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Research paper

The bioflavonoid quercetin synergises with PPAR-γ agonist pioglitazone in reducing angiotensin-II contractile effect in fructose-streptozotocin induced diabetic rats

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ABSTRACT

This study investigated the effects of combined minimal concentrations of quercetin and pioglitazone on angiotensin II-induced contraction of the aorta from fructose-streptozotocin (F-STZ)-induced type 2 diabetic rats and the possible role of superoxide anions (O_2^-) and nitric oxide (NO) in their potential therapeutic interaction. Contractile responses to Ang II of aortic rings from Sprague-Dawley (SD) and F-STZ rats were tested following pre-incubation of the tissues in the vehicle (DMSO; 0.05%), quercetin (Q, 0.1 μM), pioglitazone (P, 0.1 μM) or their combination (P + Q; 0.1 μM each). The amount of superoxide anion was evaluated by lucigenin-enhanced chemiluminescence and dihydroethidium fluorescence, and NO by assay of total nitrate/nitrite, and 4-Amino-5-Methylamino-2′,7′-Difluorofluorescein (DAF-FM) diacetate. The synergistic reduction of Ang II-induced contraction of diabetic but not normal aorta with minimally effective concentrations of P + Q occurs through inhibiting O_2^- and increasing NO bioavailability. This finding opens the possibility of maximal vascular protective/antidiabetic effects with low dose pioglitazone combined with quercetin, thus minimizing the risk of adverse effects.

1. Introduction

Angiotensin II (Ang II) is an octapeptide member of the renin-angiotensin system (RAS) and it mediates a variety of physiological and pathophysiological activities, such as vasoconstriction, raised blood pressure, vascular remodeling, endothelial dysfunction and sodium retention [1]. The actions of Ang II are modulated by two major receptor subtypes, angiotensin II type 1 receptor (AT_1R) and angiotensin II type 2 receptor (AT_2R) [1,2]. Ang II has been associated with the pathophysiology of type 2 diabetes mellitus as its interaction with AT_1 receptors induces a signaling cascade that impairs insulin function at various levels [3] and causes oxidative stress that leads to macro- and microvascular complications [4]. Furthermore, studies with Ang II receptor blockers in both animals [5,6] and diabetic patients [7–9] have demonstrated improved cardiovascular complications and clinical outcomes such as reductions in congestive heart failure and stroke, thus lowering the mortality and morbidity rates. Ang II activates NAD(P)H oxidase, a crucial enzyme that generates reactive oxygen species (ROS), especially superoxide anion (O_2-), which leads to oxidative stress [2,3]. Production of excessive superoxide anions will interact with nitric oxide (NO) released from endothelial cells and subsequently reduce NO bioavailability causing endothelial dysfunction [4–6]. Thus, pharmacological/dietary agents capable of ameliorating ROS in the vessel wall may attenuate the undesirable vascular effects of Ang II in diabetes.

Improvement in endothelium-derived relaxing factors, especially endothelium-derived nitric oxide (EDNO) has been observed in various studies [10–13] in which pharmaceutical antioxidant nutrient and/or non-nutrient preparations were administered to diabetic animals. In line with that, polyphenolic flavonoid compounds, especially quercetin, found in daily human diets such as vegetables and fruits, is one of the most potent antioxidants of

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plant origin [14,15]. It has been shown to possess potent vasodilator, free radical scavenging and antioxidant actions [16]. It has also been shown to exert protective effects on nitric oxide and to improve endothelium function in both *in-vitro* and *in vivo* studies of diabetic rat models [17,18].

Thiazolidinediones, such as pioglitazone, are peroxisome proliferator-activated receptor gamma (PPAR-γ) agonists and are oral antidiabetic agents that have been effectively used in the treatment of non-insulin dependent diabetes mellitus with insulin resistance. Thiazolidinediones improve insulin-mediated glucose uptake into skeletal muscle without increasing endogenous insulin secretion [19]. Although PPAR-γ activation plays an important role in glucose metabolism, it has been shown to inhibit adhesion molecules cascades and vascular inflammatory events [20]. In addition, it plays a role in up-regulating endothelial NO synthase (eNOS), thus enhancing the generation of vascular NO [21]. It has also been shown to downregulate Ang II type 1 receptors in vascular smooth muscle cells [22] and in neonatal cardiac fibroblasts [23]. Furthermore, pioglitazone was shown to improve endothelial dysfunction by endothelium-dependent and independent vasodilation assessed using Color Doppler ultrasonography in a randomized, double-blind and prospective study among first-degree relatives of patients with type 2 diabetes [24] and in aorta from streptozotocin (STZ)-induced type 1 diabetic rats [25]. In spite of these beneficial effects, prolonged use of pioglitazone can cause adverse effects such as fluid retention, peripheral edema and congestive heart failure [26].

Finding a method for harnessing the beneficial effects of pioglitazone, while minimizing the risk of adverse effects, is thus an attractive therapeutic goal. We hypothesize that this is achievable through using reduced doses of pioglitazone in combination with dietary plant flavonoids which antioxidant effects may synergize with those of pioglitazone. Quercetin and pioglitazone have been separately shown to improve endothelial function in aortic vessels [17,18,25], but there is a lack of information on whether combining both agents may provide therapeutically beneficial interactions. We, therefore, designed an *in vitro* study to explore the effect of combined minimally effective concentrations of quercetin and pioglitazone on Ang-II-induced contraction of the isolated aorta from fructose-streptozotocin (F-STZ)-induced type 2 diabetic rat model.

2. Materials and methods

2.1. Drugs and chemicals

Acetylcholine (ACh), Ang II, diphenylene iodonium (DPI), fructose, losartan, quercetin, phenylephrine (PE), pioglitazone, ω-nitro-L-arginine methyl ester (L-NAME), superoxide dismutase (SOD) and streptozotocin (STZ) were purchased from Sigma Chemicals Company (St. Louis, MO, USA). All the drugs were dissolved in distilled water except for quercetin and pioglitazone. Quercetin and pioglitazone stock solutions (10 mM) were prepared in 5% (v/v) dimethyl sulfoxide (DMSO). The final concentrations were obtained by serial dilutions of distilled water and the final concentration of DMSO was adjusted to less than 0.05% (v/v).

2.2. Experimental animals

Male SD rats (6–7 weeks old), were obtained from the University of Malaya Experimental Unit, and all the experimental procedures were approved by the University of Malaya Animal Care and Ethics Committee (Ethics Reference No: 2013-06-07/PHAR/R/DDM) and accredited by Association for Assessment and Accreditation of Laboratory Animal Care International (AALAC). The animals were housed in a well-ventilated room and had free access to standard rat chow (Altromin, Australia) and filtered tap water. Insulin resistance model of type 2 diabetes mellitus was induced by a continuous supply of 10% fructose in drinking water for 3 weeks, followed by a single dose of STZ (30 mg kg^{-1} of body weight, i.p) freshly dissolved in distilled water [27]. Blood glucose levels were measured using an Accu-check monitor (Roche, Mannheim, Germany) at both day-7 and 7 weeks after STZ injection, the latter just prior to sacrificing the animals for isolated tissue studies. The animals were considered diabetic if the blood glucose levels exceeded 10 mmol/L. The control group was injected with distilled water and blood glucose levels below 5 mmol/L were considered normal.

2.3. Oral glucose tolerance test (OGTT)

Oral glucose tolerance test was conducted on overnight fasted animals with glucose dose at 3 g kg^{-1} [28]. Briefly, glucose load was given orally and blood glucose levels were measured at 0 min (prior to glucose load), 30, 60, 90, 120 and 180 min after oral glucose load.

2.4. Insulin tolerance test (ITT)

Insulin tolerance test was conducted on fasted (6–8 h) animals with an intraperitoneal injection of insulin (0.75 U/kg body weight) [29]. Blood glucose was measured at 0 min (prior to insulin injection), 15, 30, 45, 60 and 75 min after insulin injection.

2.5. Measurement of blood pressure

Blood pressure (BP) was measured in unanesthetized animals by the indirect tail-cuff method (PowerLab 4/S, ADInstruments, Australia). Rats were maintained at 37 °C for 10 min, and then three consecutive stable BP measurements were averaged.

2.6. Blood collection and analysis of plasma insulin, serum total cholesterol, and triglycerides

Animals were fasted overnight and sacrificed by carbon dioxide (CO_2) inhalation. Blood was collected by cardiac puncture. Serum and plasma were collected immediately and stored at −80 °C for further use. Plasma insulin level and serum total cholesterol and triglycerides levels were determined using commercial assay kits purchased from Mercodia AB, Sweden, and Cayman Chemical Company, Ann Arbor, MI respectively. All assays were done according to the manufacturer's instructions.

2.7. Vascular ring preparation

Ten weeks after the start of fructose feeding the rats were sacrificed by CO_2 inhalation. The descending thoracic aorta was isolated and cleaned of surrounding fat and connective tissue. The aorta was cut into small rings (3–5 mm) and suspended in a 5 ml organ bath containing oxygenated Krebs physiological salt solution (KPSS in mM: NaCl 119, $NAHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, glucose 11.7, and $CaCl_2 \cdot 2H_2O$ 2.5) to measure the isometric tension. The tissue-bath, maintained at 37 °C, was aerated continuously with CO_2 (5%) and O_2 (95%). The isometric tension (g) was measured using a force transducer (Grass Instrument Co, Quincy, MA, USA) connected to the Power Lab recording system (AD Instruments, Sydney, Australia). The tissue was stretched to an optimal tension of 1 g and allowed to equilibrate for 45 min before initiation of the experimental protocol. During this period of stabilization, the bath solution was replaced every 15 min. Following equilibration, the contractile responses of aortic rings were tested as a sign of viability by the addition of 10% KCl (high K^+) for 4 min

every 10 min until two consecutive equal contractions were attained. To confirm intact endothelium (ED), each tissue was contracted with phenylephrine (PE; 10^{-6} M) and then exposed to acetylcholine (ACh; 10^{-5} M) at the peak of the contraction. Only tissues that exhibited more than 70% relaxation of the PE contraction were selected for subsequent studies. Endothelium denudation was done by gently inserting blunt forceps into the aortic lumen which was then gently rubbed. The absence of ED was confirmed by the lack of a response to the ED-dependent vasodilator, ACh, in PE pre-contracted rings. Rings that exhibited <5% relaxation of the PE contraction were included for further studies [30].

2.8. Contractile responses to angiotensin II

To evaluate the role of Ang II contraction in aortic rings from F-STZ diabetic and SD rats, concentration-response curves were obtained over a range of single concentrations of Ang II (10^{-10}–10^{-6} M). These contractile responses were obtained in both endothelium-intact and -denuded aortic rings. Several concentrations of quercetin and pioglitazone were selected to investigate their inhibitory effects on contraction induced by an optimal concentration (10^{-7} M) of Ang II.

To investigate the effect of single and combination treatments, minimal concentrations of quercetin (10^{-7} M), pioglitazone (10^{-7} M) or their combination (pioglitazone 10^{-7} M + quercetin 10^{-7} M) was added to the organ bath in which the tissues were incubated for 30 min prior to a single concentration of Ang II (10^{-7} M). The protocol was repeated in another set of tissues exposed to the eNOS inhibitor, L-NAME (10^{-4} M), and in rings without endothelium. At the end of each experiment, the aortic rings were stimulated again with high K^+ to confirm tissue viability. The Ang II-induced contraction was calculated as a percentage of the initial high K^+- induced contraction.

2.9. Vascular NO metabolites measurement

Aortic rings that underwent isometric tension experiments and pre-treated with vehicle, single- or combination (pioglitazone + quercetin) treatment in the presence or absence of L-NAME for 30 min and stimulated with Ang II (10^{-7} M) were snap-frozen in liquid nitrogen. The rings were then homogenized in phosphate-buffered saline (PBS), and total nitrate/nitrite levels were measured using the standard Griess reaction method kit (Cayman Chemicals, Ann Arbor, MI) [31].

2.9.1. In situ detection of NO production

Aortic ring NO production in response to single- or combination (pioglitazone plus quercetin) treatment was determined using NO detection specific dye, 4-Amino-5-Methylamino-2′,7′-Difluorofluorescein Diacetate (DAF-FM, Invitrogen, CA, USA) [32]. Briefly, rings incubated with vehicle, pioglitazone and/or quercetin with or without L-NAME were stimulated with Ang II (10^{-7} M) prior to (DAF-FM; 10^{-5} M) incubation for 30 min. The tissues were thereafter immediately snap-frozen with optimal cutting temperature OCT embedding compound (Sakura Finetek, Netherlands) in liquid nitrogen. Frozen rings were then cut into 20 μM sections and imaged with optimized excitation and emission wavelengths (DAF-FM, 495/519) using a fluorescence microscope (Nikon eclipse Ti-S; C-HGFi, Japan). All images were captured at constant exposure time and gain. The fluorescence intensity was quantified using imageJ software (imagej.nih.gov/ij/). Four regions were randomly selected from each aortic section and quantified as mean fluorescence intensity and normalized to the control SD level.

2.10. Vascular superoxide measurement

Lucigenin-enhanced chemiluminescence assay [13,33] with slight modification was used to measure the vascular superoxide production. Aortic rings were pre-incubated for 30 min at 37 °C in Krebs-HEPES buffer containing diethylthiocarbamic acid (DETCA, 10^{-3} M) to inactivate superoxide dismutase. After the 30 min incubation the tissues were exposed to Ang II (10^{-7} M) in the presence of pioglitazone and/or quercetin, or diphenylene iodonium (DPI; 5×10^{-6} M), an inhibitor of NADPH oxidase, or superoxide dismutase (SOD, 100 U/ml), a superoxide scavenger, or losartan (10^{-10} M), an AT_1 receptor inhibitor. Prior to measurement, the 96-well Optiplate was filled with 300 μl of Krebs-HEPES buffer containing lucigenin (5×10^{-6} M) and NADPH (10^{-4} M) per well to evaluate NADPH-oxidase-driven superoxide production. The Optiplate was loaded into the Hidex plate CHAMELEON™ V (Finland) in the luminescent mode to measure the background photoemission over 20 min. Subsequently, rings incubated earlier in Krebs-HEPES with DETCA were washed with Krebs-HEPES buffer and transferred to each appropriate well of the Optiplate. The photon emission was measured every minute for 20 min. Rings were dried for 48 h at 65 °C and weighed. The data were expressed as average counts per mg of vessel dry weight and normalized to the control SD.

2.10.1. In situ detection of vascular superoxide production

The amount of *in situ* vascular superoxide formation was determined using dihydroethidium (DHE, Invitrogen, CA, USA) dye [32,34]. Briefly, aortic rings from respective treatment groups stimulated with Ang II (10^{-7} M) were frozen in OCT compound (Sakura Finetek, Netherlands) and 20 μM cross-sections were obtained. The sections were incubated in the dark for 30 min in PBS containing 5×10^{-6} M DHE fluorescence dye. The fluorescence intensity was measured at excitation/emission of 488/605 nm to visualize the signal via fluorescence microscopy (Nikon eclipse Ti-S; C-HGFi, Japan). The images were analyzed using imageJ software (imagej.nih.gov/ij/). Four regions were randomly selected from each aortic section and quantified via mean fluorescence intensity and normalized to the SD control.

2.11. Statistical analysis

Results are shown as means ± SEM for the number of rats tested. Data were analyzed for statistical significance using Student's t-test for unpaired observations and, for comparison of more than two groups, one-way ANOVA followed by Bonferroni's multiple comparison tests (Prism 5.0, GraphPad Software) was applied. A value of $P < 0.05$ was taken as statistically significant.

3. Results

Characteristics of F-STZ-induced type 2 diabetic rat.

The body weight (BW) gain was calculated by subtracting the initial weight from the final weight. Values are expressed as

Table 1
Comparison of physiological indices in SD and F-STZ rats.

Parameters	SD control	F-STZ control
Body weight (g)	335.7 ± 8.7	421.0 ± 6.7#
Body weight gain (g)	174.5 ± 2.16	260.0 ± 10.6#
Systolic BP (mm Hg)	107.4 ± 1.65	147.9 ± 0.37#
Fasting blood glucose (mmol/L)	4.55 ± 0.29	12.52 ± 0.9#
Insulin ((μIU/ml)	8.75 ± 0.25	11.70 ± 0.54#
Total cholesterol (mmol/L)	0.45 ± 0.05	0.97 ± 0.08#
Triglycerides (mmol/L)	1.54 ± 0.97	4.52 ± 0.04#

#$p<0.05$ compared to the normal group.

mean ± S.E.M (n = 5–10).

As indicated in Table 1, F-STZ diabetic rats gained more weight than SD rats, although the starting weights were similar (161.20 ± 2.16 g). Similarly, the systolic blood pressure (SBP), fasting blood glucose, plasma insulin levels were significantly higher in F-STZ than in SD rats. Plasma total cholesterol and triglycerides levels were also higher in F-STZ compared to the SD.

Oral glucose and intraperitoneal insulin tolerance tests were carried out to measure glucose and insulin sensitivities, respectively. Compared to SD rats, the F-STZ rats had significantly impaired glucose tolerance (Fig. 1a). Insulin tolerance test showed that insulin at a dose of 0.75 U/kg significantly reduced the blood glucose levels in SD rats and delayed the normalization of blood glucose level to beyond 1 h as compared to the F-STZ rats where the levels normalized within an hour to the pre-test raised blood glucose level (Fig. 1b). The percentage reduction in blood glucose level following the insulin injection in F-STZ diabetic rats was much smaller ($p < 0.05$) compared to the SD rats (Fig. 1c).

To evidence the influence and activity of RAS in our study, a concentration-contraction curve was obtained for a range (10^{-10}–10^{-6} M) of single concentrations of Ang II, in both SD and F-STZ aortic rings because tissue desensitization occurs upon repeated or cumulative stimulation with Ang II. A decrease in contractility was observed at 10^{-6} M, the highest Ang II concentration tested which may due to the tachyphylaxis effect at this high dose of Ang II [35]. Ang II-induced contraction of endothelium-intact rings from F-STZ diabetic rats was significantly higher than those of the SD rats at 10^{-8} M and 10^{-7} M (Fig. 2b). Both SD and F-STZ groups exhibited greater contractility to Ang II in endothelium-denuded compared to intact preparations, respectively (Fig. 2a and b).

Quercetin or pioglitazone concentration-dependently (10^{-6}–10^{-4} M) inhibited the contraction to Ang II in endothelium-intact aortic rings from F-STZ diabetic rats (Fig. 3a and b, respectively). However, with the endothelium-intact SD group these agents inhibited Ang II-induced contraction only at the highest concentration (10^{-4} M) tested. Both drugs demonstrated minimal inhibitory effects at 10^{-7} M concentration, thus, this concentration was chosen to evaluate the effects of combination treatment.

Quercetin or pioglitazone at a concentration of 10^{-7} M did not inhibit Ang II-induced contraction in SD (Fig. 4a) or F-STZ (Fig. 4b) endothelium-intact preparations. However, the combination of both agents (10^{-7} M) significantly inhibited Ang II contraction by more than 50% in F-STZ preparations (Fig. 4b) but had no effect on the SD aortic rings. As for endothelium-denuded preparations from both SD and F-STZ, mono and combination treatment with quercetin and pioglitazone significantly reduced the Ang II-induced contractions compared to the control. Thus, further experiments evaluated the possible mechanisms underlying the effect on both SD normal and F-STZ diabetic tissues of the combined treatment with pioglitazone and quercetin.

To understand the role of endothelium in this study, the beneficial effects of quercetin and/or pioglitazone were tested in tissues with and without endothelium. Quercetin or pioglitazone (10^{-7} M) did not affect Ang II-induced contraction of endothelium-intact SD and F-STZ preparations, but significantly inhibited contraction of the endothelium-denuded tissues (Fig. 5a and b). The combination treatment decreased Ang II contraction in F-STZ rings with or without endothelium but the reduction of Ang II-induced contraction was only seen in endothelium-denuded rings from SD rats.

On the other hand, the synergy effect of combination treatment in F-STZ was only observed in endothelium-intact tissues with 44.9% inhibition of Ang II contractile response compared to a total of 8.3% reduction when the individual effects of pioglitazone and quercetin are added (Fig. 5b). An additive (not synergistic) effect was observed with respect to pioglitazone and quercetin combination treatment in the endothelium-denuded tissues with 64.0% inhibition of Ang II contractile response compared to 68.4% when the individual effects of pioglitazone and quercetin are added. The additive but not synergistic effect was seen in response to combination treatment in SD rings without endothelium; combination treatment produced 17.1% inhibition of Ang II contractile response

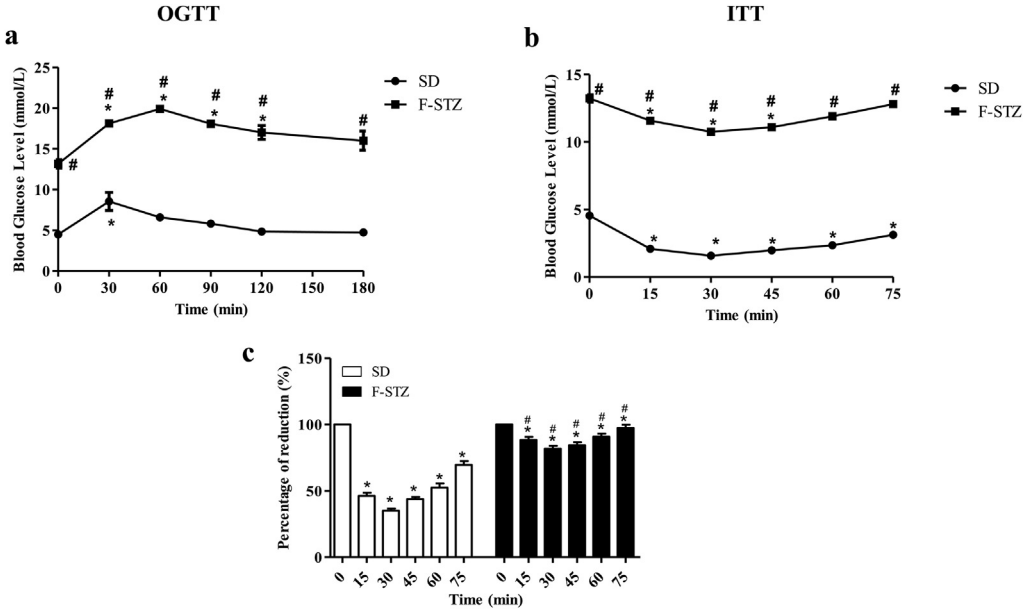

Fig. 1. Oral glucose and intraperitoneal insulin tolerance tests in SD (control) and F-STZ diabetic rats. (a) Temporal changes in blood glucose level following oral glucose (3 g/kg) feed in control and diabetic rats. Temporal changes in blood glucose level, (b) in absolute figures and (c) as % change of area under the curve, following an intraperitoneal injection of insulin (0.75 IU/kg) in control and diabetic rats. Symbols represent mean ± S.E.M. ($n = 20$). $^{\#}p < 0.05$ compared to same time point with age-matched SD control, $^{*}p < 0.05$ compared to 0 min fasting blood glucose level of the respective group.

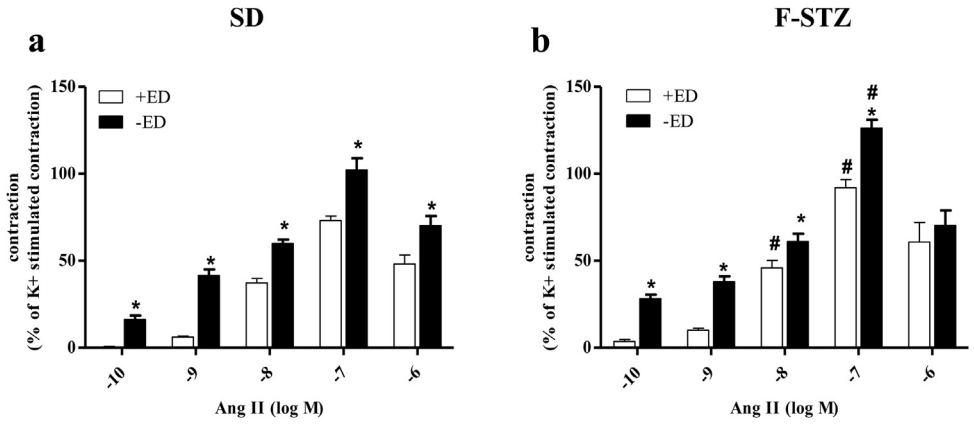

Fig. 2. Angiotensin-II-induced contraction of endothelium-intact (+ED) and denuded (−ED) aortic rings from control SD (a) and F-STZ diabetic (b) rats. Data are expressed as mean ± S.E.M ($n = 5–7$). $^{*}p < 0.05$, compared to within-group endothelium-intact tissues; $^{\#}p < 0.05$ compared to the corresponding (+ED or −ED) SD group.

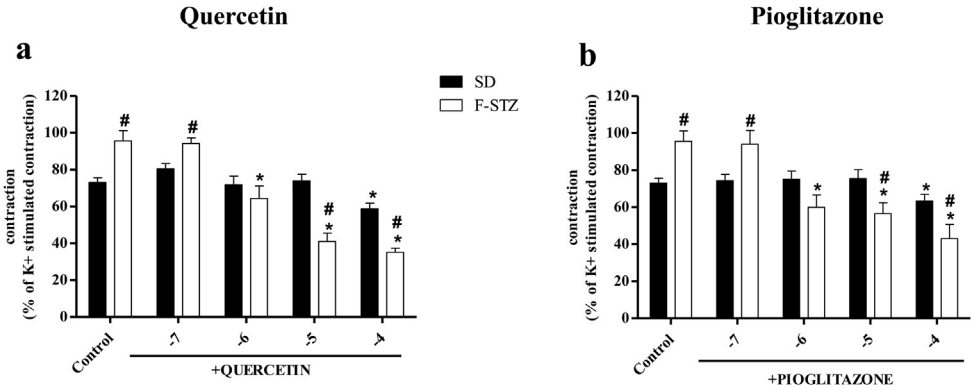

Fig. 3. Effect of different concentrations of quercetin (a) and pioglitazone (b) on Ang II (10^{-7} M)-induced contraction of endothelium-intact aortic rings from SD and F-STZ diabetic rats. Data are expressed as mean ± S.E.M ($n = 5–7$). $^{*}p < 0.05$ compared to respective control group, $^{\#}p < 0.05$ compared to the SD respective group.

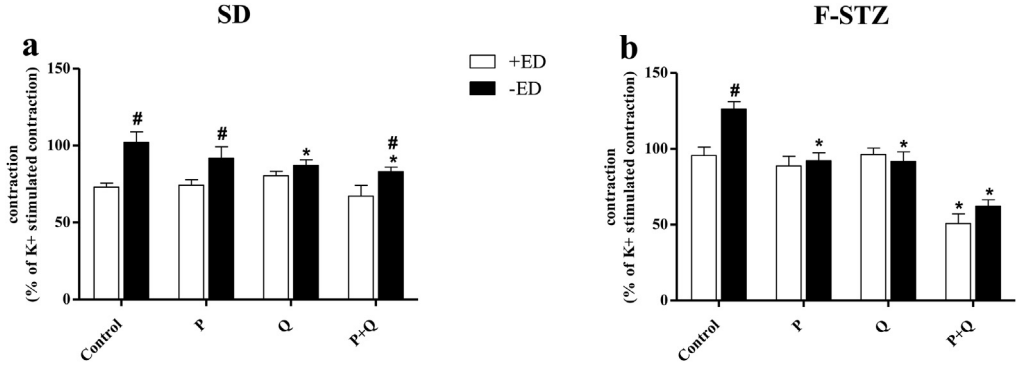

Fig. 4. Effect of treatment with pioglitazone (P, 10^{-7} M) or quercetin (Q, 10^{-7} M) or their combination (P + Q) on Ang II (10^{-7} M)-induced contraction of endothelium-intact (+ED) and endothelium-denuded (−ED) aortic rings from control SD (a) and F-STZ (b) diabetic rats. Data are expressed as mean ± S.E.M ($n = 5–7$). $^{*}p < 0.05$ compared to the respective vehicle (control) group, $^{\#}p < 0.05$ compared to the respective +ED group.

compared to a total of 18.2% reduction when the individual effects of pioglitazone and quercetin are added (Fig. 5a).

Single concentration (10^{-7} M) of quercetin or pioglitazone reduced the Ang II-induced contraction of endothelium-intact or denuded SD and F-STZ tissues treated with the non-selective eNOS inhibitor, L-NAME. Combination treatment produced an additive effect in both endothelium-intact and -denuded F-STZ tissues with 15.7% and 52.0% inhibition, respectively, of Ang II-induced contraction. As for SD tissues, combination treatment showed an additive effect in endothelium-intact tissues with 38.2% inhibition and endothelium-denuded tissues with 66.1% inhibition.

To demonstrate that the ROS-scavenging effect of pioglitazone and quercetin combination treatment contributed to their inhibitory action on Ang II contraction, we explored the effects of single (pioglitazone or quercetin) and combination (pioglitazone + quercetin) treatments on superoxide levels in aortic rings using DHE staining and lucigenin assay under various experimental condition (Fig. 6). F-STZ diabetic aortic rings

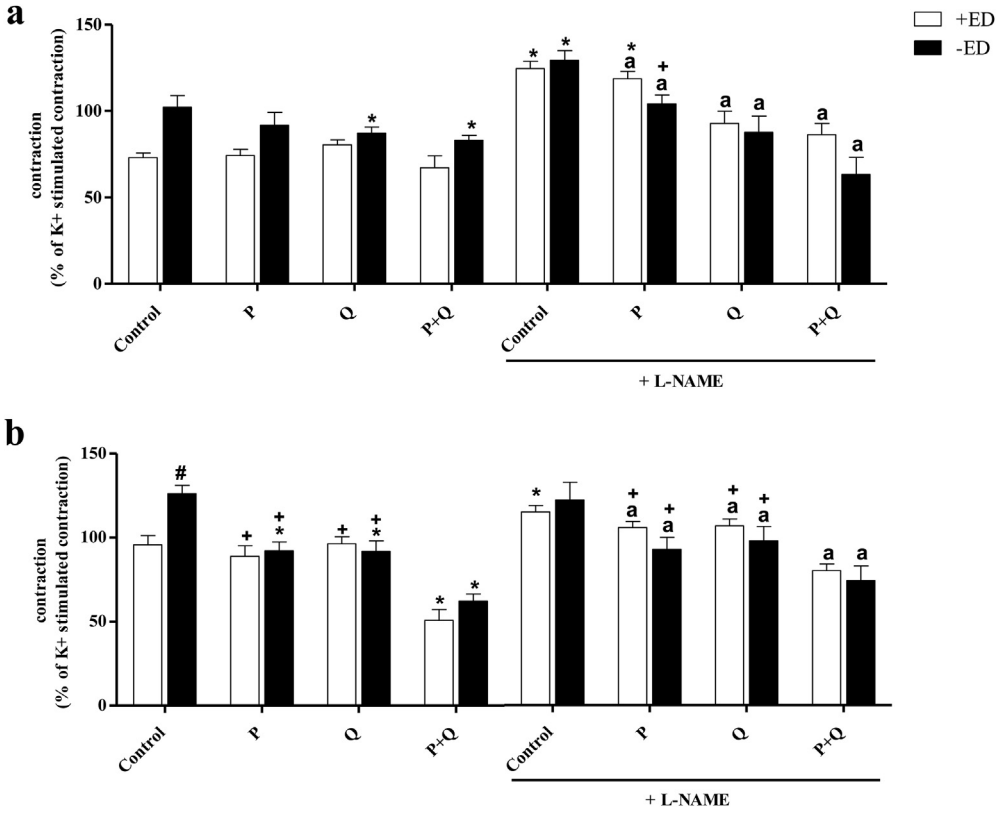

Fig. 5. The effect of treatment with 10^{-7} M quercetin (Q) and/or 10^{-7} M pioglitazone (P) on L-NAME (10^{-4} M) – treated or untreated Ang II (10^{-7} M) -contracted endothelium-intact (+ED) and endothelium-denuded (−ED) aortic rings from SD (a) and F-STZ diabetic (b) rats. Data are expressed as mean ± S.E.M ($n = 5–7$). $^{*}p < 0.05$ compared with L-NAME-untreated control, $^{+}p < 0.05$, compared with corresponding P + Q, $^{a}p < 0.05$, compared with corresponding L-NAME-treated control group, $^{\#}p < 0.05$ compared with corresponding +ED group.

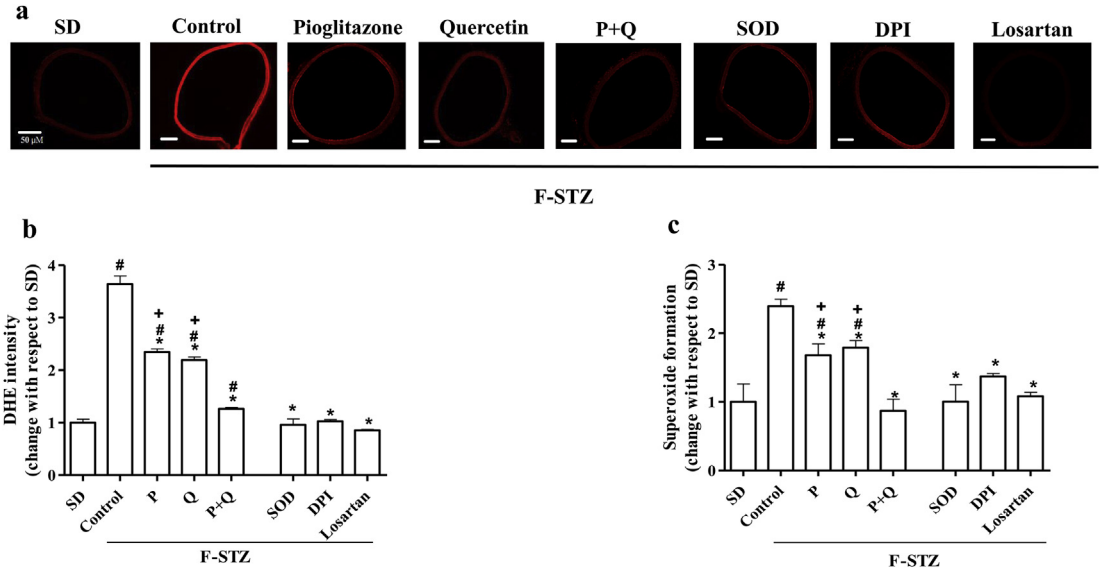

Fig. 6. The effects of treatment with quercetin (Q) and/or pioglitazone (P), or SOD, DPI or losartan on Ang II (10^{-7} M) -stimulated superoxide production in endothelium-intact aortic rings from F-STZ diabetic rats. SD and F-STZ (Control) tissues are untreated. (a) Representative fluorescence images of superoxide production as measured by dihydroethidium (DHE); (b) The quantified mean fluorescence intensity of DHE-stained aortic sections; (c) The quantitative vascular superoxide production by lucigenin-enhanced chemiluminescence method. Data are expressed as mean ± S.E.M ($n = 5–6$). $^{\#}p < 0.05$, compared to SD, $^{*}p < 0.05$ compared to F-STZ (control), $^{+}p < 0.05$ compared to P + Q.

demonstrated an increased DHE intensity staining (Fig. 6a and b) and superoxide production (Fig. 6c) compared to the SD tissues. Treatment with quercetin or pioglitazone decreased the superoxide level (Fig. 6) as evidenced by reduced DHE staining. The combination treatment further depressed the DHE intensity and vascular superoxide production compared to pioglitazone or quercetin

treatment. The decrease observed with the combination was equal to those observed in the presence of superoxide dismutase (SOD), a superoxide scavenger; diphenyleneiodonium (DPI), an NADPH inhibitor; or losartan, an AT1 receptor antagonist.

To determine the contribution of NO in the combination (pioglitazone + quercetin) treatment, we imaged aortic sections loaded with DAF-FM DA (a fluorescent indicator of NO) and measured total vascular NO products. The fluorescence intensity for NO from the F-STZ aortic sections was significantly lower than from the SD (Fig. 7a and b). Treatment with quercetin or pioglitazone alone did not significantly enhance NO production. However, the combination treatment enhanced the NO production to above the levels seen in the control SD group as measured by DAF-FM DA fluorescence and total nitrate/nitrite (Fig. 7b and c). Pre-incubation with L-NAME significantly lowered but did not abolish the intensity in all treatment groups.

4. Discussion

Clinically pioglitazone enhances insulin sensitivity and demonstrates a beneficial effect on endothelial function. Prolonged use, however, results in adverse effects such as fluid retention, peripheral edema, liver injury and certain heart problem [24]. Therefore, this study postulated that using minimal effective concentration of pioglitazone in combination with the antioxidant, quercetin, may provide beneficial therapeutic interaction with lesser risk for the development of side effects. Interestingly, we found that pretreatment with a combination of minimal concentrations of quercetin and pioglitazone synergistically inhibited Ang II-induced contraction in endothelium-intact aorta from F-STZ diabetic but not the SD tissues. Consistent with this result is the finding that quercetin or pioglitazone alone concentration-dependently reduced the Ang II-induced contraction in F-STZ diabetic aortic rings but not in the SD aortic rings, except at the highest concentration. These results suggest that the effects of quercetin and/or pioglitazone were more pronounced in the unhealthy (F-STZ diabetic) than the healthy (SD) tissues. These results suggest that quercetin and/or pioglitazone interacts with the pathophysiologic mechanisms of Ang II (inhibition of endothelial function) in tissues from F-STZ diabetic animals. These findings are in line with previous studies where the Ang II contractile responses were higher in diabetic animal vessels [36,37] and in non-diabetic endothelium-denuded aortic rings [38–40]. Enhanced expression of AT_1 receptors and/or reduced NO bioavailability has been associated with the enhanced Ang II contractility in diabetic vessels [37,41,42].

In sync with the hypotheses of this study, quercetin and pioglitazone individually have been reported for their beneficial effects in previous studies. Quercetin has been shown to improve nitric oxide bioavailability via its antioxidant action; it releases endothelium-derived relaxing factors [17] and inhibits NADPH oxidase enzyme which is involved in the production of superoxide anions [43,44] in diabetic and hypertensive animal models. Similarly, pioglitazone has been demonstrated to reduce oxidative stress and increase nitric oxide bioavailability in diabetic vessels [45,46]. It is probable that quercetin and pioglitazone, through their ROS scavenging action and NO-releasing property, normalize Ang II-induced contraction in F-STZ aortic rings to the levels observed in SD aortic rings. This speculation is consistent with the observation

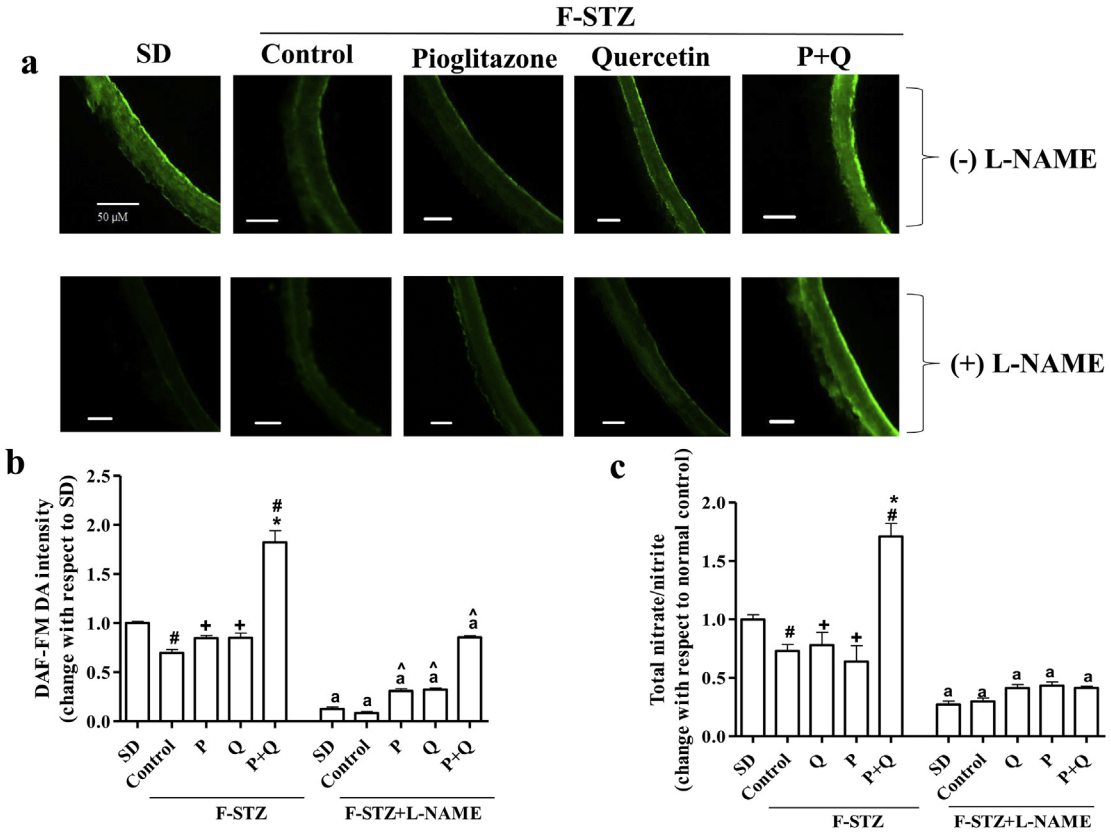

Fig. 7. Total nitric oxide levels in quercetin and pioglitazone-treated tissues. The effect of treatment with quercetin (Q) and/or pioglitazone (P) on NO production in Ang II (10^{-7} M)-stimulated endothelium-intact aortic rings from normal SD and F-STZ diabetic rats. (a) Representative fluorescence images of NO production as measured by DAF-FM DA; (b) The quantified mean fluorescence intensity of DAF-FM DA-stained aortic sections; (c) Measurement of vascular total nitrate/nitrite. Data are expressed as mean ± S.E.M (n = 5). $^{\#}p < 0.05$, compared to SD, $^{*}p < 0.05$ compared to F-STZ control, $^{+}p < 0.05$ compared to P + Q, $^{a}p<0.05$, compared to without L-NAME, $^{\hat{}}p < 0.05$ compared to F-STZ control +L-NAME.

that the effects of quercetin and pioglitazone are more pronounced in conditions of impaired EDNO bioavailability.

However, the synergy of quercetin and pioglitazone combination was lost and an additive effect was observed in rings denuded of endothelium or treated with L-NAME, suggesting that the synergistic attenuating action of quercetin and pioglitazone on Ang II contraction is, in part, dependent on endothelium-derived relaxant factor, most likely EDNO since the combination strongly promotes NO (Fig. 7). In agreement with previous studies, treatment with L-NAME or removal of endothelium enhanced Ang II contraction [11,17,18], indicating eNOS-mediated NO release inhibits Ang II contractility of F-STZ or SD aortic rings. However, in endothelium-denuded or L-NAME treated F-STZ and SD rings, the monotherapy also decreased Ang II-induced contraction to a similar extent as in SD endothelium-intact aortic rings (Fig. 5). These findings raise the possibility that the anticontractile effect of pioglitazone and quercetin is partly mediated by some endothelium- or EDNO-independent mechanism, probably an endothelium-independent NO pool which has been previously described [47]. The observation that pioglitazone plus quercetin treatment raised NO in L-NAME-treated tissues (Fig. 7) supports this view.

Ang II has been shown to release superoxide anions via NADH/NADPH oxidase activity in diabetic tissues [48]. Elevated ROS and superoxide anions production were observed in Ang II-stimulated F-STZ aortic rings and this was decreased on treatment with quercetin or pioglitazone alone and much more in combination. Moreover, the reduction seen with combination treatment was to a similar extent as observed with known inhibitors of ROS, like SOD, DPI, and losartan. This suggests that combination of minimal effective concentrations of quercetin and pioglitazone is able to achieve the maximal therapeutic effect in this model. In addition, treatment with pioglitazone and quercetin reversed the decreased NO levels seen in the F-STZ tissues, raising NO to a level two times more than that observed in the SD control tissues. This suggests an additive interaction between quercetin and pioglitazone on NO production in the F-STZ tissues. Although the presence of L-NAME decreased the elevated NO level in the pioglitazone plus quercetin treated F-STZ tissues, it was not fully suppressed, suggesting the possibility of additional eNOS-independent NO involvement as discussed above. This speculation requires further investigation although the eNOS-independent release of NO has been demonstrated in hypertensive aorta [47]. Although monotherapy of quercetin or pioglitazone decreased ROS level and improved the impaired NO production in F-STZ aortic rings, this was not sufficient to decrease the elevated Ang II contractility, perhaps because the decrease in ROS and/or the improved NO production did not attain a critical level for this effect.

5. Conclusion

In conclusion, the present study demonstrates that a combination of minimally effective concentrations of pioglitazone and quercetin synergistically inhibits Ang II-induced contraction of isolated F-STZ type 2 diabetic aortic tissues. In contrast, either agent alone or doubling the concentration of each (data not shown) did not inhibit the contractile effect of Ang II in these tissues. This inhibition of Ang II-induced contraction is endothelium-dependent and involves both NO-releasing and ROS-scavenging actions of pioglitazone and quercetin. These results raise the need to explore the possibility of similar findings *in vivo*. The current data suggest that a combination of low concentrations of pioglitazone and quercetin is able to decrease oxidative stress and provide vascular protection in type 2 diabetes model and thus the possibility of a dietary/pharmaceutical supplementation of pioglitazone with quercetin for the optimal therapeutic outcome.

Competing interest

The authors declare that they have no competing interests.

Authors contribution

M.D.D and A.F.I are the co-principal investigators for this project. T.K., M.M.R., M.D.D., A.F.I. designed research; T.K. performed experiments; analyzed data; T.K., M.D.D and A.F.I wrote the paper. M.D.D and A.F.I had primary responsibility for final content. All authors read and approved the final manuscript.

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