Luteolin reduces high glucose-mediated impairment of endothelium-dependent relaxation in rat aorta by reducing oxidative stress

Ling-Bo Qian, Hui-Ping Wang, Ying Chen, Fang-Xia Chen, Yan-Yan Ma, Iain C. Bruce, Qiang Xia*

Department of Physiology, Zhejiang University School of Medicine, 388 Yuhangtang Road, Hangzhou 310058, China

ARTICLE INFO

Article history:
Received 28 June 2009
Received in revised form 23 October 2009
Accepted 23 October 2009

Keywords:
Luteolin
Aorta
Endothelium
Vasorelaxation
High glucose
Oxidative stress

ABSTRACT

While luteolin, a flavone rich in many plants, has some cardiovascular activity, it is not clear whether luteolin has beneficial effects on the vascular endothelial impairment in hyperglycemia/high glucose. Here, we reveal the protective effect of luteolin on endothelium-dependent relaxation in isolated rat aortic rings exposed to high glucose. The thoracic aorta of male Sprague–Dawley rats was rapidly dissected out and the effect of luteolin on the tension of aortic rings pretreated with high glucose (44 mM) for 4 h was measured in an organ bath system. The levels of nitric oxide (NO), hydroxy radical (OH·) and reactive oxygen species (ROS), and the activity of superoxide dismutase (SOD) and nitric oxide synthase (NOS) were measured in aortas. The vasorelaxation after treatment with luteolin for 8 weeks in aortic rings from diabetic rats was also determined. We found that exposure to high glucose decreased acetylcholine-induced endothelium-dependent relaxation. However, high mannitol had no effect on vasorelaxation. Luteolin evoked a concentration-dependent relaxation in aortic rings previously contracted by phenylephrine, and the pD2 value was 5.24 ± 0.04. The EC50 of luteolin markedly attenuated the inhibition of relaxation induced by high glucose, which was significantly weakened by pretreatment with l-NAME (0.1 mM), but not by indomethacin (0.01 mM). Luteolin significantly inhibited the increase of ROS level and OH· formation, and the decrease of NO level, NOS and SOD activity caused by high glucose. The improving effect of luteolin on endothelium-dependent vasorelaxation in diabetic rat aortic rings was reversed by pretreatment with l-NAME or methylene blue. The results indicate that the decrease of endothelium-dependent relaxation in rat aortic rings exposed to high glucose is markedly attenuated by luteolin, which may be mediated by reducing oxidative stress and enhancing activity in the NOS–NO pathway.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

Diabetes, a common metabolic disease characterized by chronic hyperglycemia, affects 4% of the population. It leads to cardiovascular disease and, worldwide, more than 171 million in 2000 and an expected 366 million in 2030 will be at risk [1]. Hyperglycemia-induced vascular disease is the principal cause of morbidity and mortality in patients with diabetes, and endothelial dysfunction is a potential initiating factor for hyperglycemic vascular diseases [2]. The endothelium plays a crucial role in regulating vascular tone and structure by producing vasoactive mediators including nitric oxide (NO), prostacyclin, and endothelin, and impaired endothelium-dependent relaxation has been demonstrated in various vascular beds of different models of diabetes or hyperglycemia [2–4]. Prolonged exposure to high glucose in vitro or in vivo generates reactive oxygen species [2], impairs the bioactivity of NO [5,6], and inhibits acetylcholine (ACh)-induced endothelium-dependent relaxation [4,7]. It has been suggested that oxidative stress induced by hyperglycemia plays a key role in the pathogenesis of both microvascular and macrovascular complications [2,8], and an early marker of such damage is the development of endothelial dysfunction [2,8,9]. However, the role of oxidative stress in diabetic cardiovascular diseases is questioned by the results of intervention studies with antioxidants such as vitamins C and E, which are unclear or unsuccessful [10–12]. These results indicate that the role of oxidative stress in hyperglycemic endothelial dysfunction is complex and certain vasoactive agents with anti-oxidative characteristics may help to reduce such dysfunction.

Flavonols and flavones are plant-derived polyphenolic compounds that are commonly consumed in the diet. Epidemiological studies indicating that high dietary intake of flavonols reduces the risk of mortality due to coronary heart disease have provoked interest in the mechanism of this protective effect [13]. Luteolin, a flavone rich in the Chinese herb Flos Chrysanthemi (FC, the flower of Chrysanthemum morifolium Ramat.) and many other plants, has
a variety of pharmacological activities, including antihypertensive [14], anti-inflammatory [15], and anti-oxidative [16] effects.

Our previous studies showed that an aqueous extract of FC attenuates the decrease of contractile function and coronary flow caused by ischemia-reperfusion injury in isolated rat heart [17], and that an ethyl acetate extract of FC [18] causes endothelium-dependent relaxation in rat thoracic aorta at least partly through the NO-cyclic 3′-5′-guanosine monophosphate (cGMP) axis. Recently, we found that luteolin from FC protects ACh-induced endothelium-dependent relaxation against superoxide anion injury in resistance arteries [16], implying that luteolin is effective in therapy for vascular diseases associated with oxidative stress. However, it is not known whether luteolin has beneficial effects on vascular endothelial function in hyperglycemia. We hypothesized that luteolin may attenuate endothelial dysfunction induced by high glucose via its modulation of the vascular NO pathway and oxidative stress.

Therefore, the aim of this study was to explore the effect of luteolin on endothelial dysfunction induced by high glucose in aortic rings isolated from rats, and clarify the underlying mechanism. The effects of luteolin on ACh–induced vasorelaxation, NO level, reactive oxygen species (ROS) level, hydroxy radical (OH•) formation, and activity of nitric oxide synthase (NOS) and superoxide dismutase (SOD) in aortas isolated from non-diabetic rats and incubated in high glucose were measured. We also determined the effects of luteolin on ACh-induced vasorelaxation in aortas from streptozotocin (STZ)-induced diabetic rats.

2. Materials and methods

2.1. Chemicals

Luteolin was from Hangzhou Skyherb Technologies Co., Ltd. (Hangzhou, China), and the purity was 99.4% by HPLC. Streptozotocin (STZ), ACh, phenylephrine (PE), Nω-nitro-l-arginine methyl ester hydrochloride (l-NAME), methylene blue (MB) and indomethacin (Indo) were from Sigma–Aldrich Inc. (Saint Louis, MO, USA). 2′,7′-dichlorofluorescin-diacetate (DCFH-DA) was from Molecular Probes (Eugene, OR, USA). The kits for measurement of NO, OH• formation, and activity of NOS and SOD were from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Indo was prepared in distilled water containing 0.7% (wt/vol) sodium carbonate. Stock solutions of luteolin were prepared in dimethyl sulfoxide (DMSO) and diluted in Krebs’ solution, and the final concentration of DMSO was less than 0.03% (v/v). Preliminary experiments ascertained that none of the solvents at the final concentrations used had any effect on rat thoracic aortic rings. All other reagents were of analytical purity.

2.2. Animals

Male Sprague–Dawley rats (four to six months old and weighing on average 250 g) were obtained from the Experimental Animal Center of Zhejiang University. 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.

2.3. Preparation of rat thoracic aortic rings and bioassay of vasoreactivity

Rats were anesthetized with chloral hydrate (400 mg kg−1, i.p.) 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 cut. In some rings, the endothelium was mechanically removed by gentle rubbing with moistened cotton. For isometric force recording, aortic rings were mounted between two stainless steel hooks and suspended in a 10 ml organ bath containing Krebs’ solution at 37 °C bubbled with 95% O₂ + 5% CO₂ (pH 7.4). After equilibration under no tension for 30 min, the aortic rings were allowed to equilibrate for 1 h at a resting tension of 2 g. During the equilibration period, Krebs’ solution was changed every 15 min. Changes in tension were recorded by isometric transducers connected to a data acquisition system (PowerLab, ADInstruments Shanghai Trading Co., Ltd., China). Before each experiment, rings were stimulated three times with 60 mM KCl until a reproducible contractile response was obtained. The presence of functional endothelium was verified by the ability of ACh (10 μM) to induce more than 80% relaxation of aortic rings previously contracted by PE (1 μM). The model of high glucose-induced aortic endothelial impairment was based on the methods of our previous study [7]. Briefly, after 4 h incubation in high glucose (44 mM), the ring was contracted by PE (1 μM) in fresh Krebs’ solution. When the developed tension attained its peak value, the ring was relaxed by ACh. Cumulative concentration–response curves to ACh (0.001–10 μM) were generated.

2.4. Assay of NO level, OH• formation, NOS and SOD activity

After 4 h incubation, the aortic rings were blotted dry and weighed, then made into a 10% tissue homogenate in ice-cold homogenizing 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 tissue homogenate by centrifugation (3000 rpm, 10 min, 4 °C). Following the commercial kit manual, in this supernatant, the NO level was assayed by the Griess method [19]; OH• formation was evaluated by the Fenton reaction [20]; and SOD activity was assayed by the xanthine–xanthine oxidase method [7]. Aortic total NOS (tNOS) (endothelial NOS [eNOS] + neuronal NOS [nNOS]) and iNOS activity were assayed following the kit manual [7,21]. The tNOS activity minus the iNOS activity gave the constitutive (cNOS) activity (in rat aortas the main cNOS is eNOS).

2.5. Measurement of ROS release using the fluorescent probe DCFH-DA

Determination of ROS was based on the methods of Ugochukwu and Cobourne [22] and Gao et al. [23] with modifications. After the supernatant was obtained from the aortic homogenate as above, it was pipetted into 64-well plates (0.18 ml well−1) loaded with 10 μM DCFH-DA for 30 min at room temperature in the dark. Levels of DCFH-DA in the supernatant were measured by spectrofluorometer using 488 nm excitation/530 nm emission. Background fluorescence (conversion of DCFH to DCF in the absence of homogenate) was corrected by the inclusion of parallel blanks. ROS production was expressed as fluorescence units [F (mg protein)−1].

2.6. Induction of diabetes

Animals were divided into four groups: normal controls, luteolin controls, diabetic rats, and diabetic rats treated with luteolin. The normal control group (n = 5) and luteolin control group (n = 5) received 0.1 M citrate buffer, the solvent for STZ, intraperitoneally. Diabetes was induced by a single dose of STZ (60 mg kg−1, i.p.). About 72 h after STZ treatment, fasted glucose levels in blood taken from the tail vein early in the morning were measured.
using OneTouch Ultra 2 glucose test strips (LifeScan, Milpitas, CA, USA). Rats with glucose levels >15 mM were considered diabetic. After the onset of diabetes, the animals were divided into two groups: a diabetic control group (n = 5), and diabetic groups intra-gastrically administered a low (10 mg kg\(^{-1}\) d\(^{-1}\), n = 5), a medium (50 mg kg\(^{-1}\) d\(^{-1}\), n = 5), or a high dose (100 mg kg\(^{-1}\) d\(^{-1}\), n = 5) of luteolin dissolved in 0.5% (w/v) of carboxymethyl cellulose for 8 weeks.

2.7. Protocols of experiments

The first series of experiments was designed to evaluate the effect of luteolin on vasorelaxation. Steady contraction was induced in endothelium-intact aortic rings with 1 μM PE, and then luteolin (0.5–90.0 μM) was added cumulatively. The half-maximum effective concentration (EC\(_{50}\)) was defined as the concentration of luteolin that induced 50% of maximum relaxation from the contraction elicited by PE (1 μM), was calculated.

The second series of experiments was designed to evaluate the protective effects of luteolin against the ACh-induced endothelium-dependent relaxation of aortic rings incubated with high glucose. Experiments were carried out in 7 groups with 8–10 aortic rings from 8 rats in each group. First, a normal control bioassay of vasoreactivity was performed in Krebs’ solution. Rings that contracted in response to PE (1 μM) and relaxed more than 80% in response to ACh (10 μM) were considered to have intact endothelium and were used in the study. As in our previous study [7], with modifications, the aortic rings in each group were then incubated for 4 h in the following media: (1) control: 11 mM glucose in Krebs’ solution; (2) luteolin alone: 5.74 μM luteolin in Krebs’ solution with 11 mM glucose; (3) mannitol: 33 mM mannitol and 11 mM glucose in Krebs’ solution; (4) high glucose: 44 mM glucose in Krebs’ solution; (5) luteolin-treated: 5.74 μM luteolin in Krebs’ solution with 44 mM glucose; (6) L-NAME + luteolin: 0.1 mM L-NAME and 5.74 μM luteolin in Krebs’ solution with 44 mM glucose; and (7) Indo + luteolin: 0.01 mM Indo and 5.74 μM luteolin in Krebs’ solution with 44 mM glucose. The incubation media were changed every 30 min, and luteolin was introduced for the last 30 min of incubation. L-NAME or Indo was added for 30 min then washed before luteolin administration. After 4 h incubation, the perfusion solution was changed to Krebs’ solution with 11 mM glucose, and bioassay for vasoreactivity was performed (Fig. 1).

After 4 h incubation the NO and ROS levels, OH• formation, and NOS and SOD activity in the rings were measured. Experiments were carried out on 5 groups with 8 aortic segments of 3 cm from 8 rats in each group as follows: control, luteolin alone, mannitol, high glucose, and luteolin-treated. The incubation media and procedures were as above. When tissues were incubated with high glucose solution for biochemical assays, PE was not added and all tissues were under 0 g tension. At the end of 4 h, the segments were incubated in normal Krebs’ solution in the presence of ACh (10 μM) for 30 min before biochemical parameter measurements.

A third set of experiments was designed to evaluate the effects of chronic treatment with luteolin for 8 weeks on ACh-induced endothelium-dependent relaxation in aortic rings isolated from diabetic rats. After the 8 weeks of treatment, animals were anesthetized with chloral hydrate (400 mg kg\(^{-1}\)), killed by cervical dislocation, and the thoracic aortas were rapidly dissected out. Experiments were performed on 6 groups with 5 aortic rings from 5 rats in each group as follows: (1) control: age-matched normal rats; (2) luteolin alone: normal rats treated with luteolin (100 mg kg\(^{-1}\) d\(^{-1}\)); (3) diabetes: diabetic rats; (4) low dose luteolin + diabetes: diabetic rats treated with luteolin at 10 mg kg\(^{-1}\) d\(^{-1}\); (5) medium dose luteolin + diabetes: diabetic rats treated with luteolin at 50 mg kg\(^{-1}\) d\(^{-1}\); (6) high dose luteolin + diabetes: diabetic rats treated with luteolin at 100 mg kg\(^{-1}\) d\(^{-1}\). After the presence of aortic functional endothelium was determined, when required, L-NAME (0.1 mM) or MB (0.01 mM) was added for 30 min before PE administration.

2.8. Statistical analysis

All data are expressed as mean±S.D. The ACh-induced maximal relaxation (E\(_{\text{max}}\)) in aortic rings was calculated as a percentage of the contraction in response to PE (1 μM). The half-maximum effective concentration (EC\(_{50}\)) was defined as the concentration of luteolin or ACh that induced 50% of maximum vasorelaxation of the contraction elicited by PE (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. The p < 0.05 was considered to be statistically significant.

3. Results

3.1. Effect of luteolin on relaxation in aortic rings previously contracted by PE

In Krebs’ solution with normal glucose concentration, luteolin (0.5–90.0 μM) evoked a concentration-dependent relaxation in endothelium-intact aortic rings previously contracted by PE (Fig. 2), the E\(_{\text{max}}\) reached 81.6 ± 4.6%, and the pD\(_{2}\) value was 5.24 ± 0.04. We chose the value of pD\(_{2}\) as the experimental concentration of luteolin for our subsequent experiments. In endothelium-denuded aortic rings previously contracted by PE, luteolin (0.5–90.0 μM) also evoked slight vasorelaxation, the E\(_{\text{max}}\) reached 25.1 ± 7.6% (p < 0.01 vs endothelium-intact group; data not shown).

3.2. Effect of luteolin on endothelium-dependent relaxation of aortic rings incubated with high glucose

After 4 h incubation in control glucose (11 mM), ACh (0.001–10 μM) still evoked a normal concentration-dependent relaxation (Fig. 3A and C), the E\(_{\text{max}}\) reached 72.6 ± 6.0%, and
the pD2 value was 7.36 ± 0.09. After 4 h exposure to high glucose (44 mM), the E_{max} fell to 44.8 ± 3.2%, and the pD2 value decreased to 7.05 ± 0.11. Luteolin alone enhanced the ACh-induced endothelium-dependent relaxation. However, no significant differences of E_{max} and EC50 were found between rings incubated in mannitol (33 mM mannitol +11 mM glucose) and those in control glucose (Fig. 3A and C). Treatment with the EC50 concentration of luteolin (5.74 μM) significantly attenuated the inhibition of endothelium-dependent relaxation induced by high glucose (Fig. 3B and C). The E_{max} was 63.7 ± 6.8%, and the pD2 was 7.22 ± 0.11. However, the ameliorating effect of luteolin was reversed by pretreatment with l-NAME, the inhibitor of NOS, but not by pretreatment with Indo, a cyclooxygenase inhibitor (Fig. 3B and C). In addition, luteolin (0.5–90.0 μM) had no effect on basal tension in the endothelium-intact aorta (Fig. 3D), and as shown in Fig. 3E, preincubation with luteolin for 30 min at the EC50 (5.74 μM) had no significant effect on PE-induced contraction in rat aorta with endothelium, the maximal increment of tension was 1.706 ± 0.311 g (p > 0.05 vs 1.712 ± 0.324 g in the group without luteolin).

3.3. Effect of luteolin on ROS level, OH• formation, and SOD activity in aortic segments

We used the fluorescent probe DCFH-DA to assess the release of ROS in aorta segments and found that incubation of isolated segments in high glucose for 4 h markedly increased the fluorescence intensity of DCF, indicating significant ROS-induced stress in the model. Treatment with luteolin (5.74 μM) in the high glucose (44 mM) group prevented the enhancement of DCF fluorescence intensity (Fig. 4A). Similarly, the increase of OH• formation and the decrease of SOD activity induced by high glucose were also inhibited by luteolin (p < 0.01; Fig. 4B and C). High concentration mannitol had no effects on ROS level, OH• formation, and SOD activity, while luteolin alone significantly decreased OH• formation and increased SOD activity compared to the control glucose group (Fig. 4).

3.4. Effect of luteolin on NO level and NOS activity in aortic segments

Incubation of isolated aortic segments in high glucose for 4 h resulted in a decrease of NO level (p < 0.01 vs control glucose group) and cNOS activity (p < 0.01 vs control glucose group), while the iNOS activity was increased (p < 0.05 vs control glucose group). Treatment with luteolin (5.74 μM) in the high glucose (44 mM) group markedly attenuated the decrease of NO level and cNOS activity (Fig. 5). Mannitol (44 mM) had no effects on NO level and NOS activity, while luteolin alone markedly increased NO level and cNOS activity (Fig. 5).

3.5. Effect of chronic luteolin treatment on endothelium-dependent relaxation of aortic rings from diabetic rats

The endothelium-dependent relaxation induced by ACh in the aortic rings from diabetic rats previously contracted with PE was significantly impaired compared with normal controls. The E_{max} of aortic rings fell to 46.4% ± 6.0% (Fig. 6). Though 100 mg kg⁻¹ d⁻¹ luteolin treatment of normal rats also significantly increased the ACh-induced endothelium-dependent relaxation compared with normal controls, the E_{max} did not significantly differ between these two groups (Fig. 6A). Chronic treatment with luteolin (10, 50, or 100 mg kg⁻¹ d⁻¹) for 8 weeks improved the vasorelaxation over that in the untreated diabetic group, the E_{max} of aortic rings reaching 52.6% ± 2.3%, 57.7% ± 2.8%, and 65.7% ± 3.0% (Fig. 6A). However, the ameliorating effect of luteolin was reversed by pretreatment with l-NAME or MB, a guanylyl cyclase inhibitor (Fig. 6B).

4. Discussion

High glucose has many toxic effects on endothelial cells, such as impairing endothelium-dependent relaxation, decreasing NO bioactivity, generating free radicals, and increasing apoptosis [24]. Endothelial dysfunction is thought to be the major cause of vascular disease due to hyperglycaemia and diabetes [2]. In this study, we demonstrated for the first time the beneficial effect of luteolin in isolated rat aortas exposed to high glucose or diabetes. We used a model of pronounced hyperglycemic injury, in which isolated aortic rings were exposed to high glucose (44 mM) for 4 h [7]. The same concentration of mannitol had no effect on the endothelium markedly attenuated the luteolin-induced relaxation in aortic rings previously contracted by 1 μM PE. Treatment with luteolin markedly inhibited the impairment of ACh-induced endothelium-dependent relaxation in aortic rings exposed to high glucose, which was cancelled by pretreatment with l-NAME, an inhibitor of NOS, but not by Indo, a cyclooxygenase inhibitor. Furthermore, we found that the increase of ROS production and OH• formation, and the reductions of NO level, cNOS and SOD activity in aortas incubated with high glucose were all reversed following treatment with luteolin. At the same time, we found that chronic treatment with luteolin for 8 weeks attenuated the impaired endothelium-dependent relaxation caused by STZ-induced diabetes. Moreover, such effects of luteolin in diabetic rat aortas were dose-dependent and were inhibited by l-NAME or MB. At the end of 8 weeks, the blood glucose levels in the luteolin-treated diabetic rats were not reduced compared to the untreated diabetic group (10, 50, 100 mg kg⁻¹ d⁻¹ luteolin-treated groups: 26.4 ± 4.0, 25.3 ± 5.5, 25.5 ± 5.0 mM; diabetic group: 30.2 ± 4.4 mM). Treatment with luteolin alone had no effect on blood glucose level in non-diabetic rats (data not shown). These results suggest that luteolin is effective in reducing the impairment of endothelium-dependent relaxation induced by high glucose or diabetes mainly through reducing oxidative stress and maintaining the NOS–NO pathway, but not through reducing blood glucose.

Luteolin and luteolin-7-O-glucopyranoside, two available flavonoids known to be present in artichoke leaf extract, were confirmed to be responsible for the eNOS up-regulation in endothelio-
Fig. 3. Acetylcholine (ACh)-induced endothelium-dependent relaxation (A and B) and pD2 values (C) in rat aortic rings previously contracted by phenylephrine (PE) after incubation in high glucose (44 mM) for 4 h, effect of luteolin (0.5–90.0 μM) on basal tension (D) and effect of pretreatment with EC50 luteolin (5.74 μM) for 30 min on PE (1 μM)-induced contraction in endothelium-intact rat aortic rings (E). Tension was measured and calculated as an absolute value and ACh (0.001–10 μM)-induced relaxation was measured and calculated as a percentage of the contraction in response to PE (1 μM); pD2 is the negative logarithm of the EC50. CG: control glucose (11 mM); luteolin: 5.74 μM luteolin for 30 min; mannitol: mannitol (33 mM) plus glucose (11 mM); HG: high glucose (44 mM); HG + luteolin: high glucose (44 mM) plus luteolin (5.74 μM for 30 min); HG + luteolin + l-NAME: high glucose (44 mM) plus l-NAME (0.1 mM for 30 min) and luteolin (5.74 μM for 30 min); HG + luteolin + Indo: high glucose (44 mM) plus indomethacin (0.01 mM for 30 min) and luteolin (5.74 μM for 30 min). All data are expressed as mean ± S.D.; n = 8–10 rings from 8 rats per group. *p < 0.05, **p < 0.01 vs control glucose group (CG); ##p < 0.01 vs high glucose group (HG).

lial cells caused by artichoke extracts [26]. Furthermore, such extract-induced endothelium-dependent vasorelaxation in rat aortas is cancelled by l-NAME [26]. Other previous studies reported that luteolin or its derivative induce NO-dependent vasorelaxation in rat aortic rings [27,28], which is reduced in the presence of L-arginine [26]. NO synthase inhibitor [28]. In the present study, we also found that luteolin-induced vasorelaxation in high glucose-treated or diabetic rat aortic rings was cancelled by pretreatment with l-NAME. All of these results indicate that luteolin-induced endothelium-dependent vasorelaxation in rat thoracic aorta injured by high glucose is l-NAME-sensitive. NO, a vasodilator formed in the endothelium by the activation of eNOS, is vital for maintaining the tone of underlying vascular smooth muscle. Endothelium-derived NO binds to and activates soluble guanylyl cyclase (sGC), which catalyzes the conversion of GTP to cGMP. The rise of cGMP initiates the dilatation of vascular smooth muscle [29,30]. The fact that in the present experiment MB, an inhibitor of GC, reversed the improving effect of luteolin on vasorelaxation in diabetic rat aorta further indicates that luteolin reduces the impairment of endothelium-dependent relaxation in rat aorta exposed to high glucose at least by the NOS–NO–sGC pathway. It has been demonstrated that endothelial dysfunction induced by hyperglycemia or diabetes is related to decreased release of NO from endothelial cells [31]. Hyperglycemia itself inhibits the endogenous synthesis of NO [31], and decreases the activity of vasodilator factors including NO, thus leading to impairment of endothelium-dependent relaxation [32]. Our study showed that luteolin reversed the impairment of endothelium-dependent relax-
Luteolin markedly inhibited the alteration of iNOS and cNOS activity and maintained the homeostasis of total NOS activity. As a result, the NO level in aorta incubated with high glucose was maintained by luteolin. It is believed that the main cNOS in aorta is eNOS [33]. Therefore, it is possible that the effect of luteolin against high glucose-induced endothelial dysfunction in rat aorta is related to inhibiting the decrease of NOS activity, then maintaining the release of endothelium-derived NO to activate sGC, and finally raising the cGMP level to initiate the relaxation of aortic rings.

Our previous study showed that luteolin, a flavone rich in the Chinese herb Flos Chrysanthemi and many other plants, reduces superoxide anion-mediated impairment of endothelium in rat mesenteric arteries [16], indicating that luteolin may be effective in ameliorating the endothelial impairment induced by overproduction of ROS. In the present study, we showed that the increase of ROS production and OH• formation, and the decrease of SOD activity in aorta induced by high glucose were all markedly inhibited by treatment with luteolin, suggesting that reducing oxidative stress may be one of the key mechanisms by which luteolin reduces the endothelial impairment induced by high glucose. Hyperglycemic endothelium produces an increase in both O2•− and H2O2 leading to enhanced intracellular production of OH• [34], and enhanced OH• formation is considered to be a significant contributor to diabetes-induced endothelial dysfunction [35]. Such excessive ROS produced by hyperglycemia damages cellular proteins, membrane lipids, and nucleic acids, and eventually results in cell death [34]. More importantly, NO readily reacts with superoxide, generating the highly reactive molecule peroxynitrite (ONOO•), to trigger a cascade of harmful events [36,37]. ONOO• not only decreases NO bioavailability, causing impaired vasorelaxation [34], but also oxidizes tetrahydrobiopterin, an important cofactor for NOS, and causes uncoupling of NOS, especially the iNOS activated by hyperglycemia, which produces superoxide instead of NO [34]. All these reactions contribute to the pathogenesis of endothelial dysfunction, including impairment of endothelium-dependent relaxation in hyperglycemia [38]. In our study, the fact that luteolin reduced ROS production and enhanced SOD activity in aortas incubated in high glucose indicates that luteolin strongly enhances antioxidative defenses and reduces oxidative stress in aortas injured by high glucose. Furthermore, luteolin is cell-permeable [15]. So, like other flavones [39], it is probable that the beneficial effects of luteolin on endothelial function in hyperglycemia are due to its rapid entry into cells, especially endothelial cells, to inhibit intracellular as well as extracellular ROS, and maintain NO bioavailability. We suggest that the improving effect of chronic treatment with luteolin on diabetic rat aorta also involves reducing oxidative stress and maintaining the bioavailability of the NOS–NO–sGC pathway, though the exact mechanism needs further investigation.

To sum up, our experimental findings suggest that luteolin, a flavone rich in ordinary vegetable and fruits, effectively protects endothelium-dependent relaxation against high glucose injury through reducing oxidative stress and enhancing activity in the NOS–NO pathway.

Fig. 4. Effect of luteolin (5.74 μM) on reactive oxygen species (ROS) release by 10 μM DCFH-DA loading (A), hydroxyl radical (OH•) formation (B) and activity of superoxide dismutase (SOD) (C) in endothelium-intact rat aortic rings after exposure to high glucose (44 mM) for 4 h. CG: control glucose (11 mM); luteolin: 5.74 μM luteolin for 30 min; mannitol: mannitol (33 mM) plus glucose (11 mM); HG: high glucose (44 mM); HG + luteolin: high glucose (44 mM) plus luteolin (5.74 μM) for 30 min. All data are expressed as mean ± S.D.; n = 8 aortas from 8 rats per group. *p < 0.05, **p < 0.01 vs control glucose group (CG); #p < 0.05 vs high glucose group (HG).

Fig. 5. Effect of luteolin (5.74 μM) on nitric oxide (NO) level (A), and activity of constitutive nitric oxide (cNOS) and inducible nitric oxide synthase (iNOS) (B) in endothelium-intact rat aortic rings after exposure to high glucose (44 mM) for 4 h. CG: control glucose (11 mM); luteolin: 5.74 μM luteolin for 30 min; mannitol: mannitol (33 mM) plus glucose (11 mM); HG: high glucose (44 mM); HG + luteolin: high glucose (44 mM) plus luteolin (5.74 μM) for 30 min. All data are expressed as mean ± S.D.; n = 8 aortas from 8 rats per group. *p < 0.05, **p < 0.01 vs control glucose group (CG); *p < 0.05, **p < 0.01 vs high glucose group (HG).
Fig. 6. Acetylcholine (ACh)-induced endothelium-dependent relaxation (A), which was inhibited by pretreatment with L-NAME (0.1 mM for 30 min) or methylene blue (MB, 10 μM for 30 min) (B), in diabetic rat aortic rings previously contracted by phenylephrine (PE). ACh (0.001–10 μM)-induced relaxation was measured and calculated as a percentage of the contraction in response to PE (3 μM). Con: age-matched control group; 100 luteolin: luteolin alone group (100 mg kg⁻¹ luteolin for 8 weeks); D + 100 luteolin: diabetes treated with 100 mg kg⁻¹ luteolin for 8 weeks; D + 50 luteolin: diabetes treated with 50 mg kg⁻¹ luteolin for 8 weeks; D + 100 luteolin + L-NAME: D + 100 luteolin group pretreated with L-NAME (0.1 mM for 30 min); D + 100 luteolin + MB: D + 100 luteolin group pretreated with methylene blue (MB, 0.01 mM for 30 min). All data are expressed as mean ± S.D.: n = 5 rings from 5 rats per group. *p < 0.05, **p < 0.01 vs control group (Con); *p < 0.05, **p < 0.01 vs diabetes group (D); *p < 0.05, **p < 0.01 vs diabetes treated with 100 mg kg⁻¹ luteolin group (D + 100 luteolin).

Acknowledgements

We acknowledge the excellent technical assistance of Yuan Lu and Xu-yun Li in Zhejiang University School of Medicine. This work was supported by the Zhejiang Provincial Natural Science Foundation of China (Y206179).

References