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Inhibition of superoxide anion-mediated impairment of endothelium by treatment with luteolin and apigenin in rat mesenteric artery

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ABSTRACT

This study was designed (i) to test the hypothesis that the endothelium-derived hyperpolarizing factor (EDHF) component of ACh-induced vasorelaxation and hyperpolarization of smooth muscle cells (SMCs) are impaired following exposure to superoxide anion, and (ii) to further investigate whether luteolin and apigenin induce vasoprotection at the vasoactive concentrations in rat mesenteric artery. Rat mesenteric arterial rings were isolated for isometric force recording and electrophysiological studies. Perfusion pressure of mesenteric arterial bed was measured and visualization of superoxide production was detected with fluorescent dye. 300 μM pyrogallol significantly decreased the relaxation and hyperpolarization to ACh. Luteolin and apigenin both induced vasoprotection against loss of the EDHF component of ACh-induced relaxation and attenuated the impairment of hyperpolarization to ACh. Oxidative fluorescent microtopography showed that either luteolin or apigenin significantly reduced the superoxide levels. The results suggest that superoxide anion impairs ACh-induced relaxation and hyperpolarization of SMC in resistance arteries through the impairment of EDHF mediated responses. Luteolin and apigenin protect resistance arteries from injury, implying that they may be effective in therapy for vascular diseases associated with oxidative stress.

Introduction

Endothelium regulates vascular tone by releasing at least three relaxing factors including nitric oxide (NO) (Palmer et al., 1987), prostacyclin (prostaglandin I_2, PGI_2) (Gryglewski et al., 1986) and endothelium-derived hyperpolarizing factor (EDHF); these factors have variable distributions in different types of blood vessels (Chen et al., 1988). For example, rat mesenteric arteries contribute substantially to regulation of vascular resistance and systemic circulation (Christensen and Mulvany, 1993) and endothelium-dependent relaxation in these arteries is mediated predominantly by EDHF and NO (McCulloch and Randall, 1998), although EDHF plays the key role in the response (Hwa et al., 1994).

Oxidative stress refers to a condition in which cells are subjected to excessive levels of molecular oxygen or its chemical derivatives, termed reactive oxygen species (ROS) (Loscalzo, 2003). Overproduction of ROS under pathophysiological conditions is integral to the development of cardiovascular diseases. This increased oxidative stress may lead to endothelial cell dysfunction and alterations in the release of vasodilator substances (Khalil and Granger, 2002). Endothelium-dependent hyperpolarization and the associated relaxation are reduced in mesenteric arteries from diabetic, hypertensive or aged rats (Onaka et al., 1998; Kansui et al., 2002). Incubation for 6 h with elevated glucose impairs EDHF-mediated relaxation, an observation which allowed us to propose that the release of ROS such as superoxide anion may play a role in this impairment (Ozkan and Uma, 2005). Overall vascular function depends upon a fine balance of oxidant and anti-oxidant mechanisms, which determine endothelial functions. The intracellular oxidant milieu is also involved in several redox-sensitive cellular signaling pathways such as ion transport systems, protein phosphorylation, and gene expression, and thus also plays important roles as modulator of vascular cell functions such as cell growth, apoptosis, migration, angiogenesis and cell adhesion (Rojas et al., 2006). Clearly, factors that can modify ROS release have the potential to protect against cardiovascular disease.

It has been suggested that such protective and anti-oxidant effects may be responsible for the decreased incidence and risk of cardiovascular disease associated with consumption of fruits and vegetables (Rajaram and Sabate, 2000). Flos Chrysanthemi (FC) is the flower of *Chrysanthemum morifolium* Ramat. It is a popular traditional Chinese medicinal herb and designated as a healthy food by the State Ministry of Health of China (Zhao and Ma, 1996). Our previous study showed that an aqueous extract of FC attenuates the decrease of contractile function and coronary flow caused by

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ischemia–reperfusion injury in isolated rat heart (Jiang et al., 2004). It can also increase the coronary flow in isolated rat hearts with high levels of cholesterol (Department of Physiology of Zhejiang Medical University, 1978). A clinical trial demonstrated that FC has beneficial effects on coronary heart diseases, can retard the onset of angina and improves the ECG changes caused by ischemia (Zhao and Ma, 1996). Specifically, the main flavonoids of FC, luteolin-7-*O*-β-d-glucoside and apigenin-7-*O*-β-d-glucoside, can be biotransformed to their aglycone forms – luteolin (Lut, 3′, 4′, 5, 7-tetrahydroxyflavone) and apigenin (Api, 4′, 5, 7-trihydroxyflavone) – by microorganisms in the intestine (Dai et al., 2001; Day et al., 1998). Recently, several experiments demonstrated that an ethyl acetate extract of FC (Jiang et al., 2005b), luteolin (Jiang et al., 2005a) and apigenin (Zhang et al., 2000) cause vasodilatation in rat thoracic aorta. However, the exact effects of total flavonoids of FC (TFFC) and its bioactive components on resistance arteries, and on exogenous oxidant injury in such arteries, have not been clarified.

Therefore, the aim of the present study was to investigate whether luteolin and apigenin induce vasoprotection at the vasoactive concentrations in rat mesenteric artery, and if so, by what possible mechanisms. In particular, the potential vasoprotective effects of luteolin and apigenin on the impairment of endothelium by pyrogallol (PYG) were examined, with special emphasis on reducing the loss of the EDHF component of acetylcholine (ACh)-induced relaxation and hyperpolarization.

Materials and methods

Animals

Male Sprague–Dawley rats (200–300 g, 6–8 weeks old) 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.

Superoxide arterial injury model

Superoxide arterial injury was accomplished through the auto-oxidation of pyrogallol (PYG) added to the organ baths (Marklund and Marklund, 1974; Bell et al., 2002). Additional experiments were performed to determine the effect of exposure to PYG (10, 100, 300 and 1000 μM) for 15 min on ACh-induced (1 nM–1 μM) relaxation in mesenteric arteries precontracted by phenylephrine (PE), and to define the appropriate incubation concentration of PYG. At this incubation concentration, superoxide anion from PYG impaired but did not abolish the endothelium-dependent vasorelaxation. This allowed testing whether any agent exacerbated or attenuated superoxide anion-mediated damage to the artery. Relaxation induced by ACh following exposure to PYG was studied in rings preconstricted by PE, the concentration of which (3–5 μM) was titrated to produce contractions of similar magnitude.

Isometric tension measurement and experimental protocols

Rats were deeply anesthetized with chloral hydrate (400 mg/kg) and then killed by cervical dislocation. The small intestine was removed and pinned to a silastic-filled Petri dish in cold (4 °C) physiological salt solution (PSS). Segments of third-order branches of the mesenteric artery were rapidly excised and dissected into about 2 mm rings as described previously (Watts, 2002; Tsang et al., 2003), and periadventitial fat and connective tissue were removed. Each ring was suspended between two stainless steel wires (diameter 0.0394 mm) in a 5 ml organ bath. One wire was connected to a force transducer (small vessel myograph, DMT 610 M, Danish Myo Technology, Aarhus, Denmark), and the other wire was fixed to the lift arm of the myograph. The organ bath PSS solution was continuously oxygenated with a gas mixture of 95% O_2 and 5% CO_2, and kept at 37 °C (pH 7.4). The vessels were allowed to equilibrate for 30 min and then were set to a tension equivalent to that generated at 90% of the diameter of the vessel at 100 mmHg (Mulvany and Halpern, 1977). The rings were equilibrated for 60 min during which the bath solution was changed every 15 min with readjustment of baseline tension when necessary until a stable resting tension was acquired. Each ring was contracted three times with 60 mM KCl in order to establish a reproducible contractile response.

At the beginning of the experiments, the presence of functional endothelium in mesenteric arterial rings was verified by the ability of ACh (1 μM) to induce more than 90% relaxation in arteries precontracted with PE (3 μM).

After the appropriate incubation concentration of pyrogallol was determined, and 30 min incubation with the NO synthase inhibitor, N^{w}-nitro-l-arginine methyl ester (l-NAME, 100 μM), and the cyclooxygenase inhibitor, indomethacin (Indo, 10 μM) (Cohen and Vanhoutte, 1995; Onaka et al., 1998), experiments were performed to test the effect of exposure to PYG for 15 min on the EDHF component of ACh-induced relaxation of rings precontracted by PE. To assure that the responses were mediated by EDHF, ACh-induced relaxation were tested in the presence of charybdotoxin (ChTX, 200 nM) and apamin (100 nM) which are known to inhibit the intermediate- and small-conductance calcium activated potassium channels (IK_{Ca} and SK_{Ca}, respectively) localized in the endothelium. These inhibitors were added to the bath medium 15 min before the second application of ACh.

The concentration-dependent relaxation induced by luteolin, or apigenin, in endothelium-intact rat mesenteric arteries precontracted by PE (3 μM) was evaluated. The endothelium was mechanically removed by rubbing the luminal surface of the ring several times with a small stainless steel wire (40 μm in diameter). The functional removal of the endothelium was verified if the ring failed to relax in response to 1 μM ACh (Huang et al., 2003). And the effect of luteolin and apigenin on artery were tested in the presence of ChTX (200 nM) and apamin (100 nM).

In additional experiments, mesenteric arterial rings were used to determine whether endothelium-dependent vasorelaxation that had been impaired by exposure to superoxide anion was altered by concurrent exposure to luteolin or apigenin. After these exposures, the rings were rinsed repetitively with PSS and precontracted with PE. For luteolin or apigenin, experiments were performed to determine whether they act *via* the EDHF pathway to attenuate impaired ACh-induced relaxation following exposure to superoxide anion from PYG for 15 min.

The response of mesenteric artery was also evaluated when it was exposed to PYG with an ATP-sensitive K^+ channel opener, pinacidil (0.1 nM–10 μM) (Ozkan and Uma, 2005) or a NO donor, sodium nitroprusside (SNP) (0.1 nM–5.5 μM) (Edwards et al., 1998) which induced endothelium-independent relaxation. To avoid the possibility of time-dependent changes in vascular responsiveness, a single dose–response curve was performed on each rat mesenteric arterial ring.

Measurement of perfusion pressure in mesenteric arterial bed

Rats were given TFFC orally (in a 0.5% sodium carboxymethylcellulose aqueous solution) at the dosage of 200 mg/kg.

About 2 h later, rats were anaesthetized with ether and given an intravenous injection of 1000 units/kg of heparin and then killed by cervical dislocation. Following the injection, a midline incision was made, and the mesenteric arterial bed rapidly dissected out and placed into PSS solution. The mesenteric artery and vein were tied off