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Quercetin and pioglitazone synergistically reverse endothelial dysfunction in isolated aorta from fructose-streptozotocin (F-STZ)-induced diabetic rats

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ABSTRACT

Pioglitazone is an anti-diabetic drug with potential to cause adverse effects following prolonged use. This study, therefore, investigated the effects of combination treatment of a subliminal concentration of pioglitazone and quercetin, a potent antioxidant, on vascular reactivity of aorta isolated from fructose-streptozotocin (F-STZ)-induced diabetic rats. Relaxation to acetylcholine and sodium nitroprusside, and contraction to phenylephrine were tested in organ bath chambers following pre-incubation with vehicle (DMSO; 0.05%), quercetin (10−7 M), pioglitazone (10−7 M), or their combination (P+Q; 10−7 M each drug). Subliminal concentration of quercetin or pioglitazone did not alter the acetylcholine- induced relaxation nor the phenylephrine-induced contraction in both normal rat and diabetic F-STZ induced tissues. However, P+Q combination synergistically improved the impaired acetylcholine-induced relaxation and decreased the elevated phenylephrine-induced contraction in aortic rings from diabetic, but not in the normal rats. Neither mono nor combination treatment altered sodium nitroprusside-induced relaxation. The combination also synergistically decreased superoxide anion and increased nitric oxide production compared to the individual treatments in aorta from diabetic rats. Overall, these data demonstrated a synergistic effect, in which, a combination (P+Q; 10−7 M each drug) caused a significantly greater effect than 10−6 M of either agent in improving endothelial function of isolated diabetic aorta. In conclusion, a combination of subliminal concentrations of pioglitazone and quercetin is able to decrease oxidative stress and provide synergistic vascular protection in type 2 diabetes mellitus and thus the possibility of using quercetin as a supplement to pioglitazone in the treatment of diabetes with the goal of reducing pioglitazone toxicity.

1. Introduction

The pathophysiological risk factors of obesity, insulin resistance, hypertension, hyperlipidemia, and hyperglycemia are clustered as metabolic syndrome. These factors either alone or collectively lead to the onset of type 2 diabetes mellitus and increase the incidence of macro- and microvascular complications (Guerci et al., 2001; Scheen, 2003; Zimmet et al., 2001). In view of this, endothelial dysfunction portrayed as an imbalance between endothelium-derived relaxing and contracting factors further contributes to the progression of vascular damage (Hadi and Suwaidi, 2007). Deficiency in the endothelium-derived relaxing factors, especially endothelium-derived nitric oxide links insulin resistance and endothelial dysfunction (Cersosimo and DeFronzo, 2006). Deficiency of endothelium-derived nitric oxide results from reduced synthesis and/or release, and the presence of high levels of reactive oxygen species, which are produced by a cellular disturbance in glucose and lipid metabolism (Fatehi-Hassanabad et al., 2010). Thus, pharmacological agents capable of restoring the nitric oxide balance may result in better health in diabetes mellitus.

Antioxidants from nutrient and non-nutrient preparations administered to diabetic animals have been shown to improve endothelial dysfunction and diabetes mellitus via their antioxidant property (Leo and Woodman, 2015; Montero et al., 2014; Perez-Vizcaino and Duarte, 2010; Sena et al., 2007). Among the class of such nutrients that are gaining interest are the polyphenolic flavonoid compounds (Bohm et al., 1998). Quercetin, one of the major flavonoids of plant origin, is commonly found in human diet in the form of vegetables and fruits and is widely accepted for its potent vasodilator, free radical-scavenging and antioxidant action (Chen et al., 1990; Woodman and Malakul, 2009). Moreover, quercetin has been shown to improve endothelium-dependent vasodilatation through its protective effect on nitric oxide in both *in-vitro* and *in-vivo* studies of diabetic rat models (Ajay et al., 2006; Leo and Woodman, 2015; Machha et al., 2007; Woodman and Malakul, 2009).

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Pioglitazone, is an oral antihyperglycemic agent which enhances insulin sensitivity and is referred to as “insulin sensitizers” (Yki-Jarvinen, 2004). It acts as ligand for the peroxisome proliferator-activated receptor gamma (PPARγ). The activation of PPARγ suppresses lipolysis, decreases the plasma free fatty acids, leptin, and tumor necrosis factor α, and increases adiponectin level, all of which lead to increased insulin sensitivity (Ikeda et al., 1990; Tozzo et al., 2015). Besides, activation of PPARγ also inhibits detrimental vascular inflammatory events. It has a distinctive role in up-regulating the expression of endothelial nitric oxide synthase (eNOS), thus resulting in the enhanced generation of vascular nitric oxide (Balakumar and Kathuria, 2012). Furthermore, pioglitazone has been shown to ameliorate endothelial dysfunction in diabetic mice (Huang et al., 2008), aorta from fructose-fed (Kotchen et al., 1997) and streptozotocin-induced (Majithiya et al., 2005) diabetic rats, as well as in type 2 diabetic patients (Yu et al., 2013). Despite these positive effects, prolonged use of pioglitazone can produce adverse effects, such as fluid retention, peripheral edema, and precipitate congestive heart failure (Nesto et al., 2003).

Finding a way to exploit the beneficial effects of pioglitazone while minimizing the risk of adverse effects would be a desirable therapeutic goal in the management of type 2 diabetes mellitus. We hypothesize that this is achievable through reduced doses of pioglitazone combined with nutrient antioxidant. The current study, therefore, investigated the effects and mechanism of single or combination of subliminal concentrations of quercetin and pioglitazone on vascular reactivity of isolated aorta from fructose-streptozotocin (F-STZ)-induced diabetic rat model.

2. Materials and methods

2.1. Drugs and chemicals

Acetylcholine, sodium nitroprusside, diphenyleneiodonium, fructose, quercetin, phenylephrine, pioglitazone, ω-nitro-L-arginine methyl ester (L-NAME), indomethacin, superoxide dismutase and streptozotocin (STZ) were purchased from Sigma Chemicals Company (St. Louis, MO, USA). All the drugs were dissolved in distilled water, except for quercetin, pioglitazone, and indomethacin. Indomethacin was prepared stock (10 mmol/l) in 0.5% w/v sodium carbonate and diluted with distilled water. Quercetin and pioglitazone stock solutions (10 mM) were prepared in 5% (v/v) dimethyl sulfoxide (DMSO). The final concentrations were prepared by serial dilutions with distilled water and the final concentration of DMSO was adjusted to less than 0.05% (v/v).

2.2. Experimental animals

Male Sprague-Dawley rat (SD, 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 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 of body weight, i.p). 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 level exceeded 11 mmol/l.

2.3. Vascular ring preparation

Ten weeks after the start of fructose feeding, the rats were killed by carbon dioxide (CO_2) inhalation. The descending thoracic aorta was isolated and cleaned from surrounding fat and connective tissue. To measure the isometric tension, the aorta was cut into small rings (3–5 in width) and suspended in a 5 ml organ bath containing oxygenated (5% CO_2 and 95% O_2) Krebs physiological salt solutions (KPSS in mM: NaCI 119, $NAHCO_3$ 25, KCI 4.7, KH_2PO_4 1.2, $MgSO_{4.7}H_2O$ 1.2, glucose 11.7, and $CaCI_{2.2}H_2O$ 2.5) and maintained at 37 °C. 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 tension and allowed to equilibrate for 45 min before initiation of experimental protocols. During this period of stabilization, the bath solution was replaced every 15 min. Following equilibration, the contractile responses of aortic rings were tested for 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, each tissue was contracted with phenylephrine (10^{-6} M) and then exposed to acetylcholine (10^{-5} M) at the peak of the contraction. Only tissues that exhibited more than 70% relaxation of the phenylephrine contraction were selected for subsequent studies as endothelium-intact tissues. In some preparations endothelium was removed by gently rubbing the lumen of aortic vessels with blunt forceps. The absence of endothelium was confirmed by the lack of a response to the endothelium-dependent vasodilator, acetylcholine, in phenylephrine -precontracted rings. Rings that exhibited < 5% relaxation of the phenylephrine contraction were included for further studies as endothelium-denuded tissues (Subramaniam et al., 2009).

2.4. Minimally effective concentrations of quercetin and pioglitazone

To choose the subliminal concentration for both quercetin and pioglitazone, aortic rings from age-matched SD and F-STZ diabetic rats were mounted in the organ bath and concentration-response curves to acetylcholine (10^{-10}–10^{-5} M), sodium nitroprusside (10^{-11}–10^{-6} M), and phenylephrine (10^{-9}–10^{-5} M) were recorded in the presence or absence of various single concentrations of quercetin or pioglitazone in the concentration range of 10^{-7}–10^{-4} M. The tissues were incubated in pioglitazone and/or quercetin for 30 min prior to generating the concentration-response curves. The responsiveness to phenylephrine (10^{-6} M)-induced pre-contraction was not significantly different between SD and F-STZ. The subliminal concentration of pioglitazone and quercetin that did not significantly alter ACh, SNP and PE responses were chosen for the combination studies.

2.5. Effect of quercetin and/or pioglitazone treatment on vascular reactivity

Aortic rings with and without endothelium from both SD and diabetic rats were incubated for 30 min with and without single or combination treatment with a subliminal concentration of quercetin (10^{-7} M) and pioglitazone (10^{-7} M) determined from the earlier experiment. After the incubation, concentration-response curves obtained from tissues exposed to increasing concentrations of acetylcholine, sodium nitroprusside or phenylephrine were recorded. All experiments were carried out in the presence of indomethacin (a non-selective cyclooxygenase inhibitor; 10^{-5} M) to exclude the involvement of prostaglandins. The phenylephrine-induced contraction was calculated as a percentage of the initial high K^+-induced contraction. The responses to acetylcholine and sodium nitroprusside were calculated as percentage inhibition of the phenylephrine (10^{-6} M) induced contractions.

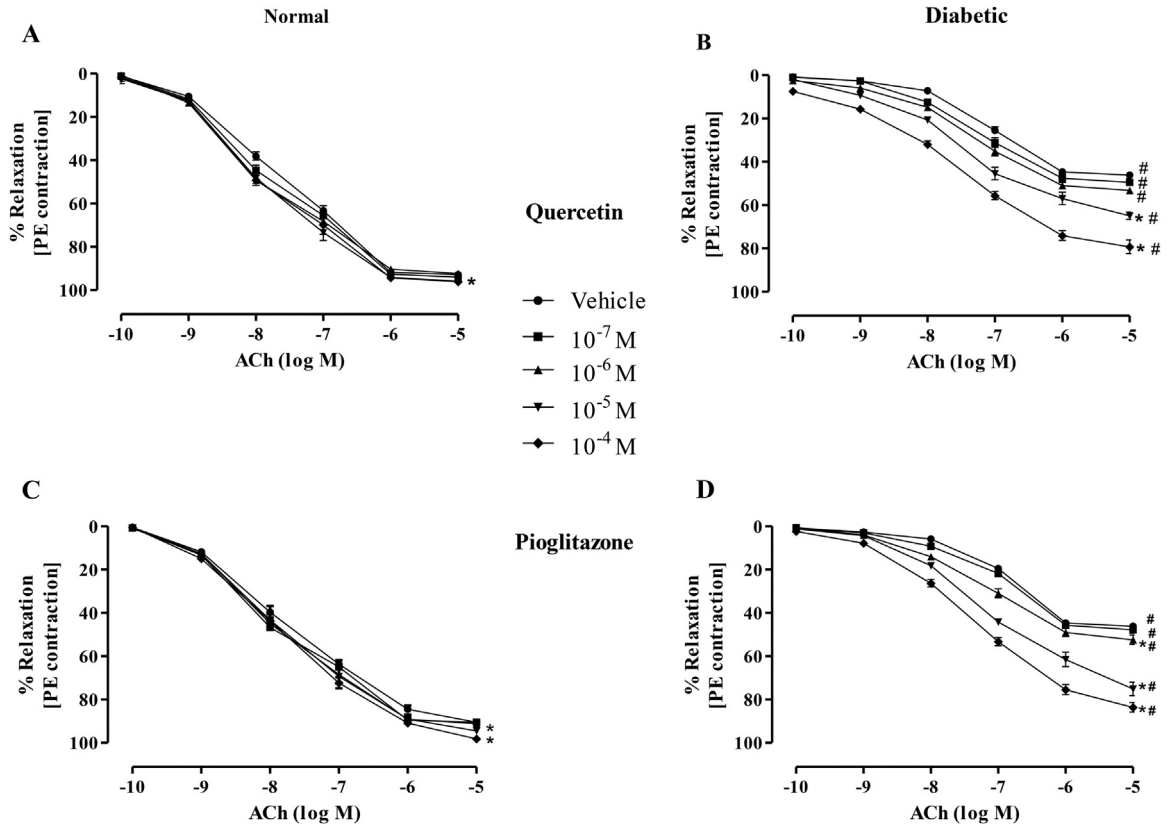


Fig. 1. Effect of different concentrations of Quercetin (upper panel) and Pioglitazone (lower panel) on acetylcholine-induced relaxation of phenylephrine-contracted endothelium-intact aortic rings from normal and diabetic rats. Data are expressed as mean ± S.E.M (n=6). *P < 0.05 compared to vehicle-treated group. #P < 0.05 compared to the normal group.

Table 1
Effect of different concentrations of Quercetin and Pioglitazone on acetylcholine-induced relaxation of phenylephrine-contracted endothelium-intact aortic rings from normal and diabetic rats. Data are expressed as mean ± S.E.M (n=6).

Quercetin	Normal		Diabetic	
	pEC_{50}	R (max) %	pEC_{50}	R (max) %
Vehicle(DMSO)	7.61 ± 0.075	92.83 ± 1.32	7.06 ± 0.043[b]	46.17 ± 1.37[b]
10^{-7} M	7.83 ± 0.082	94.00 ± 0.51	7.28 ± 0.028[b]	49.60 ± 1.60[b]
10^{-6} M	8.04 ± 0.088[a]	92.33 ± 0.61	7.31 ± 0.057[b]	53.17 ± 0.70[b]
10^{-5} M	7.95 ± 0.082	95.83 ± 0.40	7.47 ± 0.011[ab]	66.00 ± 1.43[ab]
10^{-4} M	7.95 ± 0.113	96.17 ± 0.54[a]	7.51 ± 0.100[ab]	79.33 ± 3.14[ab]
Pioglitazone	**Normal**		**Diabetic**	
	pEC_{50}	R (max) %	pEC_{50}	R (max) %
Vehicle(DMSO)	7.80 ± 0.082	90.5 ± 0.76	6.85 ± 0.042[b]	46.2 ± 1.37[b]
10^{-7} M	7.78 ± 0.091	90.5 ± 0.84	6.89 ± 0.072[b]	47.8 ± 1.74[b]
10^{-6} M	7.87 ± 0.056	91.1 ± 0.70	7.22 ± 0.097[b]	52.5 ± 2.18[ab]
10^{-5} M	7.88 ± 0.090	94.5 ± 0.56[a]	7.27 ± 0.090[ab]	75.1 ± 3.08[ab]
10^{-4} M	7.89 ± 0.082	98.1 ± 0.98[a]	7.35 ± 0.073[ab]	83.7 ± 2.18[ab]

[a] P < 0.05 compared to respective vehicle-treated.
[b] P < 0.05 compared to normal group.

2.6. *Effect of quercetin and/or pioglitazone treatment on nitric oxide level*

2.6.1. *Vascular NO metabolites measurement*

Aortic rings that underwent isometric tension experiments and were pre-treated with vehicle, single- or combination pioglitazone/quercetin in the presence or absence of L-NAME for 30 min, and stimulated with acetylcholine, 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) (Silswal et al., 2014).

2.6.2. *In situ detection of nitric oxide production*

Nitric oxide production in response to single- or combination (pioglitazone plus quercetin) treatments was assessed and imaged using nitric oxide detection specific dye, 4-Amino-5-Methylamino-2', 7'- Difluorofluorescein Diacetate (DAF-FM, Invitrogen, CA, USA (Young et al., 2012). Briefly, rings were incubated with vehicle, mono- or combination treatment with or without L-NAME, stimulated with

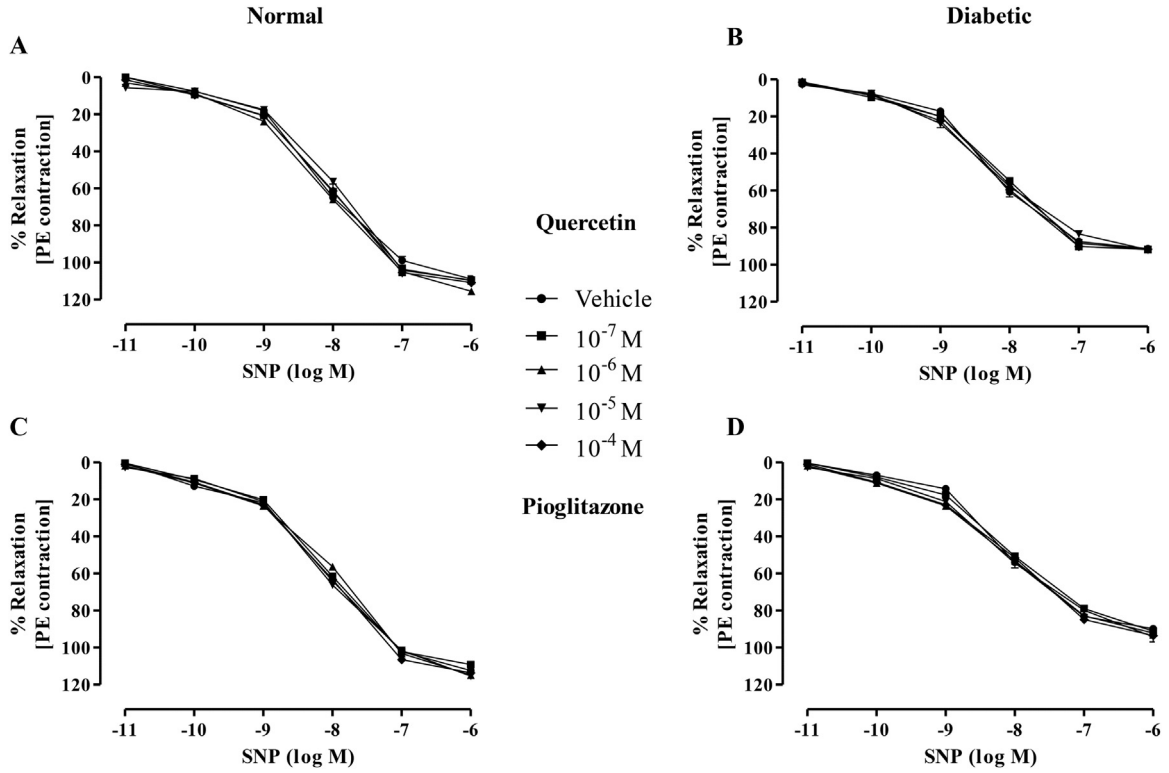


Fig. 2. Effect of different concentrations of Quercetin (upper panel) and Pioglitazone (lower panel) on the sodium nitroprusside-induced relaxation of phenylephrine-contracted endothelium-intact aortic rings from normal and diabetic rats. Data are expressed as mean ± S.E.M (n=6). *P < 0.05 compared to normal group.

Table 2
Effect of different concentrations of Quercetin and Pioglitazone on sodium nitroprusside-induced relaxation of phenylephrine-contracted endothelium-intact aortic rings from normal and diabetic rats. Data are expressed as mean ± S.E.M (n=6).

Quercetin	Normal		Diabetic	
	pEC_{50}	R (max) %	pEC_{50}	R (max) %
Vehicle(DMSO)	8.15 ± 0.029	111.5 ± 1.87	8.25 ± 0.035	91.33 ± 1.08[b]
10^{-7} M	8.09 ± 0.034	109.8 ± 0.79	8.20 ± 0.037	91.83 ± 0.94[b]
10^{-6} M	8.10 ± 0.032	115.5 ± 0.34	8.21 ± 0.037	91.83 ± 0.87[b]
10^{-5} M	7.97 ± 0.033[a]	115.3 ± 0.55	8.26 ± 0.047	92.00 ± 1.00[b]
10^{-4} M	8.07 ± 0.047	112.2 ± 1.32	8.28 ± 0.034	91.67 ± 0.76[b]
Pioglitazone	**Normal**		**Diabetic**	
	pEC_{50}	R (max) %	pEC_{50}	R (max) %
Vehicle(DMSO)	8.09 ± 0.045	112.3 ± 2.10	8.15 ± 0.030	89.83 ± 1.72[b]
10^{-7} M	8.09 ± 0.033	109.2 ± 1.35	8.06 ± 0.038	91.17 ± 0.47[b]
10^{-6} M	7.92 ± 0.041	114.8 ± 0.54	8.12 ± 0.070	92.17 ± 2.81[b]
10^{-5} M	8.09 ± 0.020	115.3 ± 0.42	8.09 ± 0.075	94.17 ± 2.92[b]
10^{-4} M	8.08 ± 0.038	113.7 ± 1.02	8.06 ± 0.055	93.5 ± 1.60[b]

[a] P < 0.05 compared to vehicle treated group.
[b] P < 0.05 compared to normal group.

acetylcholine (10^{-5} M) prior to DAF-FM dye (10^{-5} M) incubation for 30 min. The tissues were immediately snap frozen with 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) via fluorescence microscope (Nikon eclipse Ti-S; C-HGFi, Japan). All images were captured under 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 via mean fluorescence intensity and normalized to the average of SD control.

2.7. Effect of quercetin and/or pioglitazone treatment on oxidative stress

2.7.1. Effect of quercetin and/or pioglitazone treatment on β-NADH-induced oxidative stress

To determine the effect of mono-and combination treatment on oxidative stress, aortic rings from SD rats were incubated for 30 min with β-NADPH (inducer of superoxide anion through NADH/NADPH oxidase; 3×10^{-4} M) to mimic oxidative stress condition as in diabetic aortic tissues. Following that, the tissues were exposed to single and combination treatment with quercetin and pioglitazone, and apocynin (an NADPH oxidase inhibitor; 3×10^{-4} M) for 30 min prior to generat-

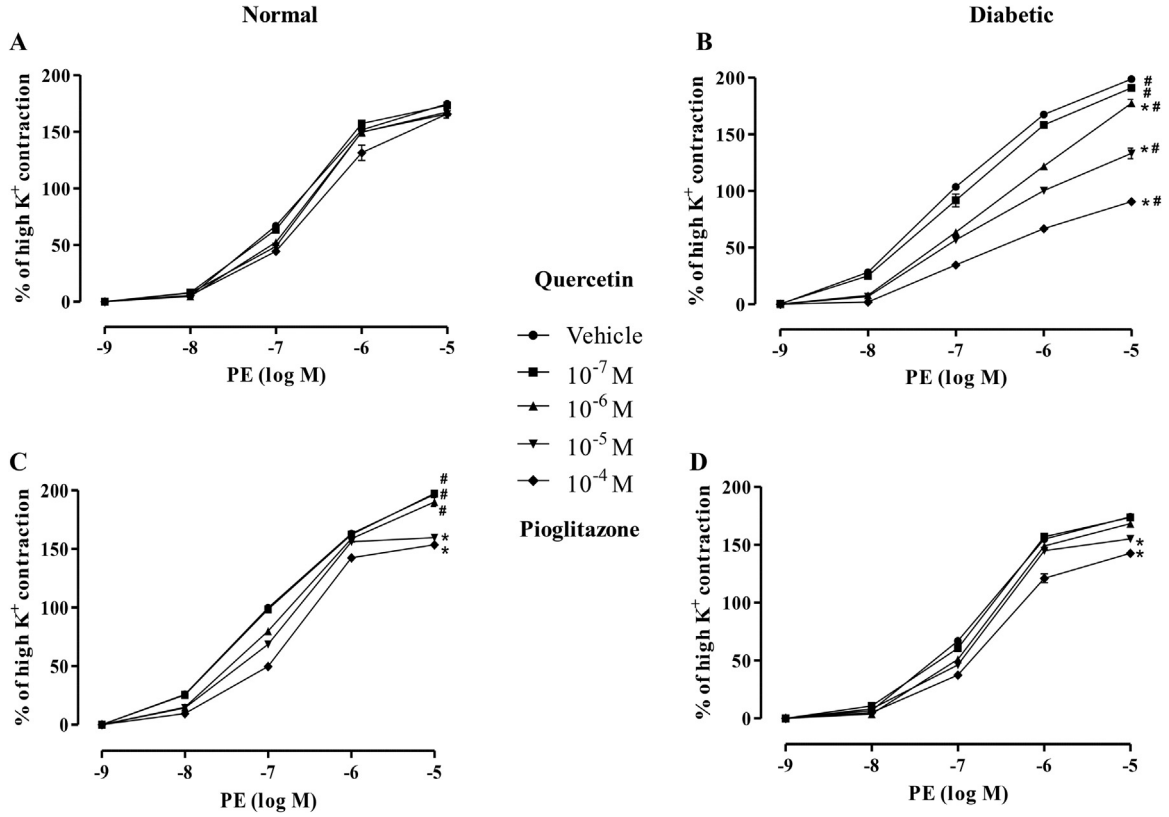


Fig. 3. Effect of different concentrations of Quercetin (upper panel) and Pioglitazone (lower panel) on phenylephrine-induced contraction of endothelium-intact aortic rings from normal and diabetic rats. The percentage of area under curve (AUC) was calculated from the highest peaks of the total area for all treatment groups and was normalized to the vehicle group. Data are expressed as mean ± S.E.M (n=6). *P < 0.05 compared to vehicle treated group. #P < 0.05 compared to the normal group.

Table 3
Effect of different concentrations of Quercetin and Pioglitazone on phenylephrine-induced contraction of endothelium intact aortic rings from SD and F-STZ diabetes rats. Data are expressed as mean ± S.E.M (n=6).

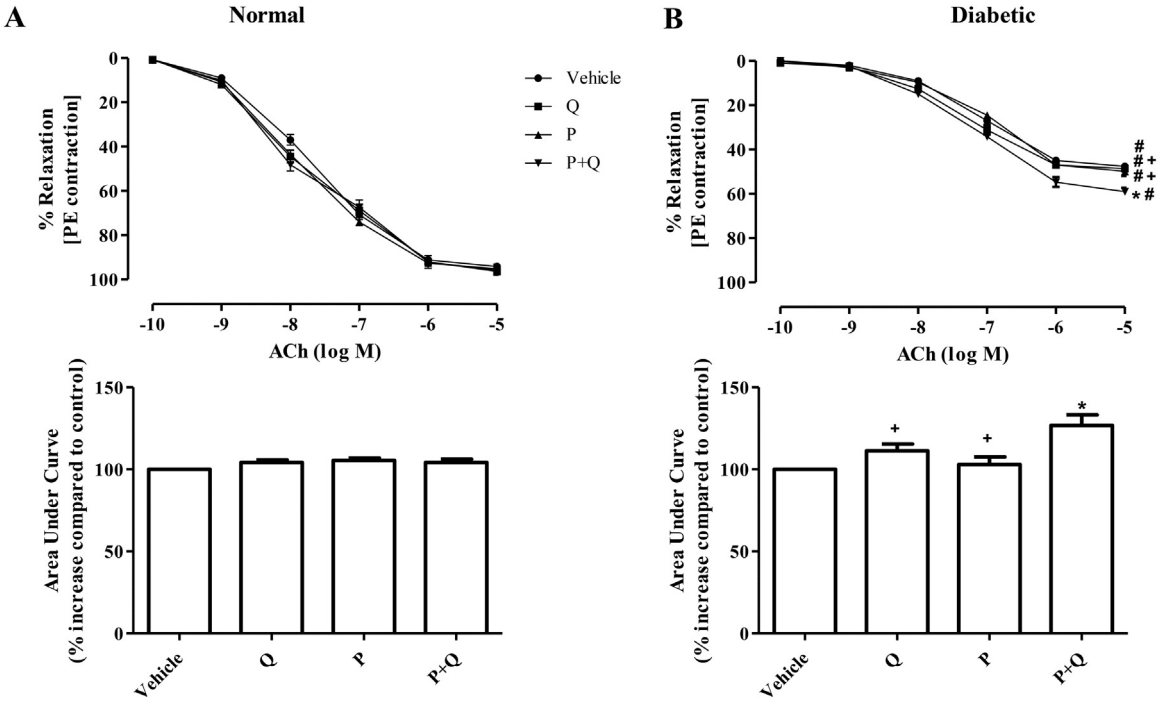
Quercetin	Normal		Diabetic	
	pEC_{50}	E (max) %	pEC_{50}	E (max) %
Vehicle(DMSO)	6.80 ± 0.028	174.7 ± 2.62	7.05 ± 0.023	198.8 ± 1.25[b]
10^{-7} M	6.79 ± 0.019	173.4 ± 1.72	6.92 ± 0.053	190.9 ± 2.56[b]
10^{-6} M	6.72 ± 0.018	167.4 ± 2.24	6.66 ± 0.086[ab]	177.5 ± 3.23[ab]
10^{-5} M	6.69 ± 0.031	165.8 ± 2.23	6.71 ± 0.084[a]	133.2 ± 4.69[ab]
10^{-4} M	6.53 ± 0.057[a]	165.9 ± 3.84	6.60 ± 0.074[a]	90.57 ± 1.89[ab]
Pioglitazone	**Normal**		**Diabetic**	
	pEC_{50}	E (max) %	pEC_{50}	E (max) %
Vehicle(DMSO)	6.81 ± 0.029	174.1 ± 1.97	6.99 ± 0.030[b]	196.5 ± 2.25[b]
10^{-7} M	6.76 ± 0.016	173.5 ± 0.763	6.97 ± 0.023[b]	197.4 ± 2.21[b]
10^{-6} M	6.69 ± 0.015	168.2 ± 2.93	6.80 ± 0.029[ab]	190.1 ± 3.75[b]
10^{-5} M	6.73 ± 0.019	155.2 ± 1.30[a]	6.80 ± 0.021[a]	159.8 ± 3.12[a]
10^{-4} M	6.60 ± 0.031[a]	142.7 ± 2.3[a]	6.74 ± 0.015[a]	153.5 ± 2.36[a]

[a] P < 0.05, compared to respective group vehicles.
[b] P < 0.05 compared to normal group.

ing concentration-response curves to acetylcholine (10^{-10}–10^{-5} M).

2.7.2. Vascular superoxide measurement

Lucigenin-enhanced chemiluminescence assay (Polizio et al., 2007; Woodman and Malakul, 2009) 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. Following that, the tissues were exposed to single and combination treatments with quercetin and pioglitazone, and diphenylene iodonium (DPI; 5×10^{-6} M, an inhibitor of NADPH oxidase) for a period of 30 min. Thereafter, acetylcholine (10^{-5} M) was added to all groups to mimic experimental conditions of the organ chamber studies. Prior to measurement, 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 then loaded into the Hidex plate CHAMELEON™ V (Hidex, 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



Groups (+ED)	Vehicle	Q	P	P+Q
Average ACh relaxation	100.0	104.1	105.4	104.3
% Change (+/-) in relaxation	-	+4.1	+5.4	+4.3
		P + Q = +9.5		

Groups (+ED)	Vehicle	Q	P	P+Q
Average ACh relaxation	100.0	110.8	102.2	127.2
% Change (+/-) in relaxation	-	+10.8	+2.2	+27.2
		P + Q = +12.9		

Fig. 4. Effect of treatment with quercetin (Q, 10^{-7} M) and/or pioglitazone (P, 10^{-7} M) on acetylcholine-induced relaxation of phenylephrine-contracted aortic rings from normal and diabetic rats. The percentage of area under curve (AUC) was calculated from the highest peaks of the total area for all treatment groups and was normalized to the vehicle group. Data are expressed as mean ± S.E.M (n=6). *P < 0.05 compared to vehicle treated group. #P < 0.05 compared to the normal group. +P < 0.05 compared to P+Q group.

Krebs-HEPES buffer and transferred to each appropriate well of the Optiplate. The photon emission was measured every minute over a period of 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 average of control SD.

2.7.3. *In situ* detection of vascular superoxide production

The amount of *in situ* vascular superoxide formation was determined using dihydroethidium (DHE, Invitrogen, CA, USA) dye (Young et al., 2012; Sutliff et al., 2002). Briefly, aortic rings pre-incubated with pioglitazone and/or quercetin were stimulated with acetylcholine to mimic experimental conditions of the organ chamber studies. The aortic rings 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 with DHE fluorescence dye (5×10^{-6} M) dissolved in phosphate buffered saline. 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 average of SD control.

2.8. Statistical analysis

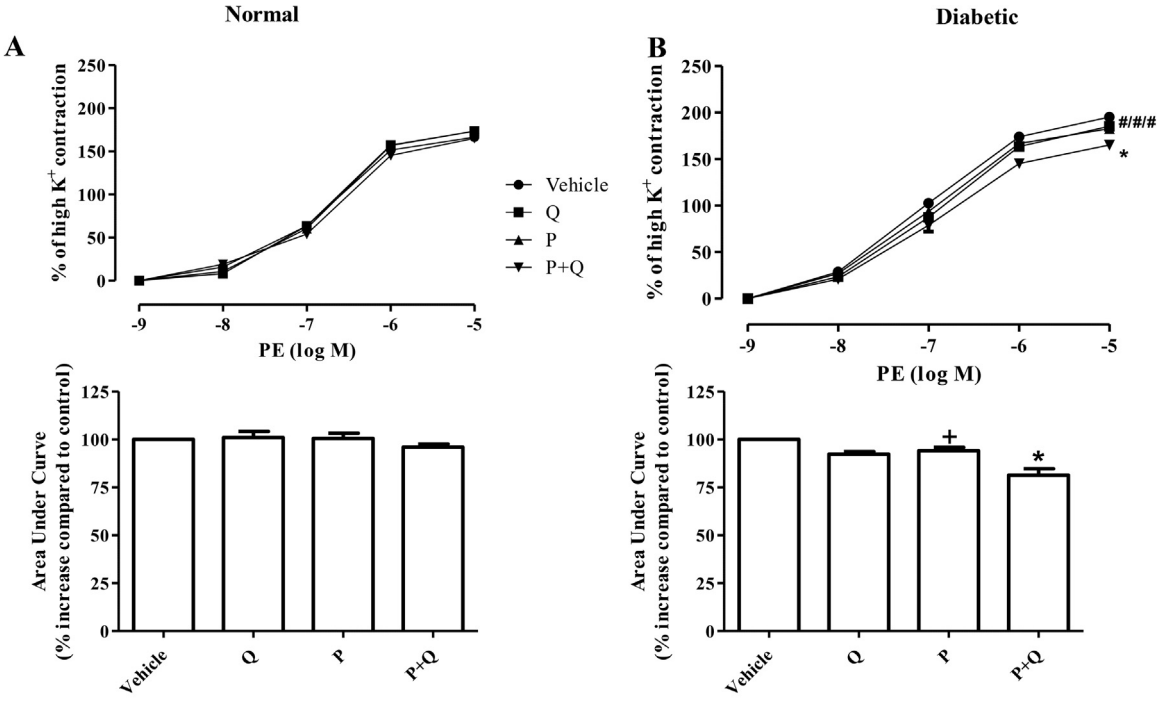
Results are shown as means ± SEM from the number of rats (n) studied. GraphPad Prism 5, (GraphPad Software La Jolla, CA, USA) was used to analyze the concentration-response curves by non-linear regression fitting. The concentrations that produce the maximal response (Emax/Rmax) and the concentration required to produce 50% of the maximal response (EC50) were derived from the analysis of the non-linear regression fitting of the individual concentration-response curves. Data were analyzed for statistical significance using Student's *t*-test for unpaired observations of two group and, for comparison of more than two groups, one-way ANOVA followed by Bonferroni's multiple comparison tests using the same statistical software. A value of P < 0.05 was taken as statistically significant.

3. Results

3.1. Effects of quercetin and/or pioglitazone on vascular reactivity

Aortic rings from diabetic rats demonstrated a significant reduction in their maximal relaxation response and pEC_{50} to acetylcholine compared to rings from normal rats (Fig. 1 and Table 1). Pre-treatment with quercetin significantly improved the acetylcholine –induced relaxation in a concentration-dependent manner in diabetic rat aortic rings (Fig. 1B), but not in the normal aortic ring, except for 10^{-4} M concentration (Fig. 1A). Pre-treatment with increasing concentrations of quercetin also enhanced the tissue sensitivity to acetylcholine in diabetic aortic tissues (Table 1). Similarly, increasing concentrations of pioglitazone improved the impaired relaxation to acetylcholine in the diabetic aorta (Fig. 1D) and a slight increase in relaxation to 10^{-5} M acetylcholine was observed following pre-incubation with 10^{-5} M and 10^{-4} M pioglitazone in normal rats (Fig. 1C). The pEC_{50} was also altered in diabetic aortic tissues (Table 1).

The maximal relaxation to sodium nitroprusside was significantly decreased in aorta from diabetic rats compared to normal rats (Fig. 2 and Table 2). However, the pEC50 was not altered in diabetic rings compared to rings from normal (Table 2). Pre-treatment with increasing concentrations of quercetin (Fig. 2A and B) and pioglitazone (Fig. 2C and D) did not affect the maximal relaxation and sensitivity



Groups (+ED)	Vehicle	Q	P	P+Q
Average PE contraction	100.0	100.5	100.2	95.8
% Change (+/-) in contraction	-	0.5	0.2	-4.2
		P + Q = -0.7		

Groups (+ED)	Vehicle	Q	P	P+Q
Average PE contraction	100.0	92.2	94.0	81.3
% Change (+/-) in contraction	-	-7.8	-6.0	-18.7
		P + Q = -13.8		

Fig. 5. Effect of treatment with quercetin (Q, 10^{-7} M) and/or pioglitazone (P, 10^{-7} M) on the phenylephrine-induced contraction of endothelium-intact aortic rings from normal and diabetic rats. The percentage of area under curve (AUC) was calculated from the highest peaks of the total area for all treatment groups and was normalized to the vehicle group. Data are expressed as mean ± S.E.M (n=6). *P < 0.05 compared to vehicle treated group. #P < 0.05 compared to the normal group. +P < 0.05 compared to P+Q group.

to sodium nitroprusside in diabetic aortic tissues (Table 2).

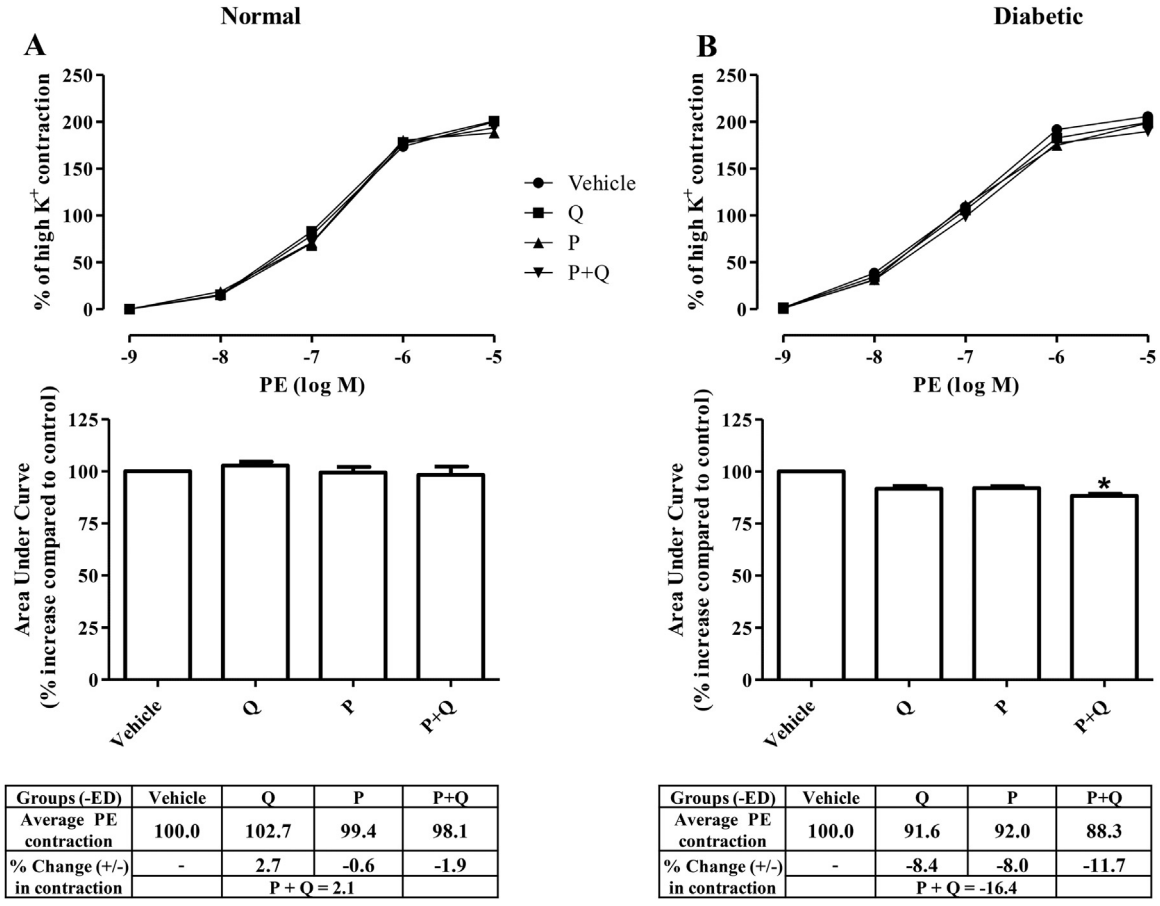
Phenylephrine-induced contraction was enhanced in diabetic compared to normal aortic rings (Fig. 3 and Table 3). Quercetin (10^{-7} M–10^{-4} M) significantly decreased peak phenylephrine -induced contraction in diabetic but not the normal aortic rings (Fig. 3A and B). The pEC50 of phenylephrine in diabetic tissues was decreased in the presence of 10^{-6} M–10^{-4} M quercetin compared to the vehicle control (Table 3). Pre-treatment with pioglitazone (10^{-5} M and 10^{-4} M) decreased the maximal contraction to phenylephrine in both normal (Fig. 3C) and diabetic aortic tissues (Fig. 3D). The pEC_{50} for phenylephrine was decreased in diabetic tissues pre-treated with pioglitazone (10^{-5} M and 10^{-4} M) compared to the vehicle control (Table 3).

3.2. Effect of quercetin and/or pioglitazone treatment on vascular reactivity

Combination of subliminal concentrations of quercetin (10^{-7} M) and pioglitazone (10^{-7} M) significantly enhanced acetylcholine-induced relaxation in aortic rings from diabetic rats much more than the relaxation observed with quercetin or pioglitazone. This effect was not seen in normal aortic rings (Fig. 4). The percentage of increase to ACh-induced relaxation in the diabetic aortic rings by the combination treatment was higher than the cumulative effect of the individual treatment as indicated by the area under curve (AUC), suggesting a synergistic effect of the combination (Fig. 4). The combination of 10^{-7} M each of pioglitazone and quercetin caused a significantly greater relaxation to acetylcholine than 10^{-6} M of either agent (R_{max}: 53.17% and 52.5% for quercetin and pioglitazone at 10^{-6} M, respectively vs 59.0% for the combination). However, the synergistic effect in the diabetic aorta was abolished with L-NAME pre-treatment (Suppl. 1). Compared to their untreated controls, treatment with quercetin and pioglitazone did not alter the vasodilation responses to sodium nitroprusside in normal and diabetic rat aortas (Suppl. 2). The combination of pioglitazone and quercetin significantly decreased the maximal contraction to phenylephrine compared to quercetin or pioglitazone alone in the diabetic, but not in the normal tissues (Fig. 5A and B). The combination of the 10^{-7} M each of pioglitazone and quercetin caused a significantly greater inhibition on phenylephrine-induced contraction than 10^{-6} M of either agent (E_{max}: 177.5% and 190.1% for quercetin and pioglitazone at 10^{-6} M, respectively vs 165.1% of the combination). The reduction of phenylephrine- induced contraction in diabetic rat aortic rings by the combination treatment was lost in rings without endothelium (Fig. 6A and B) and in endothelium-intact rings pre-treated with L-NAME (Suppl. 3). The percentage change of area under curve (AUC) with the combination treatment was smaller than the cumulative effect of the individual treatment in response to PE-induced contraction in diabetic rings with endothelium, suggesting a possible synergy (Fig. 5). However, percentage changes in PE-induced contraction were similar with either combination or individual treatments in diabetic rings with endothelium (Fig. 6).

3.3. Effect of quercetin and/or pioglitazone treatment on nitric oxide level

To determine the contribution of nitric oxide in the combination (pioglitazone + quercetin) treatment, aortic sections loaded with DAF-FM DA (a fluorescent indicator of nitric oxide) were imaged and total vascular nitric oxide products were measured. Nitric oxide production in diabetic rat aortic rings was significantly lowered compared to the rings from normal rats (Fig. 7A and B). Treatment with subliminal concentrations of quercetin or pioglitazone did not enhance the reduced nitric oxide production in rings from diabetic rats. In contrast, the combination significantly increased nitric oxide production com-



Groups (-ED)	Vehicle	Q	P	P+Q
Average PE contraction	100.0	102.7	99.4	98.1
% Change (+/-) in contraction	-	2.7	-0.6	-1.9
		P + Q = 2.1		

Groups (-ED)	Vehicle	Q	P	P+Q
Average PE contraction	100.0	91.6	92.0	88.3
% Change (+/-) in contraction	-	-8.4	-8.0	-11.7
		P + Q = -16.4		

Fig. 6. Effect of treatment with quercetin (Q, 10^{-7} M) and/or pioglitazone (P, 10^{-7} M) on phenylephrine-induced contraction of endothelium-denuded aortic rings from normal and diabetic rats. The percentage of area under curve (AUC) was calculated from the highest peaks of the total area for all treatment groups and was normalized to the vehicle group. Data are expressed as mean ± S.E.M (n=6). *P < 0.05 compared to vehicle treated group. #P < 0.05 compared to the normal group. +P < 0.05 compared to P+Q group.

pared to the control. In the presence of L-NAME, the enhancement observed with the combination was abolished (Fig. 7B). Similarly, the total vascular nitric oxide product (nitrate/nitrite) was decreased in F-STZ rings compared to control. Pre-treatment with quercetin or pioglitazone alone did not increase the nitric oxide product but the combination significantly enhanced the level (Fig. 7C). This enhancement was abolished with L-NAME treatment (Fig. 7C).

3.4. Effect of quercetin and/or pioglitazone treatment on oxidative stress

To demonstrate the effect of the combination of the subliminal concentrations of pioglitazone and quercetin on oxidative stress, normal aortic rings were pre-treated with β-NADPH to mimic oxidative stress condition. Pre-treatment with β-NADPH in normal rat aortic rings significantly reduced the acetylcholine-induced relaxation compared to the control without β-NADPH treatment (Fig. 8). Treatment with quercetin or pioglitazone alone did not significantly increase the acetylcholine-induced relaxation in β-NADPH pre-treated rings. However, the combination significantly enhanced the relaxation to acetylcholine but the improvement was less than that observed in the apocynin-treated tissues.

To demonstrate the effectiveness of superoxide anion scavenging with pioglitazone and quercetin combination, vascular superoxide levels were measured using DHE staining and lucigenin assay under various experimental conditions. Diabetic aortic rings demonstrated an increased DHE intensity staining (Fig. 9A and B) and superoxide production (Fig. 9C) compared to the normal tissues. Quercetin but not pioglitazone mono treatment significantly reduced the DHE intensity (Fig. 9A and B) and superoxide production (Fig. 9C). The combination treatment depressed the DHE intensity and vascular superoxide production to a similar extent as quercetin. DPI, a superoxide scavenger reduced the superoxide anion signaling as well as superoxide anions level.

4. Discussion

Although pioglitazone has beneficial effects on endothelial function in addition to its effect in enhancing insulin sensitivity, suppressing lipolysis and decreasing free fatty acids, prolonged use of this drug may give rise to adverse effects, such as fluid retention, peripheral edema, liver injury and certain heart problem (Radenković, 2014). On the other side, quercetin has been demonstrated to have an anti-diabetic effect without decreasing the body fats in insulin resistance subject (Arias et al., 2014). Thus, an ability to achieve these same effects using significantly reduced doses of pioglitazone and quercetin in combination would potentially enhance the therapeutic benefits of these drugs. Using a subliminal concentration of pioglitazone in combination with quercetin, an antioxidant, the present study demonstrated that in the diabetic aorta: (i) quercetin and pioglitazone combination synergistically improved the impaired endothelium-dependent relaxation and decreased the phenylephrine-induced contraction; (ii) the action of the combination treatment is endothelium dependent; and is (iii) due to reduced superoxide anion production and increased nitric oxide bioavailability.

In the present study, blunted responses to acetylcholine, which demonstrated a significant impairment of endothelial function, was observed in the aorta of the diabetic compared to the normal tissues.

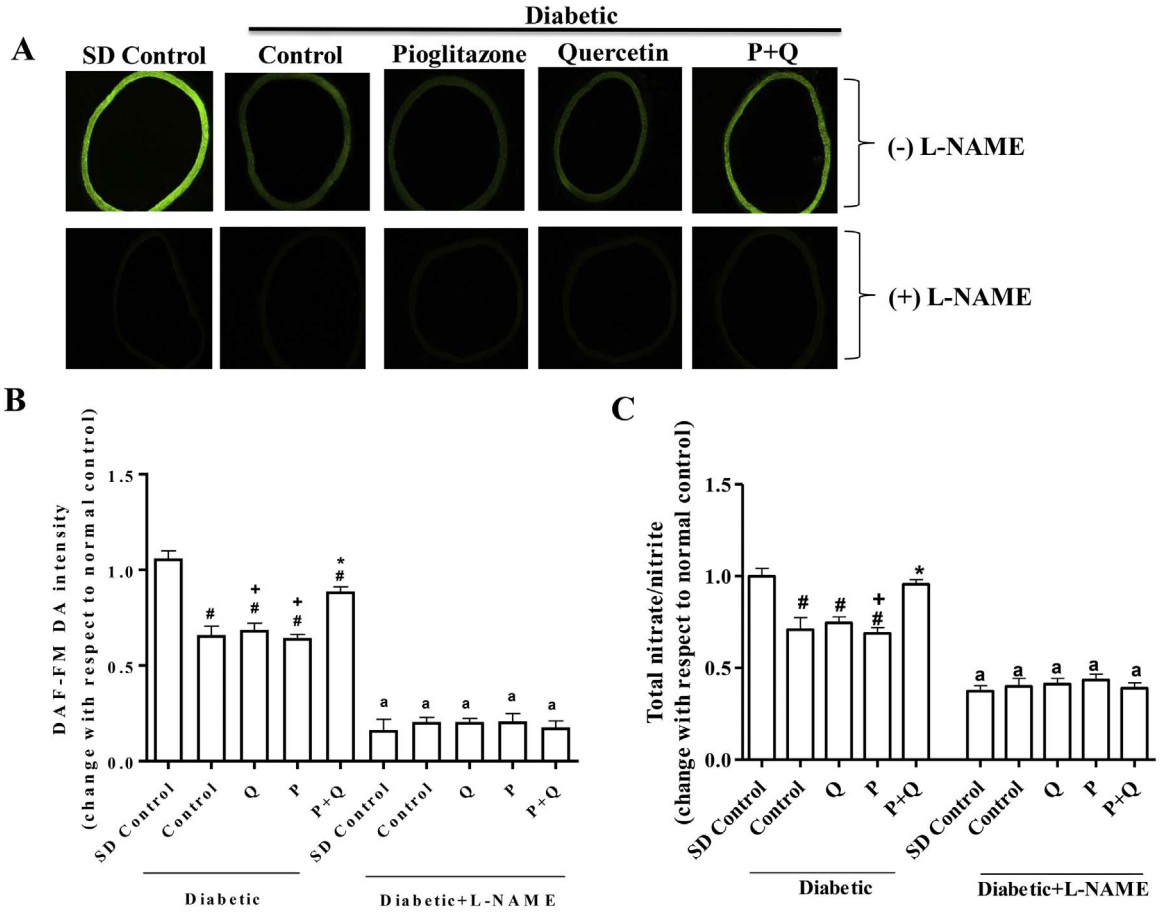


Fig. 7. Effect of treatment with quercetin (Q, 10^{-7} M) and/or pioglitazone (P, 10^{-7} M) on nitric oxide production in endothelium-intact aortic rings from normal and diabetic rats. (A) Representative fluorescence images of nitric oxide production as measured by DAF-FM DA. (B) The quantified mean fluorescence intensity of DAF-FM DA-stained aortic sections. (C) Total vascular nitrate/nitrite measurement. Data are expressed as mean ± S.E.M (n=5). $^{\#}P < 0.05$ compared to SD control group. $^{*}P < 0.05$ compared to diabetic control group. $^{+}P < 0.05$ compared to P+Q group. $^{a}P < 0.05$ compared to the respective L-NAME untreated control group.

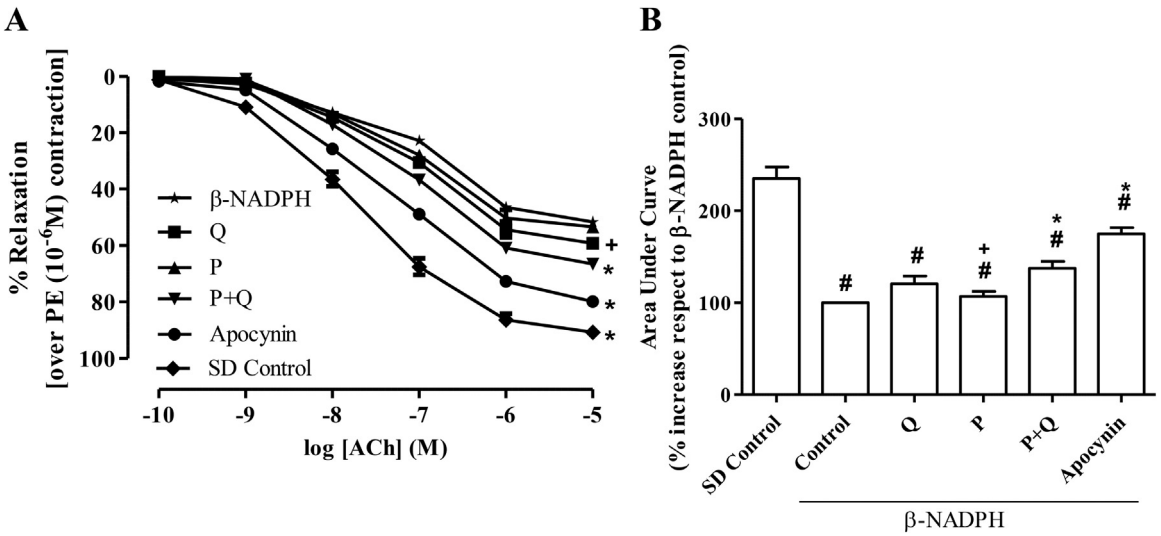


Fig. 8. Effect of treatment with quercetin (Q, 10^{-7} M) and/or pioglitazone (P, 10^{-7} M) on acetylcholine- induced relaxation of β-NADPH (superoxide inducer)-stimulated endothelium-intact aortic rings from normal rat (A). The % change of the area under the curve (B). Values are expressed as mean ± SEM (n=6). $^{*}P < 0.05$ compared to β-NADPH group. $^{+}P < 0.05$ compared to P+Q group. $^{\#}P < 0.05$ compared to SD control group.

This is consistent with previous studies in which quercetin and pioglitazone were shown to concentration-dependently improve the impaired endothelium-dependent relaxation in diabetic rat aorta (Ajay et al., 2006; Majithiya et al., 2005). Using subliminal concentrations of quercetin and pioglitazone, a synergistic effect of the combination in improving acetylcholine-induced relaxation and decreasing phenylephrine-induced contraction was observed in diabetic vessels. The combination of 10^{-7} M each of pioglitazone and quercetin caused a significantly greater effect than 10^{-6} M of either agent. The synergistic effect was lost upon inhibition of endothelial nitric oxide synthase and

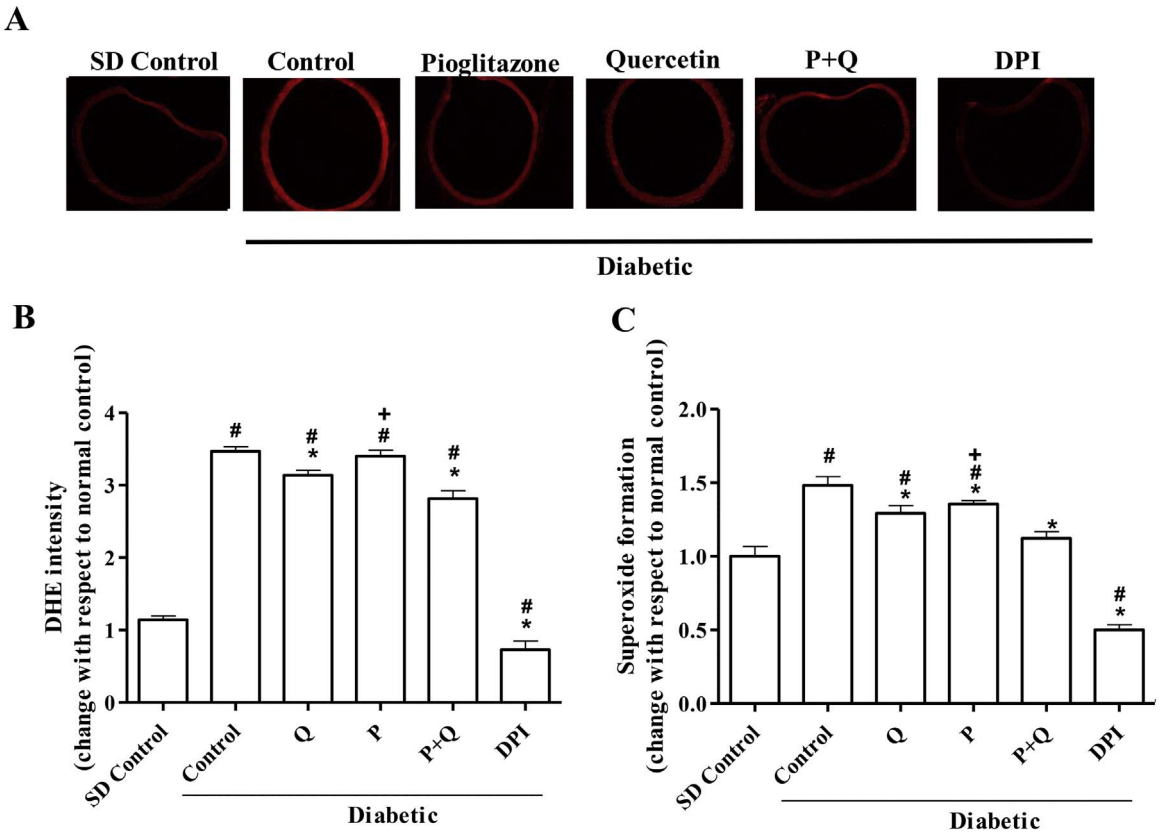


Fig. 9. Effect of treatment with quercetin (Q, 10^{-7} M) and/or pioglitazone (P, 10^{-7} M) on superoxide anion production in endothelium-intact aortic rings from diabetic rats. (A) Representative fluorescence images of reactive oxygen species production as measured by DHE. (B) The quantified mean fluorescence 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). $^{\#}P < 0.05$ compared to SD control group. $^{*}P < 0.05$ compared to diabetic control group. $^{+}P < 0.05$ compared to P+Q group.

removal of endothelium, suggesting the synergy was dependent on endothelium derived nitric oxide. This is further supported by the increase in nitric oxide level observed with DAF-FM fluorescence and the total nitrate/nitrite assay. As the experiments were carried out in the presence of the cyclooxygenase inhibitor, indomethacin, the improvement in endothelial function of the diabetic aorta caused by the combination treatment cannot be attributed to vasodilator prostaglandins.

Impaired endothelium-independent relaxation has been demonstrated in the aorta of diabetic rats (Yakubu et al., 2012) and in diabetic patients (Okon et al., 2005). These findings are consistent with result of this study in which the sensitivity and maximal relaxation to the endothelium-independent nitric oxide donor, sodium nitroprusside, were decreased in the diabetic aorta. Put together, these findings point to a disruption in diabetic tissues of the relaxation-contraction mechanism that is endothelium-independent; possibly a disruption of the effector cells. Quercetin and pioglitazone combination treatment had no effect on the relaxation induced by this nitric oxide donor, indicating that the improvement in acetylcholine-induced relaxation caused by this combination in diabetic aortic rings was most likely due to the release of endothelium-derived nitric oxide rather than the changes in the sensitivity of the effector cells to nitric oxide.

Many *in vitro* and *in vivo* studies have shown quercetin to ameliorate endothelial dysfunction by improving nitric oxide bioavailability via its antioxidant action, releasing endothelium-derived relaxing factors, and inhibiting NADPH oxidase enzyme which is involved in production of superoxide anions (Choi et al., 2016; Romero et al., 2009; Sanchez et al., 2006). Similar to quercetin, pioglitazone has been shown to reverse endothelial dysfunction by reducing oxidative stress and by increasing nitric oxide bioavailability (Huang et al., 2008; Kotchen et al., 1997; Matsumoto et al., 2007). Taken in conjunction, the present data demonstrate that subliminal concentrations of quercetin and pioglitazone in combination, protects endothelial function through the reversal of β-NADPH-induced oxidative stress. In diabetic aorta, the combination decreased the elevated reactive oxygen species and superoxide anions production to the level observed following treatment with DPI, an NADPH oxidase inhibitor. The synergistic effect of the quercetin and pioglitazone combination, therefore, may be partly due to their ability to reduce NADPH oxidase-induced superoxide anion production in diabetic rat aorta. This action mainly contributed by quercetin (Fig. 9) leads to increase nitric oxide bioavailability.

5. Conclusion

In summary, a combination of subliminal concentrations of quercetin and pioglitazone synergistically increased endothelium-dependent vasorelaxation, and reduced phenylephrine- induced contraction compared to treatments with the subliminal or higher concentrations of the individual agent, in isolated aorta from type 2 diabetic rats. This observed vasorelaxation is endothelium-dependent and involves a synergistic promotion of nitric oxide-releasing and superoxide anion scavenging actions. Thus, the present study suggests that a combination of pioglitazone and quercetin at subliminal concentrations is able to produce positive therapeutic interaction in decreasing oxidative stress and reversing impaired vascular reactivity in patients with type 2 diabetes mellitus.

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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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejphar.2017.02.022.

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