College of Pharmaceutical Sciences[1], Zhejiang University; Department of Physiology[2], Zhejiang University School of Medicine, Hangzhou, China; Department of Physiology[3], the University of Hong Kong, Hong Kong, China

Luteolin induces vasorelaxion in rat thoracic aorta via calcium and potassium channels

HUIDI JIANG[1], QIANG XIA[2], XIAOXIA WANG[1], JIANFENG SONG[1], I. C. BRUCE[3]

Received July 19, 2004, accepted July 29, 2004

Huidi Jiang, College of Pharmaceutical Sciences, Zhejiang University, 353 Yan'an Road, Hangzhou 310031, China
hdjiang@zju.edu.cn

Pharmazie 60: 444–447 (2005)

The aims of the present study were to investigate the vasoactive effects of luteolin and its mechanisms of action on the rat thoracic aorta. Luteolin (4.5–36 μmol/L) caused a concentration-dependent relaxation of endothelium-intact or endothelium-denuded aortic rings precontracted with phenylephrine (PE, 10^{-6} mol/L) or a high level of K^+ (6×10^{-2} mol/L). Luteolin induced a shift of the PE concentration-response curve to the right and downward. L-NAME and propranolol did not influence the vascular effect of luteolin. However, 5-hydroxydecanoate, tetraethylammonium, $BaCl_2$ and 4-aminopyridine significantly attenuated the vasorelaxant effect of luteolin. In Ca^{2+}-free medium, medium with graded concentrations of Ca^{2+}, or K^+-free solution, luteolin reduced PE-induced contraction. It is concluded that luteolin induces endothelium-independent relaxation in rat thoracic aorta. The mechanism involves the inhibition of sarcolemmal Ca^{2+} channels, release from intracellular Ca^{2+} stores and activation of K^+ channels.

1. Introduction

Luteolin, a flavone derived from Chinese herbs such as *Chrysanthemum morifolium* Ramat., *Elsholtzia blanda* Benth., and *Euphorbia humifusa* Willd., has a variety of pharmacological actions, including inhibition of proliferation and collagen synthesis in hepatic cells (Zhao et al. 2002), an antioxidant effect (Areias et al. 2001), and shows inhibition of protein kinase C activity (Agullo et al. 1997). Luteolin lowers blood pressure in rats (Wang et al. 1986) and increases coronary flow by reducing coronary artery resistance in dogs (Wang et al. 1992). A direct vasodilation is induced by luteolin in rat isolated thoracic aorta (Chan et al. 2000). However, the mechanism by which luteolin causes vasorelaxation has not been elucidated. In the present study, we investigated this vasorelaxant effect and the mechanism underlying it.

2. Investigations and results

2.1. Vasorelaxant effect of luteolin

Luteolin reduced the vascular tension induced by PE equally in endothelium-intact and endothelium-denuded aortic rings in a concentration-dependent manner (Fig. 1). Pretreatment with L-NAME did not influence luteolin-induced relaxation in endothelium-intact aorta.

Rapid application of a high extracellular concentration of KCl (60 mmol/L) induced aortic contraction. Luteolin also inhibited the KCl-induced contraction in endothelium-denuded aortic rings in a concentration-dependent manner (Fig. 2).

Phenylephrine contracted the rat isolated aortic rings in a concentration-dependent manner. After a control curve for the phenylephrine-induced response was constructed, the rings were incubated for 30 min with different concentrations of luteolin and a concentration-response curve for phenylephrine was again obtained. As shown in Fig. 3, luteolin at 9 μmol/L and 36 μmol/L caused a shift of the PE concentration-response curve downward and to the right.

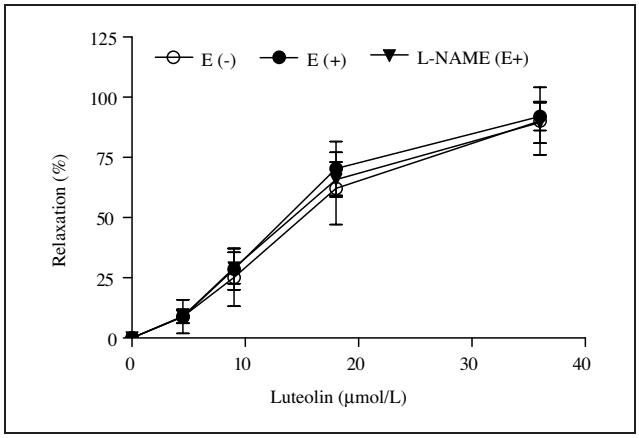

Fig. 1: Effect of luteolin on tension in phenylephrine (10^{-6} mlo/L) precontracted aortic rings with endothelium [E(+)] (n = 15) or without endothelium [E(−)] (n = 15) and the effect of 10^{-4} mol/L L-NAME on luteolin-induced relaxation in endothelium-intact aortic rings [L-NAME(E+)] (n = 10). Values are expressed as $\overline{X} \pm s$

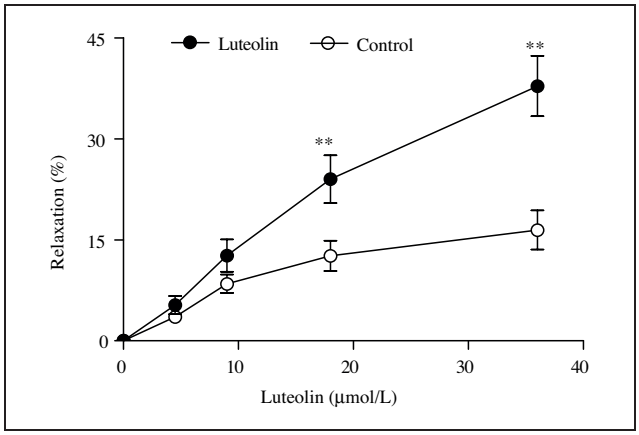

Fig. 2: Effect of luteolin on KCl-induced contraction. KCl at a concentration of 60 mmol/L was applied. Control: same volume of solvent as luteolin group. Values are expressed as $\overline{X} \pm s$, $n = 10$
* $P < 0.05$, ** $P < 0.01$ compared with control

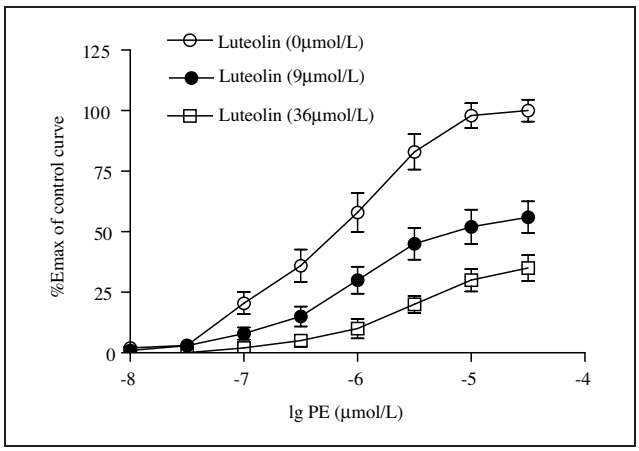

Fig. 3: Concentration-response curves of rat aortic rings to phenylephrine (PE) in the absence or presence of luteolin. Data are expressed as percentages of the maximum contraction obtained in the control concentration-response curve (Luteolin 0 μmol/L). Values are expressed as $\overline{X} \pm s$, $n = 8$

2.2. Effect of propranolol on luteolin-induced vasorelaxation

Pretreatment of the endothelium-intact rings with 10^{-6} mol/L propranolol, a β-adrenoceptor antagonist, did not attenuate the vasorelaxation induced by luteolin (Fig. 4).

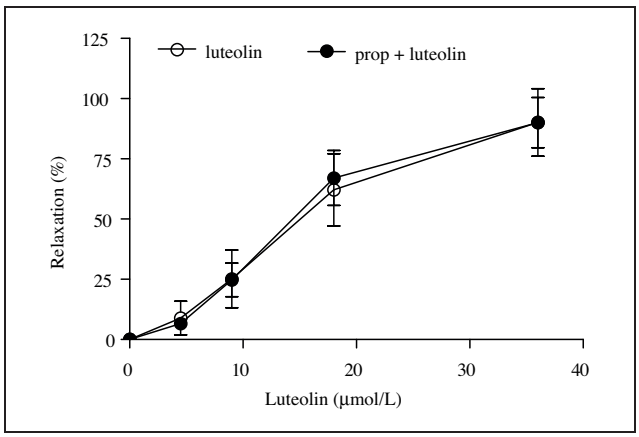

Fig. 4: Effect of propranolol (prop, 10^{-6} mol/L) on luteolin-induced relaxation in the endothelium-intact aorta. Values are expressed as $\overline{X} \pm s$, $n = 10$

2.3. Relationship between luteolin-induced relaxation and Ca^{2+} channels

In Ca^{2+}-free solution, after PE induced a stable aortic contraction, Ca^{2+} was cumulatively added to the bath, the tension generated by the aortic rings increased with the concentration of Ca^{2+}. Incubation of the aortic rings with 18 μmol/L luteolin for 10 min before PE application significantly inhibited the Ca^{2+}-dependent contraction (Fig. 5).

In K^+-free solution, luteolin also significantly inhibited the contraction induced by 10^{-6} mol/L PE ($P < 0.05$) (Fig. 6).

In endothelium-denuded rings, a transient contractile response in Ca^{2+}-free solution was elicited by 10^{-6} mol/L PE. A second contraction was then induced by PE in the absence or presence of luteolin. Pretreatment of the aortic rings with 18 μmol/L luteolin for 10 min clearly reduced the ratio (con2/con1) of the responses to PE (0.853 ± 0.0141 vs 0.987 ± 0.0119, $n = 8$, $P < 0.01$).

2.4. Effect of K^+ channel blockers on luteolin-induced relaxation

In endothelium-denuded aortic rings, luteolin induced a concentration-dependent relaxation, which was signifi-

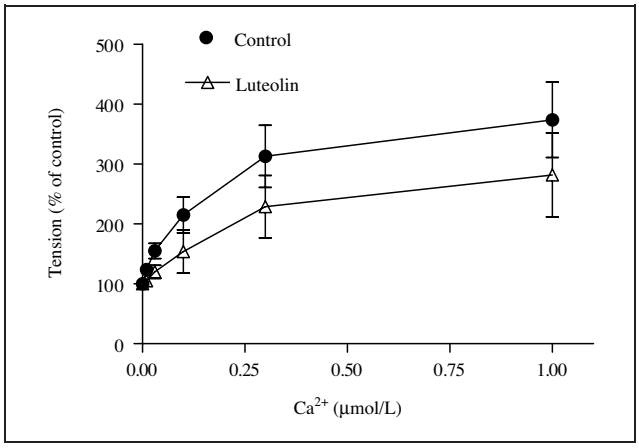

Fig. 5: Concentration-response curves for the effect of Ca^{2+} on PE-contracted endothelium-denuded aortic rings in the presence and absence of luteolin. All values are expressed as $\overline{X} \pm s$, $n = 10$
* $P < 0.05$, ** $P < 0.01$ compared with control

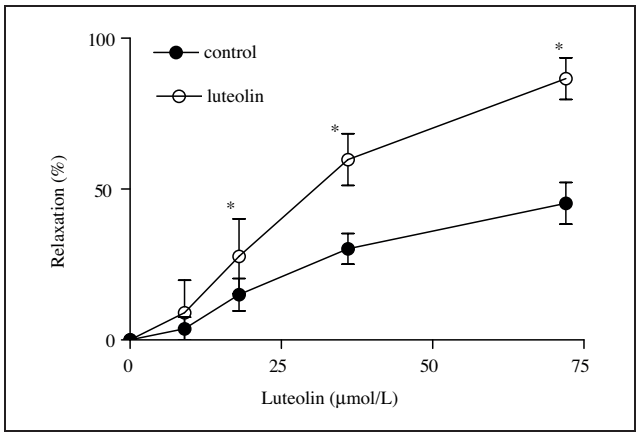

Fig. 6: Effect of luteolin on PE-contracted endothelium-denuded aortic rings in K^+-free K-H solution. All values are expressed as $\overline{X} \pm s$, $n = 10$
* $P < 0.05$, ** $P < 0.01$ compared with control

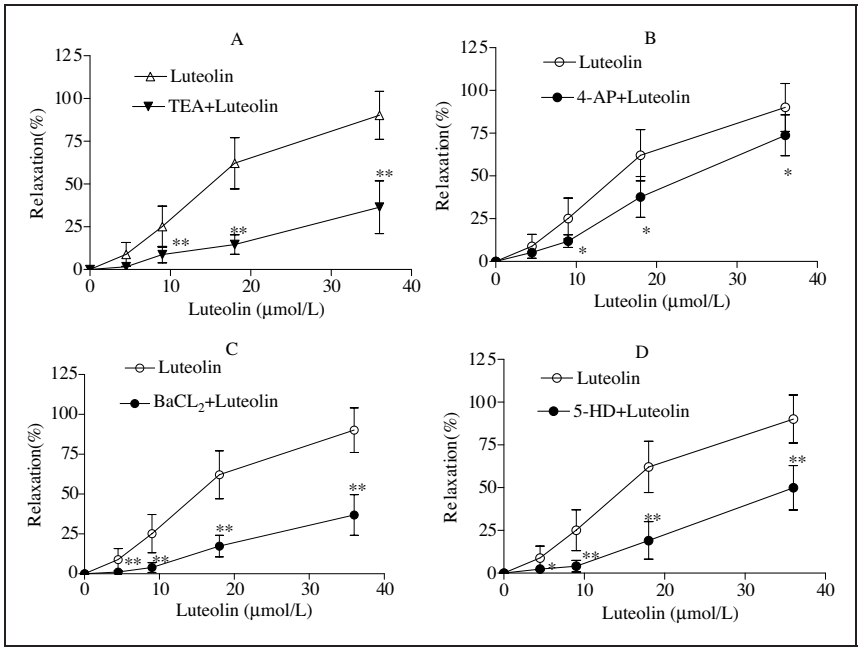

Fig. 7:
Effect of 5×10^{-3} mol/L TEA (A), 10^{-4} mol/L 4-AP (B), 10^{-4} mol/L $BaCl_2$ (C), and 10^{-4} mol/L 5-HD (D) on luteolin-induced relaxation in endothelium-denuded aortic rings. Values are expressed as $\overline{X} \pm s$, $n = 10$
* $P < 0.05$, ** $P < 0.01$ compared with luteolin group

cantly inhibited by 5×10^{-3} mol/L TEA (Fig. 7A), 10^{-4} mol/L 4-AP (Fig. 7B), 10^{-4} mol/L $BaCl_2$ (Fig. 7C) and 10^{-4} mol/ L 5-HD (Fig. 7D).

3. Discussion

The present study revealed a relaxant action of luteolin both in endothelium-intact and -denuded rat aortic rings precontracted by PE. Preincubation of endothelium-intact aortic rings with L-NAME, an inhibitor of nitric oxide synthase, did not affect the luteolin-induced vasorelaxation. Therefore, luteolin exerted an endothelium-independent relaxation.

Phenylephrine activates α-adrenoreceptors in vascular smooth muscle to cause muscle contraction in a concentration-dependent manner. Luteolin at 9 μmol/L and 36 μmol/L caused a right-shift and a downshift of the PE concentration-response curve, suggesting that luteolin acts as a non-competitive antagonist against the phenylephrine-induced contraction. In other words, luteolin had no specific antagonistic effect on α-adrenoreceptors. On the other hand, propranolol, a β-adrenoreceptor antagonist, also had no effect on the luteolin-induced relaxation, indicating that this relaxation was not related to activation of β-adrenoreceptors in vascular smooth muscle.

There are two kinds of Ca^{2+} channels in the vascular smooth muscle: receptor-operated Ca^{2+} channels (ROCC) and voltage-dependent Ca^{2+} channels (VDCC), which can be activated by PE and high extracellular K^+, respectively. Influx of extracellular Ca^{2+} through ROCC and VDCC and release of Ca^{2+} from the sarcoplasmic reticulum result in increased intracellular Ca^{2+} concentration, which causes vascular smooth muscle contraction. PE caused aortic contraction through release of Ca^{2+} from the sarcoplasmic reticulum and by Ca^{2+} influx through ROCC. Luteolin inhibited the contraction of aortic rings induced by PE, implying that luteolin inhibits the release of intracellular Ca^{2+} and/or blocks ROCC. In K^+-free solution, VDCC are inactivated. Luteolin reduced the tension induced by PE in K^+-free medium, which also reveals that luteolin acts on ROCC and/or inhibits release of Ca^{2+} from sarcoplasmic reticulum.

Luteolin reduced the contraction of aortic rings exposed to PE in solutions of gradually increasing Ca^{2+} concentration. This suggests that luteolin can inhibit the Ca^{2+} influx caused by PE, which means that luteolin blocked ROCC. On the other hand, luteolin decreased the ratio of second to first contraction caused by PE in Ca^{2+} free solution, indicating that luteolin reduced the release of Ca^{2+} from the sarcoplasmic reticulum.

KCl-induced contraction mainly results from the influx of Ca^{2+} upon depolarization of the cell membrane, which activates VDCC (Xiong and Sperelakis 1995). In the present study, luteolin attenuated KCl-induced vasoconstriction in endothelium-denuded rat aorta, indicating that luteolin may inhibit VDCC.

K^+ channels play important roles in the regulation of muscle contractility and vascular tone. In many instances, the vasodilation mediated by membrane hyperpolarization is attributed to a rise in K^+ permeability (Nelson and Quayle 1995). Direct activation of K^+ channels on arterial smooth muscle cells normally hyperpolarizes the cell membrane and thus inhibits Ca^{2+} influx through VDCC. There are several types of K^+ channels present in the vascular smooth muscle that can be modulated by various factors. ATP-sensitive K^+ channels (K_{ATP}), Ca^{2+}-activated K^+ channels (K_{Ca}), voltage-dependent K^+ channels (K_V) and inward-rectifier K^+ channels (K_{IR}) can be blocked by glibenclamide, TEA, 4-AP and $BaCl_2$, respectively (Ferrer and Marin et al. 1999). 5-HD is the inhibitor of the mitochondrial ATP-sensitive K^+ channel (mito-K_{ATP}). The results of the present study show luteolin-induced relaxation in endothelium-denuded arteries was reduced by 5-HD, TEA, 4-AP and $BaCl_2$, suggesting that luteolin's mechanism of action is related to an activation of various sarcolemmal K^+ channels including mito-K_{ATP}, K_{Ca}, K_V and K_{IR}. It is known that K_{ATP} plays an important role in the cardiovascular system, especially in antagonizing the effects of ischemia-reperfusion injury (Seino and Takashi 2003), so the effect of luteolin on ischemia-reperfusion in the heart deserves further investigation.

In conclusion, the present results show that luteolin induced endothelium-independent relaxation in the rat thoracic aorta via a series of mechanisms, including the inhibition of

sarcolemmal calcium channels, release of intracellular Ca^{2+} stores and activation of K^+ channels.

4. Experimental

4.1. Materials

Luteolin, N^G-nitro-L-arginine methyl ester (L-NAME), 4-aminopyridine (4-AP), tetraethylammonium (TEA), 5-hydroxydecanoate (5-HD), $BaCl_2$, propranolol, phenylephrine (PE), acetylcholine (Ach) and EGTA were purchased from Sigma Chemical Co. All other reagents were of analytical purity.

Male Sprague-Dawley rats (230–250 g) were obtained from the Experimental Animal Center of Zhejiang Academy of Medical Sciences. All procedures were approved by the Animal Care Committee.

4.2. Preparation of rat aorta

Rats were killed by stunning and cervical dislocation, and the thoracic aorta was immediately removed and placed in 4 °C modified Krebs-Henseleit (K-H) solution (in mmol/L: 118.0 NaCl, 4.7 KCl, 1.2 K_2PO_4, 1.2 $MgSO_4$, 25.0 $NaHCO_3$, 1.25 $CaCl_2$, 10.0 Glucose). The aorta was cleaned of adhering fat and connective tissue and cut into 3 mm wide rings. Care was taken to avoid abrading the intimal surface in order to maintain the integrity of the endothelial layer. Endothelium was removed by gently rubbing the intimal space with a cotton swab. Aortic rings were suspended in organ chambers containing 10 ml K-H solution at 37 °C, aerated with 95% O_2/5% CO_2. After equilibration under no tension for 15 min, the vessel segments were allowed to equilibrate for 1 h at a resting tension of 2 g. During the equilibration period, K-H solution was changed every 15 min. Changes in tension were recorded by isometric transducers connected to a data acquisition system (PowerLab, AD Instruments). Before each experiment, rings were stimulated at least 3 times with 6×10^{-2} mol/L KCl until a reproducible contractile response was obtained. The presence of functional endothelium was verified by the ability of acetylcholine (10^{-5} mol/L) to induce more than 70% relaxation of aortic rings precontracted with phenylephrine (PE, 10^{-6} mol/L).

4.3. Experimental protocols

4.3.1. Effect of luteolin on contraction of aorta

In this series of experiments, an attempt was made to verify luteolin-induced relaxation. Phenylephrine (10^{-6} mol/L) or KCl (6×10^{-2} mol/L) was used to induce steady contraction in endothelium-intact or -denuded rings. Luteolin (4.5 to 36 μmol/L) was added cumulatively.

4.3.2. Luteolin-induced vasorelaxation and α-adrenoceptors

In this series of experiments, endothelium-intact rings were contracted with PE applied cumulatively (10^{-8} to 10^{-5} mol/L) to obtain the first concentration-response curve. Once the maximal response to PE had been obtained, the preparations were washed with K-H solution until the tension returned to the basal level. The rings were then exposed to luteolin for 30 min and another cumulative concentration-response curve to PE was obtained.

4.3.3. Luteolin-induced vasorelaxation and β-adrenoceptors

To determine whether the vasorelaxation caused by luteolin was related to the activation of β-adrenoceptors, endothelium-intact aortic rings were pretreated with propranolol at 10^{-6} mol/L for 30 mim before 10^{-6} mol/L PE was added to the bath, and then luteolin was added cumulatively.

4.3.4. Luteolin-induced vasorelaxation and calcium channels

In the first set of experiments, an attempt was made to verify that the vasorelaxation induced by luteolin involved Ca^{2+} influx. Aortic rings were washed with Ca^{2+}-free solution 4–5 times before PE (10^{-6} mol/L) application to produce a steady contraction, and then Ca^{2+} was added cumulatively to obtain a concentration-response curve (0.01–1 mmol/L). Luteolin (18 μmol/L) was added 10 min before the addition of Ca^{2+}.

In the second set of experiments, the aim was to clarify whether luteolin exerted the vasorelaxant effect through receptor-operated Ca^{2+} channels. Aortic rings were washed 4–5 times with K^+-free solution, then PE (10^{-6} mol/L) was applied to induce a stable contraction before luteolin was added cumulatively.

The third set of experiments was designed to elucidate whether the vasorelaxation induced by luteolin was related to inhibition of intracellular Ca^{2+} release. The rings were exposed to Ca^{2+}-free solution with 50 μmol/L EGTA for 15 min before the application of 10^{-6} mol/L PE to induce the first transient contraction (con1). The rings were then washed three times with normal K-H solution and incubated for at least 40 min for refilling of the intracellular stores. Subsequently, the medium was rapidly replaced with Ca^{2+}-free solution and the rings were incubated for another 15 min. The second contraction (con2) was then induced by 10^{-6} mol/L PE in the absence or presence of 18 μmol/L luteolin, which was added 10 min before PE application. The ratio of the second contraction over the first contraction (con2/con1) was calculated.

4.3.5. Luteolin-induced vasorelaxation and K^+ channels

To demonstrate the possible role of K^+ channels in luteolin-induced vasorelaxation, endothelium-denuded aortic rings were incubated with the K^+ channel blockers 5-HD, 4-AP, TEA and $BaCl_2$, for 30 min before PE was added to the bath.

4.4. Data analysis and statistics

Data were expressed as $\overline{X} \pm s$. Comparisons were made by Student's t test. P values less than 0.05 were considered significant.

Acknowledgements: This study was supported by grants from the Bureau of Zhejiang Traditional Chinese Medicine (G20010358) and the Department of Science and Technology in Zhejiang Province (G20020578).

References

Agullo G, Gamet-Payrastre L, Manenti S et al. (1997) Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: a comparison with tyrosine kinase and protein kinase C inhibition. Biochem Pharmacol 53:1649–1657.

Areias FM, Rego AC, Oliveira CR (2001) Antioxidant effect of flavonoids after ascorbate/Fe^{2+}-induced oxidative stress in cultured retinal cells. Biochem Pharmacol 62: 111–118.

Chan EC, Pannangpetch P, Woodman OL (2000) Relaxation to flavones and flavonols in rat isolated thoracic aorta: mechanism of action structure-activity relationships. J Cardiovasc Pharmacol 35: 326–333.

Ferrer M, Marin J, Encabo A et al. (1999) Role of K^+ channels and sodium pump in the vasodilation induced by acetylcholine, nitric oxide, and cyclic GMP in the rabbit aorta. General Pharmacol 33: 35–41.

Nelson MT, Quayle JM (1995) Physiological roles and properties of potassium channels in arterial smooth muscle. Amer J Physiol 268, C799-C822.

Seino S, Takashi M (2003) Physiological and pathophysiological roles of ATP-sensitive K^+ channels. Progr Biophys Mol Biol 81: 133–176.

Wang L, Han C, Wang P (1986) Experimental study on hypotensive effect of semi-synthesis luteolin. Chin Pharmacol Bull 2(2): 34–36.

Wang L, Han C, Wang Pin (1992) Experiment research on luteolin affecting coronary circulation dynamics. Chin Pharmacol Bull 8(5): 388–390.

Xiong Z, Sperelakis N. (1995) Regulation of L-type calcium channels of vascular smooth muscle cells. J Mol Cell Cardiol 27: 75–91.

Zhao WX, Liang CL, Ceng ZM (2002) Luteolin inhibits proliferation and collagen synthesis of hepatic cells. Chin J Hepatol 10: 204–206.