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Mechanisms underlying quercetin-induced vasorelaxation in aorta of subchronic diabetic rats: an in vitro study

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Abstract

In this study, the mechanisms involved in vasorelaxant effect of the flavonoid quercetin was investigated in isolated aortic rings from streptozotocin (STZ)-diabetic rats. After 4 weeks, addition of quercetin (0.1 μM–1 mM) caused a significant dose-dependent relaxation of noradrenaline (NA)- and KCl-preconstricted rings in both control and diabetic groups with a significant inter-group difference of $P<0.01$. Furthermore, both nitro-L-arginine-methyl ester (L-NAME, 100 μM) and indomethacin (10 μM) markedly attenuated the vasorelaxant responses following quercetin application. Meanwhile, endothelium removal significantly attenuated the quercetin-induced vasorelaxation. It is concluded that the quercetin can relax the preconstricted rings of aorta in subchronic STZ-diabetic rats through nitric oxide- and -prostaglandin-mediated pathways, which themselves could be considered as endothelium-dependent.

Keywords: Quercetin; Aorta; Vasorelaxation; Diabetic rat

1. Introduction

Flavonoids have been known to exhibit various beneficial effects such as platelet antiaggregant, free radical scavenger, reducing levels of low-density lipoproteins in plasma (Formica and Regelson, 1995) and modulation of the vascular tone (Ajay et al., 2003). Several epidemiological studies revealed the inverse association between the flavonoid intake and reduction in the occurrence of cardiovascular diseases including myocardial infarction (Hertog et al., 1993a). Among dietary flavonols, quercetin is by far the most abundant representing approximately 60% of the total intake (Hertog et al., 1993b). It has also been shown that quercetin causes endothelium-dependent relaxation in the rat aorta (Fitzpatrick et al., 1993; Duarte et al., 1993a). In addition, its cardiovascular protective effects has been well-documented (Duarte et al., 2001).

Since cardiovascular complications are among the major causes of mortality in diabetic patients (Garcia et al., 1974), and enhanced vascular reactivity to vasoconstrictor agents (Abebe et al., 1990) and impairment of the vascular relaxation (Tesfamariam, 1994) has been observed in diabetic condition, the aim of the present study was to evaluate the possible vasorelaxant effect of quercetin at early stages of diabetes mellitus and to investigate the underlying mechanisms in STZ-diabetic rats.

2. Materials and methods

2.1. Animal experiments

Male albino Wistar rats (Pasteur's institute, Tehran, Iran) weighing 235–275 g (8–10 weeks old) were housed in an

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air-conditioned colony room on a light/dark cycle (21±2 °C and 30–40% humidity) and supplied with standard pelleted diet and tap water ad libitum. Procedures involving animals and their care were conducted in conformity with the institutional guidelines of Shahed University (Tehran, Iran) and in accordance with the NIH guidelines for the care and use of laboratory animals.

The animals were randomly divided into two experimental groups: control (n=10) and diabetic (n=12). Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ, 60 mg/kg) dissolved in cold 0.9% saline solution immediately before use. Diabetes was verified by a serum glucose level higher than 250 mg/dl using glucose oxidation method (glucose oxidase kit, Zistchimie, Tehran).

2.2. Experimental procedure

After 4 weeks, the rats were anesthetized with diethyl ether, decapitated, and through opening the abdomen, descending thoracic aorta was carefully excised and placed in cold physiological saline solution (PSS) containing (mM): NaCl 118, KCl 4.6, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.1, $NaHCO_3$ 27.2, ethylenediaminetetraacetic acid (EDTA) 0.03, and $CaCl_2$ 1.8. Thereafter, the aorta was cleaned of excess connective tissue and fat and cut into rings of approximately 4 mm in length. One ring of each pair was left intact, and in the other ring, endothelium was mechanically removed by gently rotating it on a glass rod. Aortic rings were suspended between two triangular-shaped wires. One wire was attached to a fixed tissue support in a 50 ml isolated tissue bath containing PSS (pH 7.4) maintained at 37 °C and continuously aerated with a mixture of 5% CO_2 and 95% O_2. The other end of each wire attached by a cotton thread to a F60 isometric force transducer (Narco Biosystems, USA) coupled to a signal amplifier and connected to computer via an A/D interface. The rings were allowed to equilibrate for 90 min under a resting tension of 1.5 g before experiments were begun. This had been shown in preliminary experiments to be the optimal resting tension for all groups. During equilibration period, the rings were washed every 30 min. For examining the endothelial integrity in intact rings, preconstricted rings with noradrenaline (NA, 1 μM) were exposed to a single addition of acetylcholine (ACh, 10 μM). Successful removal of the endothelium in denuded rings was confirmed by loss of acetylcholine (10^{-5} M)-induced relaxation in preconstricted rings by NA (10^{-6} M). Furthermore, all experiments for NA were done in the presence of 1 μM timolol, 1 μM imipramine, and 1 μM prednisolone to eliminate the effects of β-adrenoceptors, neuronal uptake, and extraneuronal uptake respectively. Recording and analysis of data were performed using the software Physiograph I (Behineh Arman Co., Tehran).

After an initial equilibration, the aortic rings were allowed to achieve maximal tension by exposure to high K^+ solution (80 mM), which was prepared by replacing the NaCl concentration of PSS with an equimolar concentration of KCl. Then, the relaxant responses to different concentrations of quercetin (0.1 μM–1 mM) were recorded. After PSS rinsing (3 times within a period of 30 min), the rings were constricted with NA (1 μM) and again the relaxant responses to the same concentrations of quercetin were recorded. The quercetin-evoked vasorelaxation was expressed as a percentage of relaxation and the IC_{50} (the concentration which produced a 50% maximal relaxation) was determined from the concentration–response curves. Meanwhile, for some other rings (n=6 in each group), cumulative concentration–response curves were obtained for NA.

The involved mechanisms underlying the vasorelaxant action of quercetin was examined by pretreatment of the aortic rings with nitro-L-arginine-methyl ester (L-NAME, 100 μM) and indomethacin (10 μM) individually or in combination (data not to be presented) 30 min before addition of the vasoconstrictors and the quercetin. In order to determine the involvement of intracellular Ca^{2+} mobilization in the vasorelaxant action of the quercetin, a Ca^{+2}-free PSS prepared by replacing $CaCl_2$ in PSS with an equimolar concentration of $MgCl_2$ and the addition of ethylene-glycol-tetraacetic acid (EGTA, 0.5 μM) to chelate any free Ca^{2+} in the medium. After a 15 min preincubation period in this solution with 2–3 serial washings, NA (1 μM) was added to stimulate the release of intracellular Ca^{+2} and the contraction recorded for at least 2 min. A similar procedure was repeated with Ca^{+2}-free PSS containing quercetin (0.1 mM).

2.3. Drugs and chemicals

Noradrenaline bitartrate, acetylcholine-HCl, quercetin, indomethacin, L-NAME, and streptozotocin were purchased from Sigma Chemical (St. Louis, Mo., USA). Prednisolone, imipramine, and timolol were obtained from Temad (Tehran, Iran). All other chemicals were purchased from Merck (Germany). The flavonoid quercetin was dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO was less than 0.08%, which was shown to be devoid of any observable effects on muscle tone. Meanwhile, cumulative addition of this vehicle (DMSO) had no significant effect (at most 1.5–4% relaxation at the highest concentration of DMSO) on the thoracic aorta. Indomethacin was dissolved in 0.5% w/v sodium bicarbonate. Further dilutions of the drugs were made in PSS. Meanwhile, streptozotocin was freshly dissolved in 0.9% saline solution.

2.4. Data and statistical analysis

All values were given as means±S.E.M. and were analyzed by student's t-test or one-way analysis of variance (ANOVA) followed by Tukey post-hoc test with a significant level of $P<0.05$.

3. Results

After 4 weeks, body weight of diabetic animals significantly decreased from 241.7±5.4 g to 207.8±5.8 g (P<0.05). Regarding serum glucose level, it significantly increased to 379.8±15.7 mg/dl from the initial level of 104.7±4.7 mg/dl (P<0.001). With respect to contractile response of endothelium-intact aortic rings, NA (1 μM) induced a sustained contraction of the rat aorta with a peak tension of 758.23±36.7 and 867.17±41.09 mg in control and diabetic groups respectively. This difference was not statistically significant (P=0.53). Meanwhile, after obtaining concentration–response curves for aortic rings from some diabetic animals (n=6), it was found out that there is a leftward shift in the related curves, indicating the increased sensitivity of the tissue. This was despite no significant changes in the maximum contractile response to NA. On the other hand, removal of endothelium significantly caused an enhanced contractile response to NA in both control and diabetic groups (P<0.01) (data not shown). Addition of the quercetin to endothelium-intact rings from control and diabetic rats induced a dose-dependent relaxation of the precontracted rings with NA (Fig. 1A). In this respect, quercetin-induced vasorelaxation of rings from diabetic group was significantly lower only at concentrations higher than 100 μM as compared to control group (P<0.01). It was of interest that quercetin produces a vasorelaxation greater than 70% for both groups. In this respect, the IC_{50} (in a logarithmic scale) was −4.42±1.02 for control and −3.81±0.09 for diabetic groups and their difference did not reach a significant level. Removal of endothelium significantly attenuated, but not abolished, the quercetin-induced vasorelaxation in both control and diabetic groups (P<0.01) (data not presented).

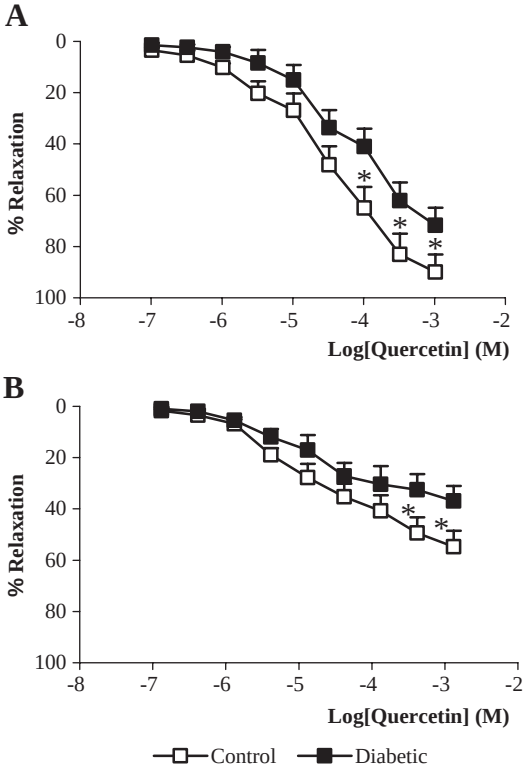

Fig. 1. Vasorelaxant effect of quercetin against NA (A) and high K^+ (B)-induced contractions in aortic rings from control (n=8) and streptozotocin-diabetic (n=9) rats. *P<0.01

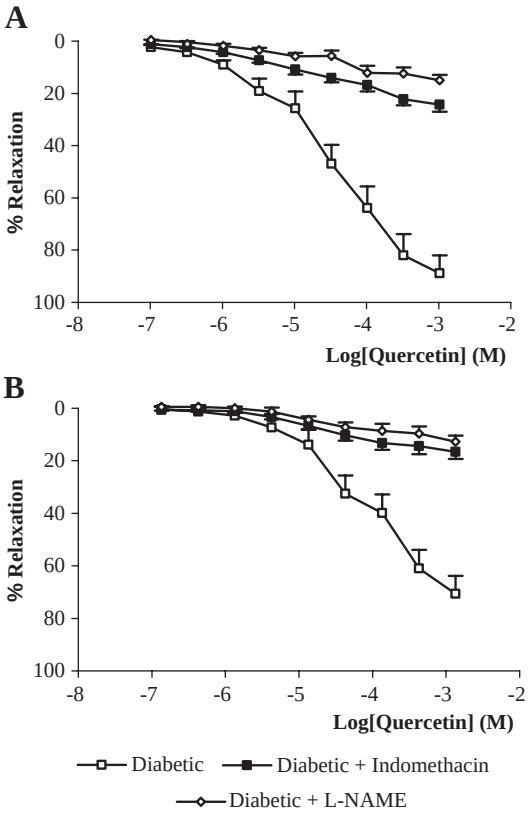

Fig. 2. Vasorelaxant effect of quercetin against NA-induced contractions in the presence of L-NAME or indomethacin in control (n=10) (A) and diabetic (n=11) (B) groups.

Addition of high K^+ (80 mM)-containing PSS to the tissue bath induced a maximal tension of 317.2±15.9 and 374.1±21.09 mg in endothelium-intact rings from control and diabetic groups respectively. This difference was statistically significant (P<0.05). The addition of quercetin produced a dose-dependent relaxation in both control and diabetic groups (Fig. 1B). The relaxation response for diabetic group was significantly lower at quercetin concentrations higher than 0.5 mM as compared to control group (P<0.01). The maximum vasorelaxation was 55.06±6.25 and 37.22±5.87 mg for control and diabetic groups respectively with a significant difference of P<0.01. Removal of endothelium significantly reduced this vasorelaxation in both control and diabetic groups (P<0.01) (data has not been shown).

To further evaluate the mechanism of the vasorelaxant response in control and diabetic groups in endothelium-intact rings, they were pre-incubated for 30 min with L-NAME (100 μM), a nitric oxide synthesis inhibitor, or

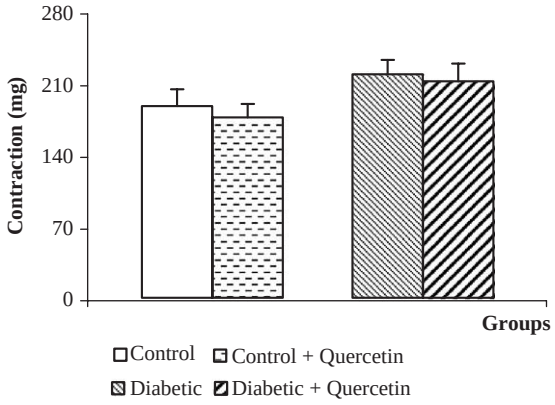

Fig. 3. Effect of quercetin on NA-induced transient contractions in normal and Ca^{+2}-free PSS in control (n=9) and diabetic (n=8) groups.

indomethacin (10 μM) a cyclooxygenase inhibitor. Pretreatment of the tissues with L-NAME or indomethacin markedly attenuated the inhibitory effect of quercetin against NA (1 μM)-induced contraction in both control (Fig. 2A) and diabetic groups (Fig. 2B). In another series of experiments, for evaluation of the role of intracellular calcium mobilization in vasorelaxant effect of quercetin, the effect of this flavonoid was studied against contractions induced by NA in Ca^{2+}-free PSS. In the absence of extracellular Ca^{2+}, NA produced a transient contraction with amplitudes of 187.23±16.25 and 218.12±14.09 mg in control and diabetic groups respectively. This difference was not statistically significant. Furthermore, pretreatment of the aortic rings with quercetin did not significantly reduce the contractions induced by NA for both groups (Fig. 3).

4. Discussion

The present work was performed to investigate the underlying mechanisms involved in quercetin-induced vasorelaxation in NA- and KCl-precontracted aortic rings from subchronic (4-week) STZ-diabetic rats. The results showed that there are an enhanced responsiveness of aortic rings from diabetic animals to KCl, an enhanced sensitivity to NA with no significant change in the maximum contractile force, a significant attenuation of the contractile force due to KCl and NA following quercetin application, and a significant diminution of the latter response following L-NAME (an inhibitor of NO synthase) and/or indomethacin (prostaglandin synthesis inhibitor) pretreatment. Furthermore, endothelial removal significantly reduced, but not completely abolished quercetin-induced vasorelaxation, and intracellular mobilization of calcium did not play a role in this response.

The increased vascular responsiveness to contractile agents in STZ-diabetic rats has been reported in most previous studies (Abebe et al., 1990; Ozcelikay et al., 1994). Although the actual responsible mechanisms have not been completely understood, some possible factors that could have been involved in the increased vascular smooth muscle responsiveness to non-specific (KCl) and receptor-mediated agonists (NA) in diabetic rats are deficient endothelial activity (Karasu and Altan, 1993), enhanced phosphoinositide (PI) metabolism (Chang et al., 1993), enhanced sensitivity of calcium channels (Abebe et al., 1990), and increased sensitivity to adrenergic agonists (Macleod and McNeill, 1982). Furthermore, oxidative stress is increased due to excessive production of oxygen-free radicals and decreased antioxidant defense systems (Oberlet, 1988) and this phenomenon could be responsible for augmented contractility together with deficient endothelial activity in diabetic state (Tesfamariam, 1994). Since at early stages of diabetes, there is an enhanced sensitivity to α-adrenergic agonists with no significant changes in the maximum contractile response, and thereafter, there is a marked accentuated contractile response (Zhu et al., 2001), this strongly supports our diabetes model in its initial stages with regard to vascular abnormalities, and for this reason there has been no significant increase in contractile responsiveness to NA, as observed in this study.

In this study, the involved mechanisms in quercetin-induced vasorelaxation was evaluated in diabetic animals. Several lines of evidence support the idea that the relaxant effect of quercetin was dependent on the production of NO from endothelial cells (Chen and Pace-Asciak, 1996; Ajay et al., 2003). The vasorelaxation induced by quercetin in this study was dependent on the presence of a functional endothelium and was reduced after inhibition of NO synthase by L-NAME. Thus, it may be unlikely that the direct activation of guanylyl cyclase or the inhibition of phosphodiesterases in smooth muscle could be accounted for the vasorelaxing effect of quercetin. The possibility that endothelial vasorelaxant factors derived from cyclooxygenase pathway (prostacyclin PGI_2 released from endothelial cells) participated in the relaxant effect of quercetin (Jaffe et al., 1982) was also evaluated. In this respect, indomethacin pretreatment also significantly attenuated the quercetin-induced vasorelaxation. Furthermore, in the rat aorta, the α_1-adrenoceptor agonists including NA and phenylephrine induce an initial transient contraction followed by a tonic contraction. The initial contraction is mediated by intracellular Ca^{2+} release, whilst the sustained tonic contraction results from Ca^{2+} influx via the receptor-operated calcium channels (Abebe et al., 1990). In the present study, the effect of quercetin on intracellular Ca^{2+} release was examined indirectly by evaluation of its effect on contractions induced by NA in calcium-free PSS. In this respect, quercetin failed to significantly attenuate the initial response to NA. This implies that at flavonoid concentrations used, the vasorelaxant actions of this flavonoid may not involve inhibition of Ca^{2+} release from the sarcoplasmic reticulum stores, which is in contradistinction with some previous reports (Chan et al., 2000).

The endothelial dependence of the vasodilator effect of quercetin in rat conductance arteries like aorta has pre-

viously been studied. The relaxation induced by quercetin and other related flavonoids have reported to be endothelium-independent (Duarte et al., 1993b; Fitzpatrick et al., 1993) or very weakly inhibited by endothelial removal (Chen and Pace-Asciak, 1996). In the present work, it was found out that the vasodilator effect of quercetin is endothelium-dependent, and its endothelium-independent effect was only observed at concentrations higher than 10^{-3} M (Data not presented). For this reason, at concentrations used in this study (equal or lower than 10^{-3} M), endothelium removal is expected to significantly attenuate the vasorelaxation response.

To conclude, the flavonoid quercetin could induce a dose-dependent vasorelaxation at early stages of diabetes development and this response follows a NO-, and prostaglandin-dependent process, the integrity of endothelium is essential for this relaxant response, and finally, intracellular mobilization of calcium may not play a role in this phenomenon. Further studies are warranted to evaluate the beneficial vascular effect of quercetin administration in diabetic animals.

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