelium-intact rat aortic rings precontracted with 1 μM phenylephrine after exposure to 500 μM pyrogallol for 15 min. Tension was measured and calculated as a percentage of the contraction in response to phenylephrine (1 μM); pD2 is the negative logarithm of the EC$_{50}$. Data expressed as mean±S.D., n=8-11 rings from 8 rats per group. Statistical comparisons were made using one-way ANOVA followed by Newman–Keuls test. *P<0.05, **P<0.01 compared with the control group; ##P<0.01 compared with the pyrogallol group.

Fig. 4. Effect of apigenin (5.0 μM) on inhibition of superoxide anion (O$_2^-$) (A), nitric oxide (NO) level (B), and activity of cNOS and iNOS (C) in endothelium-intact rat aortic rings after exposure to 500 μM pyrogallol for 15 min. Inhibition of O$_2^-$ is the arbitrary unit (U/g protein) calculated from tissue O$_2^-$ reaction system and standard O$_2^-$ reaction system in the presence of O$_2^-$ inhibitor. Data expressed as mean±S.D., n=8 rings from 8 rats per group. Statistical comparisons were made using one-way ANOVA followed by Newman–Keuls test. *P<0.05, **P<0.01 compared with the control group; ##P<0.01 compared with the pyrogallol group.
increased the cNOS activity and had no effect on the iNOS activity compared with those in the control group (Fig. 4C).

4. Discussion

At least two relaxing factors are involved in the endothelium-dependent modulation of vascular tone in rat thoracic aorta, NO and prostacyclin (PGI₂) (Bryan et al., 2005; Palmer et al., 1987). We found that apigenin markedly increased the NO level in aorta and evoked endothelium-dependent vasorelaxation in the aortic rings precontracted with phenylephrine. Pretreatment with l-NAME, an inhibitor of NOS, significantly reduced but did not abolish the apigenin-induced relaxation. Indomethacin, a cyclooxygenase (COX) inhibitor, did not influence the relaxation induced by apigenin. These results suggested that the relaxation induced by apigenin in endothelium-intact aorta involved the NOS–NO pathway, but may not involve the COX–PGI₂ pathway. The relaxation of vascular smooth muscle by NO involves a sequence of steps. NO formed in the endothelium by the activation of NOS, diffuses out of the endothelium to vascular smooth muscle where it binds to and activates soluble guanylyl cyclase (sGC). This enzyme catalyzes the conversion of GTP to cGMP. The rise of cGMP initiates the relaxation of the vascular smooth muscle (Fujita et al., 1998; Marin and Rodriguez-Martinez, 1997). It is believed that there are two types of NOS (cNOS and iNOS) in vivo and each has a particular function. The main cNOS in aorta is endothelial NOS (eNOS) (Forstermann et al., 1998), which is the main source of NO under physiological conditions, while iNOS is activated to produce NO and O₂⁻ under pathological conditions like oxidative stress injury (Maritim et al., 2003).

Considerable evidence indicates that increased vascular oxidative stress plays an important role in endothelial dysfunction (Cai and Harrison, 2000; Thomas et al., 2003). Pyrogallol rapidly auto-oxidizes in an oxygen-containing aqueous medium to generate O₂⁻ (Marklund and Marklund, 1974) and has been used to induce damage from reactive oxygen species in vitro (Bell et al., 2002). In our study, pre-incubation with pyrogallol was used to induce O₂⁻ damage in the isolated rat thoracic aorta, and decreased acetylcholine-induced relaxation in a dose-dependent manner. We selected 500 µM as the experimental concentration of pyrogallol, since at this concentration pyrogallol significantly impaired, but did not abolish the endothelium-dependent relaxation to acetylcholine. An assay of biochemical parameters also indicated that treatment with pyrogallol decreased the ability to inhibit O₂⁻ and the NO level, accompanied by a decrease of the cNOS activity and an increase of the iNOS activity in aorta, which may be responsible for the impairment of relaxation to acetylcholine. O₂⁻ is a major component of reactive oxygen species and the direct inactivation of NO by O₂⁻ is a key event in impairing the endothelium-derived NO bioactivity. For example, the addition of O₂⁻ to the vascular bioassay systems impairs NO-dependent vessel relaxation, and the exogenous superoxide dismutase (SOD) improves the vascular relaxation response to the endothelium-derived NO under acetylcholine-stimulated conditions both in normal and atherosclerotic animals (Gryglewski et al., 1986; Mugge et al., 1991). The underlying mechanism is probably that NO readily reacts with O₂⁻ to generate the highly reactive molecule peroxynitrite (ONOO⁻⁻) (Kissner et al., 1997), and triggers a cascade of harmful events, such as apoptosis, causing uncoupling of NOS, which produces O₂⁻ instead of NO (Maritim et al., 2003). All these reactions contribute to the pathogenesis of endothelial dysfunction, including impairment of the endothelium-dependent vasorelaxation.

Apigenin is as powerful antioxidants, a property common to flavonoids, that are reported to scavenge superoxide anion (O₂⁻•) (Cos et al., 1998) and peroxyl radicals (Dugas et al., 2000) and to inhibit a variety of enzymes responsible for O₂⁻ production (Pietta, 2000). An intake of parsley (Petroselinum crispum), which contains high levels of apigenin, increases erythrocyte glutathione reductase and superoxide dismutase activity in human subjects (Nielsen et al., 1999). Importantly, apigenin is cell-permeable (Choi et al., 2004). We found that, pretreat-

ment with apigenin significantly attenuated the impairment of vasorelaxation and the decrease of O₂⁻ inhibition and the cNOS activity induced by pyrogallol. Although the total NOS activity appeared similar among aortas in the control, the apigenin and the pyrogallol groups, the eNOS activity in the pyrogallol group was the lowest and treatment with apigenin attenuated such reduction. The result of the NO level is consistent with those of the eNOS activity and the inhibition of O₂⁻ in the present study. Previous studies have demonstrated that sufficient superoxide generated from pyrogallol quenches endothelium-derived NO, even under conditions of maximal endothelial stimulation (MacKenzie and Martin, 1998; Mian and Martin, 1995). Therefore, the aortic NO in the pyrogallol group may be quenched by the overproduction of superoxide in spite of the similar total NOS activity compared with the control group. It is also believed that overproduction of NO reacts with superoxide to form peroxynitrite (Kissner et al., 1997), and causes uncoupling of NOS, which produces O₂⁻ instead of NO (Maritim et al., 2003). All these reactions contribute to the low NO level in aorta treated with pyrogallol in spite of the high iNOS activity. Moreover, we found that the functional removal of the endothelium markedly attenuated the apigenin-induced relaxation in aortic rings precontracted with 1 µM phenylephrine, and the E_max decreased to 28.7% ± 7.3% (87.6% ± 6.7% in the endothelium-intact group). The E_max in the endothelium-denuded group was slightly lower than that in the endothelium-intact plus l-NAME group, but there was no significant difference between the two groups. It is suggested that inhibiting both L- and T-type voltage-gated Ca²⁺ currents in the rat thoracic aorta may contribute to the endothelium-independent vasorelaxation of apigenin (No et al., 1991). However, the mechanism responsible for the endothelium-independent vasorelaxation of apigenin needs to be further explored.

In conclusion, we have shown that oxidative stress has a pronounced deleterious effect on vasorelaxation. Apigenin concentration-dependently evoked vasorelaxation in the endothelium-intact aortic rings, and protected against the dysfunction in the relaxation from injury by O₂⁻. This vascular protective effect of apigenin may be mediated by its antioxidant ability and attenuating NO reduction. Thus, our studies suggest that apigenin may be potentially useful for the development of therapeutic treatments for cardiovascular diseases associated with oxidative stress.

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