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Lack of beneficial metabolic effects of quercetin in adult spontaneously hypertensive rats

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ABSTRACT

Insulin sensitivity is partly dependent on insulin-mediated nitric oxide (NO) release and antioxidants may decrease insulin resistance by amelioring NO bioavailability. The effects of chronic therapy with the antioxidant quercetin on blood pressure, vascular function and glucose tolerance in male spontaneously hypertensive rats (SHR), a model of genetically hypertension and insulin resistance, were analyzed. Rats were divided into four groups, WKY vehicle, WKY quercetin, SHR vehicle and SHR quercetin. Animals were daily administered by gavage for four weeks: vehicle, quercetin in vehicle (10 mg/kg body weight). Blood pressure was followed by tail-cuff plethysmography. Chronic quercetin treatment reduced systolic blood pressure, and significantly reduced left ventricular (−10%) and renal (−6%) hypertrophy. However, oral glucose tolerance test, homeostatic model assessment of insulin resistance, total cholesterol and triglycerides were unaffected by quercetin in both strains of rats. It also improved the blunted aortic endothelium-dependent relaxation to acetylcholine, without affecting both endothelium-dependent relaxation to insulin and endothelium-independent relaxation to sodium nitroprusside in SHR. In WKY rats, quercetin in vitro and in vivo, impaired the relaxation to insulin. Quercetin reduced both plasma malondialdehyde levels and aortic superoxide production in SHR. Furthermore, quercetin inhibited insulin-stimulated protein kinase B (Akt)- and endothelial NO synthase (eNOS) phosphorylation. In conclusion, quercetin reduced blood pressure, left ventricular and renal hypertrophy and improved NO-dependent acetylcholine relaxation. However, and despite its antioxidant effects, quercetin was unable to improve insulin sensitivity possibly through its specific interference with the insulin signalling pathway.

1. Introduction

The vascular endothelium contributes importantly to the regulation of cardiovascular and metabolic homeostasis (Kim et al., 2006). Reciprocal relationships between endothelial dysfunction, insulin resistance and hypertension may help coupling hemodynamic and metabolic abnormalities observed in important interrelated public health problems, including diabetes, obesity, hypertension, coronary heart disease, atherosclerosis, and the metabolic syndrome (Kim et al., 2006; Sowers, 2004). The link between hypertension and insulin resistance is also present in some animal models of hypertension. The spontaneously hypertensive rat (SHR) is not only genetically hypertensive but also shows endothelial dysfunction and increased fasting levels of insulin consistent with metabolic insulin resistance (Reaven and Chang, 1991). Thus the SHR may be a useful model to explore the relationship between metabolic and hemodynamic dysregulations. Endothelial dysfunction in the insulin-resistant state is characterized by decreased endothelial production of nitric oxide (NO) as well as excess production of superoxide anion (O_2^-), resulting in the degradation of NO before it can reach the vascular smooth muscle cells (Shinozaki et al., 1999). Moreover, oxidative stress generation impaired pancreatic β-cell insulin secretion (Bast et al., 2002) and interfered with insulin signalling pathway, thereby accelerating the progression to overt type 2 diabetes from insulin resistance (Evans et al., 2003). In fact, studies in animal models of diabetes and several clinical trials with insulin-resistant individuals indicate that antioxidants improve insulin sensitivity (Ceriello and Motz, 2004).

Flavonoids have high antioxidant activities as free radical scavengers and as inhibitors of enzymes generating reactive oxygen species (Hollman and Katan, 1999). Flavonoids may preserve β-cell

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function by reducing oxidative stress-induced tissue damage and therefore protect against the progression of insulin resistance to type 2 diabetes. In fact, quercetin, the main dietary flavonol, prevents and protects streptozotocin-induced oxidative stress and β-cell damage in rat pancreas (Coskun et al., 2005) and decreased blood glucose concentration in both alloxan- and streptozotocin-induced diabetic rats (Coskun et al., 2005; Nuraliev and Avezov, 1992). Moreover, the intake of some specific types of flavonoids, including quercetin, was inversely associated with a higher risk of incident type 2 diabetes (Knekt et al., 2002). However, in women free of cardiovascular disease, high intake of flavonols and flavones was not significantly associated with risk of type 2 diabetes and insulin resistance (Song et al., 2005). In patients with essential hypertension the consumption of a flavanol-rich dark chocolate decreased blood pressure, improved endothelial dysfunction and ameliorated insulin sensitivity (Grassi et al., 2005). However, there is no data available regarding the metabolic effects of the main dietary flavonol quercetin. Recently, we have shown that quercetin improved endothelial function in several models of experimental hypertension, including SHR (Duarte et al., 2001; Sánchez et al., 2006). In SHR chronic treatment with quercetin resulted in an enhanced endothelial NO synthase (eNOS) activity and a decreased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase mediated-O_2^- generation associated with a reduced $p47^{phox}$ expression (Sánchez et al., 2006). We hypothesized that quercetin, acting as an inhibitor of this O_2^--generating system, would improve glucose homeostasis in a genetic model of hypertension and insulin resistance.

The main aim of this study was to investigate the effects of the oral administration of quercetin in metabolism, focusing in plasmatic and vascular parameters of insulin resistance in SHR.

2. Materials and methods

2.1. Animals and experimental protocol

All the experiments were performed in accordance with Institutional Guidelines for the ethical care of animals. Twenty four-week old, male SHR and their normotensive counterparts Wistar Kyoto (WKY) rats were obtained from Harlan Laboratories (Barcelona, Spain). All rats were maintained at five per cage at a constant temperature (24 ± 1 ºC), with a 12-h dark/light cycle and on standard rat chow. An adaptation period of 2 weeks for vehicle administration and blood pressure measurements was allowed before the initiation of the experimental protocols. Sixteen WKY rats and eighteen SHR were randomly assigned to a control group (vehicle, 1 ml of 1% methylcellulosa) or a quercetin group (10 mg/kg, mixed in 1 ml of 1% methylcellulosa). Rats were treated orally by gavage for 4 weeks. During the experimental periods rats had free access to tap water and chow. Body weight was measured every week. Since it is known that after oral quercetin consumption the elimination of quercetin metabolites is quite slow, with reported half-lives ranging from 11 to 28 h (Graefe et al., 2001), the quercetin treatment was stopped 2 days before the end of the experiment in order to study the long-term effects of quercetin without the involvement of the effects of acute administration.

2.2. Blood pressure measurements

Systolic blood pressure was measured weekly 18–20 h after administration of the drugs in conscious, prewarmed, restrained rats by tail-cuff plethysmography (Duarte et al., 2001). At least seven determinations were made in every session and the mean of the lowest three values within 5 mmHg was taken as the systolic blood pressure level. At the end of the 4th week, direct blood pressure was measured in conscious SHR. For this purpose, the rats were anaesthetised with 2.5 ml/kg equitensin (500 ml contain 43% w/v chloral hydrate in 81 ml ethanol; 4.86 mg nembutal; 198 ml propylene glycol; 10.63 g $MgSO_4$; distilled water) (i.p.) and the carotid artery was cannulated to obtain direct measurements of arterial blood pressure. The catheter was exteriorised through the skin on the dorsal side of the neck and protected with a silver spring. Rats were fasted and allowed to recover for 18 h and, after connecting the catheter to a transducer and a two-channel recorder (TRA-021 and Letigraph 2000, respectively; Letica SA, Barcelona, Spain), blood pressure and heart rate were continuously recorded for 60 min. Mean arterial pressure was calculated as diastolic pressure plus one-third of pulse pressure.

2.3. Oral glucose tolerance test

After direct blood pressure recording, all rats were administered a 50% glucose solution in water by gavage at a dose of 1.75 g/kg body weight. Blood samples (0.4 ml) were collected from the carotid artery into heparinized capillary tubes at 0, 30, 60 and 120 min. The blood samples were chilled on ice and centrifuged for 20 min at 5000 *g* at 4 ºC, and the plasma frozen at −70 °C.

Plasma glucose, triglycerides, and total cholesterol concentrations were measured by colorimetric methods using Spinreact kits (Spinreact, S.A., Spain). Plasma insulin concentration was quantified using a rat insulin enzyme immunoassay system (Amersham Biosciences, Buckinghamshire, UK). Homeostatic model assessment of insulin resistance was calculated using the formula: fasting glucose (mM) × fasting insulin (μU/ml)/22.5. Plasma malonyldialdehyde (MDA) content was evaluated as previously described (Duarte et al., 2001). Briefly, 100 μl of plasma reacted with a chromogenic reagent, 1-methyl-2-phenylindole (10.3 mM) in acetonitrile and 37% aqueous HCl (10.4 M). After incubation of the reaction mixture for 40 min in a 45 °C water bath, the absorbance was measured at 586 nm in a GBC 920 spectrophotometer.

2.4. Cardiac, and renal indices

Immediately after exsanguination, the heart and kidneys were excised, cleaned and weighed. The atria and the right ventricle were then removed and the remaining tissue (left ventricle plus septum) weighed. The heart weight index, the left ventricular weight indices and the kidney weight index were calculated by dividing the heart weight, the left ventricular weight and the kidney weight by the body weight. The ratio left ventricular weight/heart weight was calculated as index of absolute cardiac hypertrophy.

2.5. Vascular function ex vivo

Descending thoracic aortic rings (3 mm) were dissected and mounted in organ chambers filled with Krebs solution (composition in mM: NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2, KH_2PO_4 1.2 and glucose 11) at 37 °C and gassed with 95% O_2 and 5% CO_2. Rings were stretched to 2 g of resting tension by means of two L-shaped stainless-steel wires inserted into the lumen and attached to the chamber and to an isometric force-displacement transducer (Letigraph 2000), respectively, as previously described (Duarte et al., 2001). The concentration–relaxation response curves to acetylcholine (10^{-9} M−10^{-4} M) and insulin (10^{-10} M–10^{-6} M) were performed in endothelium-intact rings pre-contracted by 10^{-7} M phenylephrine. The concentration–relaxation response curves to nitroprusside (10^{-9} M–10^{-6} M) were performed in the dark in endothelium-denuded rings pre-contracted by 10^{-6} M phenylephrine.

In parallel experiment, both acetylcholine- and insulin-mediated responses were tested in rings from WKY rats. For this purpose, concentration response curves to both drugs were performed in both endothelium-denuded rings and endothelium-intact rings pre-contracted by 10^{-7} M phenylephrine, with or without previous incubation for 30 min with the eNOS inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME,10^{-4} M), or the phosphatidylinositol-3 (PI3) kinase inhibitor, wortmannin (3×10^{-8} M), or the cyclooxygenase inhibitor, indomethacin (10^{-5} M),or quercetin (10^{-6} M and 10^{-5} M). Because

high concentrations of wortmannin may inhibit other lipid kinases, such as PI4-kinase, we performed the experiments using this low concentration.

2.6. In situ detection of vascular superoxide anion (O_2^-) production

Unfixed thoracic aortic rings were cryopreserved (phosphate buffer solution 0.1 mol/l, PBS, plus 30% sucrose for 1–2 h), included in optimum cutting temperature compound media, frozen (−80 ºC), and 10 µm cross sections were obtained in a cryostat (Microm International Model HM500 OM). Sections were incubated for 30 min in HEPES buffered solution containing dihydroethidium (DHE, 10^{-5} M), counterstained with the nuclear stain 4,6-diamidino-2-phenylindole 2 HCl (DAPI) and in the following 24 h examined on a fluorescence microscope (Leica DM IRB, Wetzlar, Germany). Sections were photographed and ethidium and DAPI fluorescence were quantified using ImageJ (version 1.32j, NIH, http://rsb.info.nih/ij/). O_2^- production was estimated from the ratio of ethidium/DAPI fluorescence. In preliminary experiments, DHE fluorescence was almost abolished by the O_2^- scavenger tiron, indicating the specificity of this reaction.

2.7. Western blotting analysis

Some aortic rings from both groups of rats were incubated with insulin (10^{-7} M) for 5 min and then frozen in liquid nitrogen and stored at −80 °C. We examined the state of protein kinase B (Akt) and eNOS phosphorylation in aortae homogenates. Aortae were homogenized in lysis buffer (100 mM Tris–HCl, pH 7.4, 50 mM HEPES, 10 mM EDTA, 100 mM $Na_4P_2O_7$, 100 mM NaF, 10 mM Na_3VO_4, 1% SDS). Western blottings were performed with 40 µg of protein per lane. Sodium dodecyl sulfate-polyacrilamide (8%) electrophoresis was performed in a mini-gel system (Bio-Rad Laboratories). Phosphorylated protein kinase B (Akt) (Ser-473), Phosphorylated eNOS (Ser-1177), Akt and eNOS, were detected after the membranes were incubated with the respective primary antibodies (Rabbit anti-p-eNOS-ser-1177, mouse anti-eNOS, Rabbit anti-p-Akt-ser-473 and Rabbit anti-Akt, Cell Signalling Technology, MA, USA, 1:1000 dilution) overnight at 4 °C. The membranes were then washed three times for 10 min in Tris-buffered saline (containing 0.1% Tween 20) and incubated with secondary peroxidase conjugated goat anti-rabbit or goat anti-mouse antibodies (1:2500, Santa Cruz Biotechnology, Santa Cruz, USA), respectively. All incubations were performed at room temperature for 2 h. After washing the membranes, antibody binding was detected by an electrochemiluminescent system (Amersham Pharmacia Biotech, Amersham, UK). Films were scanned and densitometric analysis was performed on the scanned images using Scion Image-Release Beta 4.02 software (http://www.scioncorp.com). Phospho-Akt/Akt and phospho-eNOS/eNOS abundance ratio was calculated and data is expressed as a percentage of the values in control aorta from the same gel.

In another set of experiments, western blot analysis was performed in rat aortic rings from WKY rats incubated for 30 min with quercetin (10^{-6} M and 10^{-5} M), or wortmannin (3×10^{-8} M), before addition of insulin (10^{-7} M) for another 5 min.

2.8. Quantification of nitric oxide release by diaminofluorescein-2 in human umbilical vein endothelial cells (HUVECs)

HUVECs were extracted from umbilical cords (modified from Vargas et al., 1994). Briefly, HUVECs were isolated by filling the lumen of fresh umbilical veins with 0.1% collagenase in physiological buffer saline, inverting the umbilical cord and washing the vein with culture medium (Medium 199+20% fetal bovine serum+Penicillin/Streptomycin 2 mM+Amphotericin B 2 mM+Glutamine 2 mM+HEPES 10 mM+endothelial cell growth supplement (30 µg/ml)+Heparin (100 mg/ml)). The collected cells were seeded in culture flasks pre-treated with gelatin 0.2%, containing culture medium.

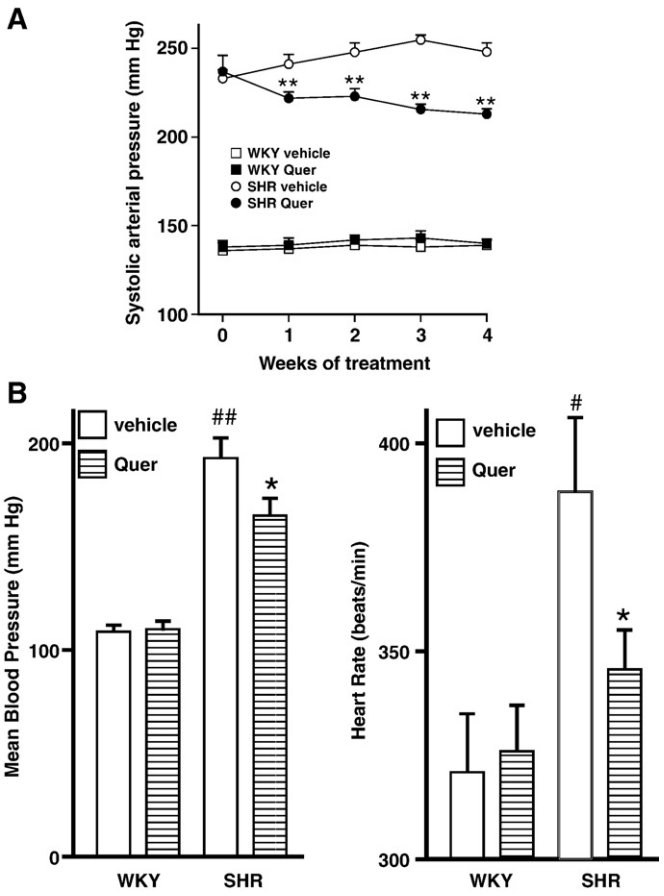

Fig. 1. Effects of quercetin (quer) on arterial blood pressure and heart rate. Panel A represents the time course of the systolic arterial pressure as measured by tail-cuff plethysmography in the WKY vehicle (□, $n=8$), WKY quer (■, $n=8$), SHR vehicle (○, $n=9$) and SHR quer (●, $n=9$) groups. Panel B shows the direct measurements of mean arterial pressure and heart rate in the vehicle (open bars) and quercetin (dashed bars) groups. Values are expressed as means ± S.E.M. *$P<0.05$, **$P<0.01$ *vs* the SHR vehicle group. #$P<0.05$, ##$P<0.01$ *vs* the WKY vehicle group.

Quantification of NO released by HUVEC was performed using the NO-sensitive fluorescent probe diaminofluorescein-2 (DAF-2) as described previously (Leikert et al., 2001) Briefly, cells were grown to confluence in 96-well plates and heparin and endothelial cell growth supplement were omitted 24 h before stimulation. Cells were washed with phosphate buffered saline and then were pre-incubated with L-arginine (100 µM in PBS, 5 min, 37 °C). In some experiments, L-NAME

Table 1
Body weight and cardiac and renal indices in WKY rats and SHR received vehicle or 10 mg/kg/day of quercetin (quer) for 4 weeks.

Group	WKY vehicle ($n=8$)	WKY quer ($n=8$)	SHR vehicle ($n=9$)	SHR quer ($n=9$)
BW initial, g	332.5 ± 4.3	335.8 ± 6	344.1 ± 7.3	345.3 ± 14.5
BW final, g	371.0 ± 5.6	366.9 ± 4.7	370.9 ± 10.7	366.6 ± 9.7
HW, mg	855 ± 10	831 ± 20	1251 ± 42[a]	1195 ± 56[a]
LVW, mg	618 ± 9	599 ± 15	982 ± 24[a]	880 ± 25[a,c]
KW, mg	866 ± 20	872 ± 27	856 ± 32	802 ± 30
LVW/ HW ratio	0.72 ± 0.01	0.72 ± 0.01	0.79 ± 0.01[a]	0.74 ± 0.02[b]
HW/BW ratio	2.31 ± 0.03	2.27 ± 0.06	3.38 ± 0.09[a]	3.26 ± 0.12[a]
LVW/BW ratio	1.67 ± 0.02	1.63 ± 0.05	2.65 ± 0.06[a]	2.40 ± 0.06[a,c]
KW/BW ratio	2.34 ± 0.06	2.38 ± 0.08	2.31 ± 0.05	2.19 ± 0.04[b]

BW indicates body weight; HW, heart weight; LVW, left ventricular weight; KW, kidney weight.
[a] $P<0.01$ *vs.* WKY vehicle.
[b] $P<0.05$ SHR quercetin vs. SHR vehicle.
[c] $P<0.01$ SHR quercetin *vs.* SHR vehicle.

Table 2
Plasma analytical determinations in WKY rats and SHR received vehicle or 10 mg/kg/day of quercetin (quer) for 4 weeks.

Group	WKY vehicle ($n=8$)	WKY quer ($n=8$)	SHR vehicle ($n=9$)	SHR quer ($n=9$)
Cholesterol (mg/dl)	53.5 ± 3.3	50.3 ± 1.8	81.4 ± 4.5[a]	81.1 ± 2.6[a]
Triglycerides (mg/dl)	39.4 ± 3.7	43.5 ± 4.7	54.0 ± 4.3[a]	55.0 ± 4.1[a]
Glucose (mmol/l)	5.7 ± 0.6	6.1 ± 0.4	7.3 ± 0.4[a]	9.0 ± 0.5[a,b]
Insulin (pmol/l)	112.0 ± 20.5	106.5 ± 14.9	448.8 ± 149.7[a]	452.2 ± 146.3[a]

[a] $P<0.01$ *vs.* WKY vehicle.
[b] $P<0.01$ SHR quercetin *vs.* SHR vehicle.

(10^{-4} M), wortmannin (3×10^{-8} M), or quercetin (10^{-6} M and 10^{-5} M) was added 30 min before the addition of L-arginine. Subsequently, DAF-2 (10^{-7} M) and insulin (10^{-7} M) were added and cells were incubated in the dark at 37 °C. Then the fluorescence was measured at 5, 15 and 30 min, respectively, using a spectrofluorimeter (Fluorostart, BMG Labtechnologies, Offenburg, Germany) with excitation wavelength set at 495 nm and emission wavelength at 515 nm. The auto-fluorescence obtained from PBS/DAF-2 was subtracted from each value.

2.9. Statistical analysis

Results are expressed as means ± standard error means (S.E.M.) of measurements. Repeated measures analysis of variance was used for multiple time point data. Group means were compared using t test for simple comparisons or the Newman–Keuls test for post hoc analyses. All analyses were carried out using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com), with statistical significance set at $P<0.05$.

3. Results

3.1. General parameters

Long-term quercetin administration induced a progressive reduction in systolic blood pressure in SHR and this effect reached statistical significance after the first week of treatment and was observed even after 48 h after the last administration of quercetin as previously reported (Duarte et al., 2001), while no changes were observed in WKY rats (Fig. 1A). At the end of the 4 weeks of treatment, direct measurements of blood pressure in conscious rats showed that quercetin induced a significant reduction (−14%) in mean arterial blood pressure and heart rate (Fig. 1B).

Treatment with quercetin did not modify the body weight in either SHR or WKY rats. The heart weight index, left ventricular weight index and left ventricular weight/heart weight ratio in SHR were significantly greater than that found in WKY rats. Absolute heart weight and left ventricular weight and relative heart and left ventricular index and left ventricular weight/heart weight ratio were significantly reduced in quercetin-treated SHR as compared to vehicle-treated SHR (Table 1).

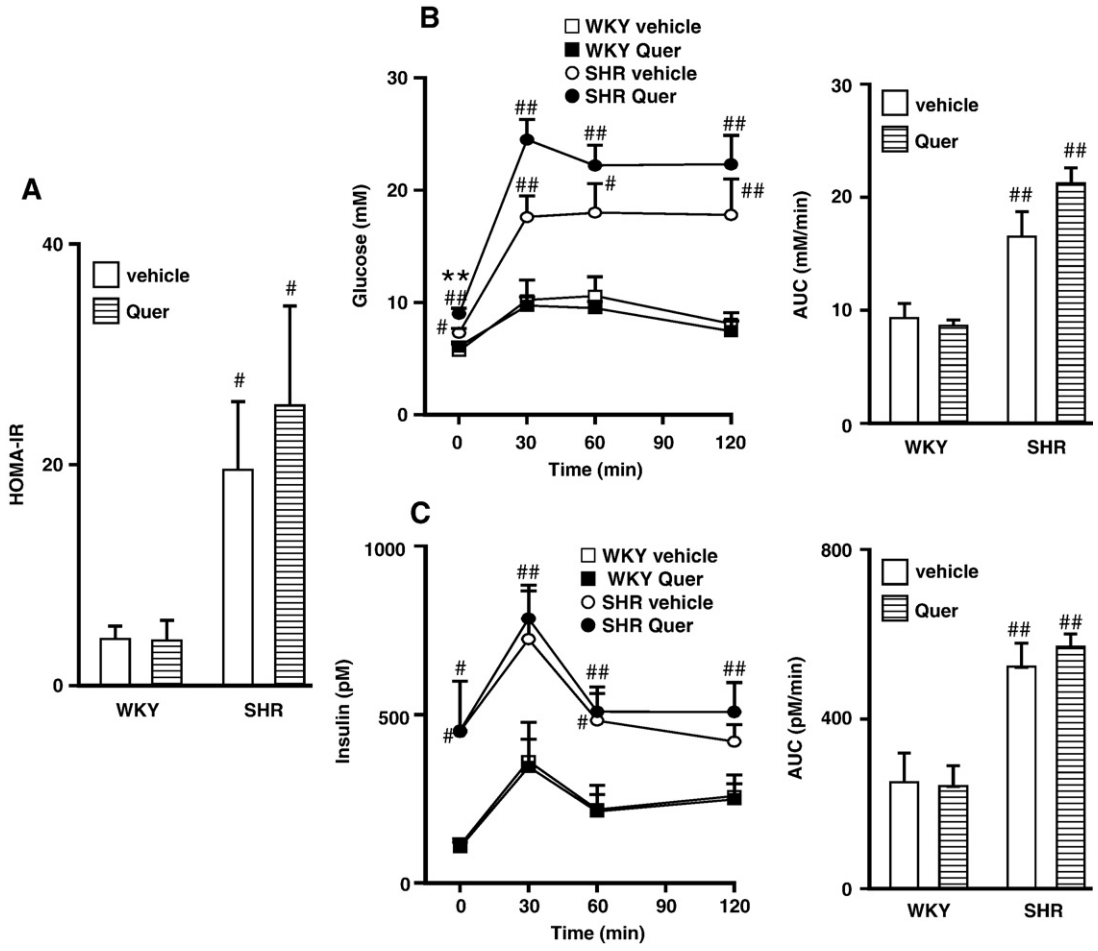

Fig. 2. Homeostasis model assessment of insulin resistance (HOMA-IR) in fasted rats (A) and glucose (B) and insulin (C) response to an oral glucose load (OGTT) in the WKY vehicle ($n=8$), WKY quercetin ($n=8$), SHR vehicle ($n=9$) and SHR quercetin ($n=9$) groups. HOMA-IR: fasting glucose (mM) × fasting insulin (μU/ml)/22.5. In OGTT, rats were fasted for 18 h prior administration of a 50% glucose solution in water by gavage at a dose of 1.75 g/kg body weight. AUC: area under curve. Values are expressed as means ± S.E.M. $^{\#}P<0.05$ and $^{\#\#}P<0.01$ *vs* WKY vehicle; $^{**}P<0.01$ *vs* the SHR vehicle group.

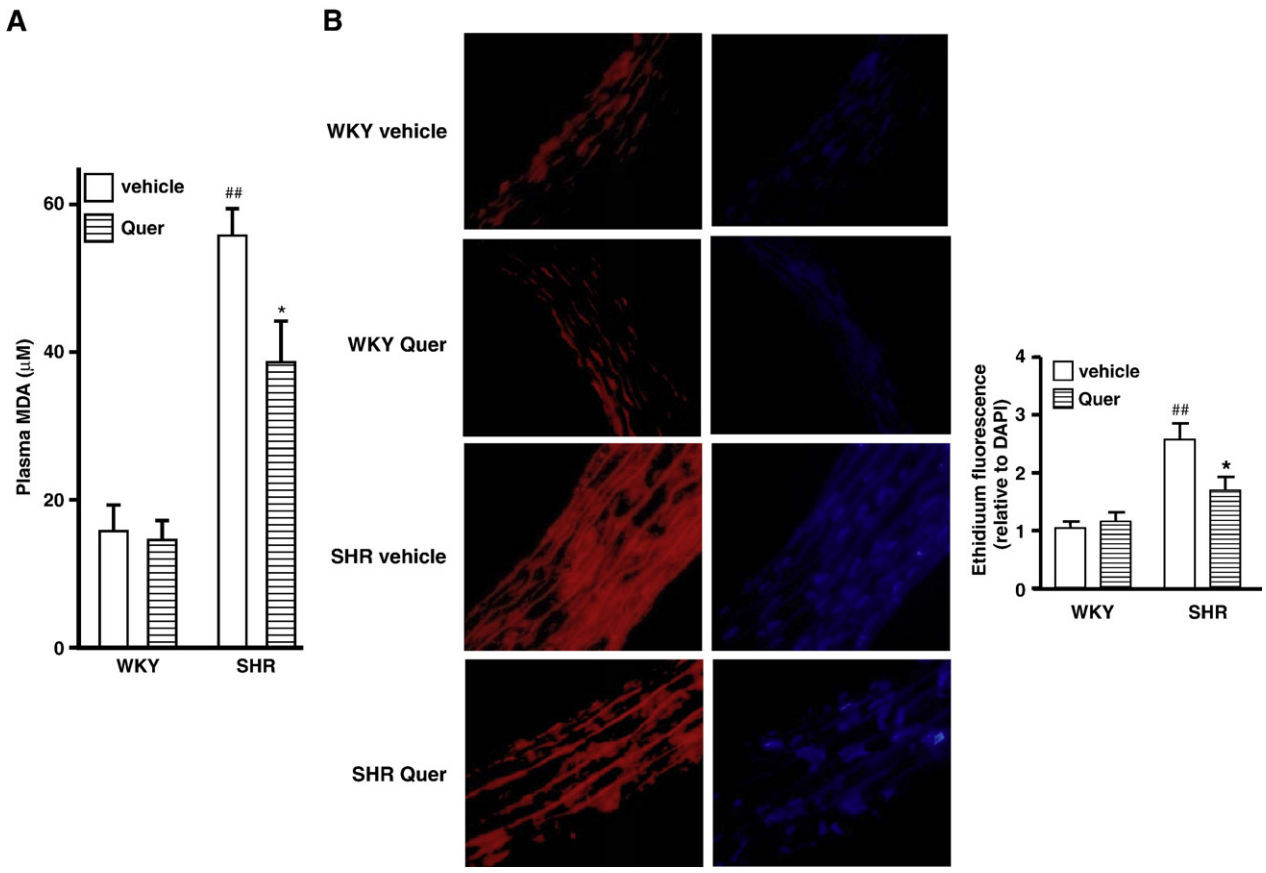

Fig. 3. Effects in systemic and vascular oxidative status. (A) Total plasma malondialdehyde (MDA) content in the WKY vehicle (open bars, $n=8$), WKY quercetin (dashed bars, $n=8$), SHR vehicle (open bars, $n=9$) and SHR quercetin (dashed bars, $n=9$) groups. (B) Effects of quercetin on the O_2^- production in aortic rings from SHR and WKY rats. In left upper pictures show arteries incubated in the presence of DHE which produces a red fluorescence when oxidized to ethidium by O_2^-. In left lower pictures show blue fluorescence of the nuclear stain DAPI (×400 magnification). In right panel averaged values, mean ± S.E.M ($n=4$ rings from different rats) of the red ethidium fluorescence normalized to the blue DAPI fluorescence. Values are expressed as means ± S.E.M. $^{*}P<0.05$ *vs* the SHR vehicle group. $^{\#\#}P<0.01$ *vs* WKY vehicle group.

3.2. Metabolic parameters in the fasted state

Total cholesterol, triglycerides, glucose and insulin levels (Table 2) and insulin resistance (Fig. 2A), expressed as homeostatic model assessment of insulin resistance, were increased in the SHR vehicle group as compared to WKY vehicle rats. In WKY rats, chronic quercetin treatment did not alter these parameters. However, fasting plasma glucose in SHR was significantly increased by quercetin (Table 2) and a weak non-significant increase was also noticed in the homeostatic model assessment of insulin resistance (Fig. 2A) indicating that there is a subtle negative effect of quercetin on glucose metabolism.

3.3. Response to a glucose load

After oral glucose, both the area under the glucose response curve (Fig. 2B) and the area under the insulin curve (Fig. 2C) were increased in control SHR as compared to control WKY rats. No statistical differences were observed between control and quercetin-treated in both SHR and WKY rats.

3.4. Systemic and vascular oxidative status

Plasma MDA levels were higher in SHR as compared to its normotensive counterparts WKY rats. Chronic quercetin treatment only significantly reduced MDA levels in plasma from SHR (Fig. 3A). To characterize and localize O_2^- production within the vascular wall, ethidium red fluorescence was analyzed in sections of aorta incubated with DHE. DHE is oxidized by O_2^- to yield ethidium which stains DNA. Positive red nuclei could be observed in adventitial, medial and endothelial cells (Fig. 3B). Nuclear red ethidium fluorescence, indicative of O_2^- production, was quantified and normalized to the blue fluorescence of the nuclear stain DAPI, allowing comparisons between different sections. Rings from SHR showed marked increased staining in adventitial, medial and endothelial cells as compared to WKY rats, which was significantly reduced by chronic quercetin (Fig. 3B).

3.5. Ex vivo vascular responses

In aortae from SHR and WKY rats treated with quercetin, no differences were observed in the endothelium-independent vasodilator responses to the NO donor sodium nitroprusside in vessels precontracted with phenylephrine (Fig. 4B), confirming our previous data (Duarte et al., 2001). Aortic rings from vehicle-SHR showed strongly reduced endothelium-dependent vasodilator responses to acetylcholine (Fig. 4A) and insulin (Fig. 4C) in arteries stimulated by phenylephrine as compared to the control aortic rings from WKY rats. In SHR, quercetin produced a significant increase in the endothelium-dependent relaxation induced by acetylcholine (Fig. 4A), being without effect in the vasodilator responses to insulin (Fig. 4C), However, in aortae from WKY rats treated with quercetin, the relaxant responses to insulin were impaired, whereas no differences were observed in the relaxations induced by acetylcholine, as compared to vehicle-WKY group.

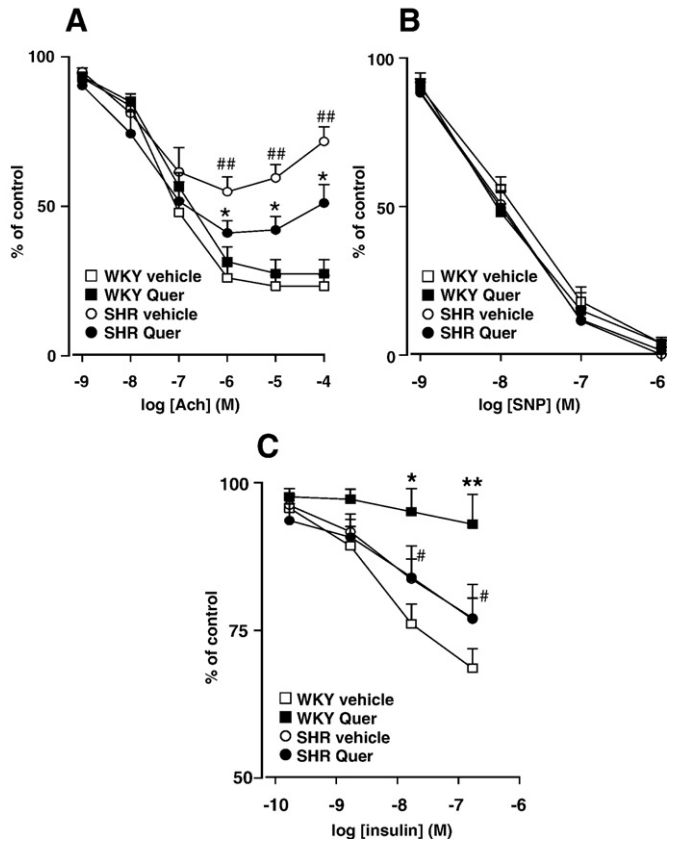

Fig. 4. Endothelium-dependent relaxation induced by both acetylcholine (ACh) (A) and insulin (C), and endothelium-independent relaxation induced by sodium nitroprusside (SNP) (B) in aortae from the WKY vehicle (□, $n=8$), WKY quercetin (■, $n=8$), SHR vehicle (○, $n=9$) and SHR quercetin (●, $n=9$) groups. Relaxations were induced in arteries contracted by phenylephrine. Values are expressed as means ± S.E.M. $^{*}P<0.05$ *vs* the SHR vehicle group. $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs* the WKY vehicle group.

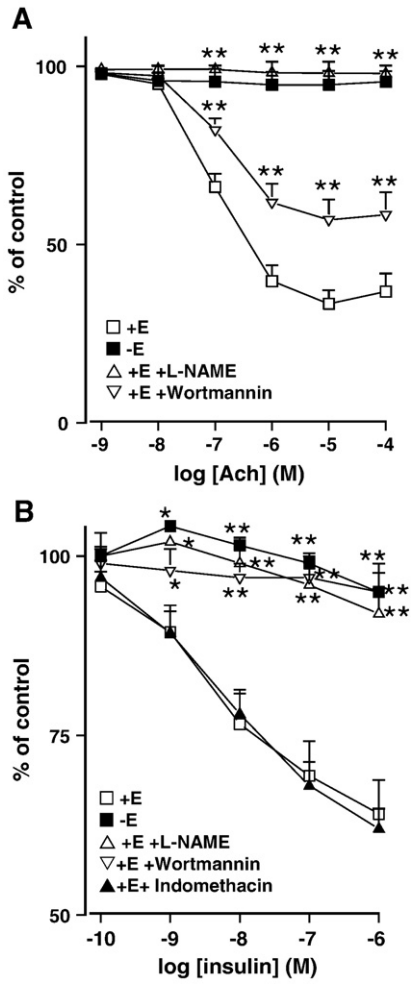

Fig. 5. Relaxations induced by both acetylcholine (Ach) (A) and insulin (B) in both endothelium-denuded (■, $n=7$) rings and endothelium-intact (□, $n=8$) rings from WKY rats pre-contracted by 10^{-7} M phenylephrine, with or without previous incubation for 30 min with the eNOS inhibitor, N^{ω}-Nitro-L-arginine methyl ester (L-NAME, 10^{-4} M) (Δ, $n=6$), the phosphatidylinositol-3 (PI3) kinase inhibitor, wortmannin (3×10^{-8} M) (▽, $n=6$), or cyclooxygenase inhibitor, indomethacin (10^{-5} M) (▲, $n=8$). Values are expressed as means ± S.E.M. $^{*}P<0.05$ and $^{**}P<0.01$ *vs* control rings with endothelium.

3.6. In vitro vascular responses

The relaxant response to both acetylcholine (Fig. 5A) and insulin (Fig. 5B) in aortic rings from WKY rats was suppressed by both endothelium denudation and incubation with the eNOS inhibitor L-NAME. The PI3-kinase inhibitor wortmannin inhibited the response induced by acetylcholine but abolished the insulin relaxation. The cyclooxygenase inhibitor, indomethacin, had no effect on the relaxant response induced by insulin.

3.7. Effects on protein phosphorylation

The ratio of phospho-eNOS/eNOS protein was significantly higher in rings from WKY rats as compared with SHR (Fig. 6A) and this difference remained highly significant after the increase induced by insulin. Insulin increased the phosphorylation of Akt (p-Ser -473) and eNOS (p-Ser-1177) in aortic rings from both WKY vehicle and SHR vehicle rats (Fig. 6B and C). However, the insulin-induced increase was almost suppressed in rings from WKY quercetin and SHR quercetin groups (Fig. 6B and C).

3.8. In vitro effects of quercetin on vascular Akt/eNOS insulin-stimulated pathway

Acute incubation of aortic rings from WKY rats with quercetin inhibited, in a concentration-dependent manner, insulin-induced phosphorylations of Akt (p-Ser -473) (Fig. 7A) and eNOS (p-Ser-1177) (Fig. 7B), which were suppressed by wortmannin. Moreover, both the wortmannin-sensitive NO production stimulated by insulin in HUVECs (Fig. 7C) and the relaxation induced by insulin in phenylephrine pre-contracted aortic rings from WKY rats (Fig. 7D) were significantly reduced by quercetin *in vitro*.

4. Discussion

The present study confirms the effects of quercetin on high blood pressure, end-organ (heart and kidney) injury and systemic and vascular oxidative status as previously described in this genetic model of hypertension (Duarte et al., 2001; Sánchez et al., 2006). Chronic quercetin treatment in SHR also restored the impaired endothelium-dependent relaxation to acetylcholine. These vascular protective effects induced by quercetin in SHR seem to be related to the reduced O_2^- production found in SHR treated with quercetin. However, unexpectedly, quercetin did not modify the plasma lipid profile, the homeostatic model assessment of insulin resistance or the oral glucose tolerance test, neither in SHR nor in WKY rats. Despite the effects of quercetin on the impaired endothelium-dependent relaxation to acetylcholine in SHR it

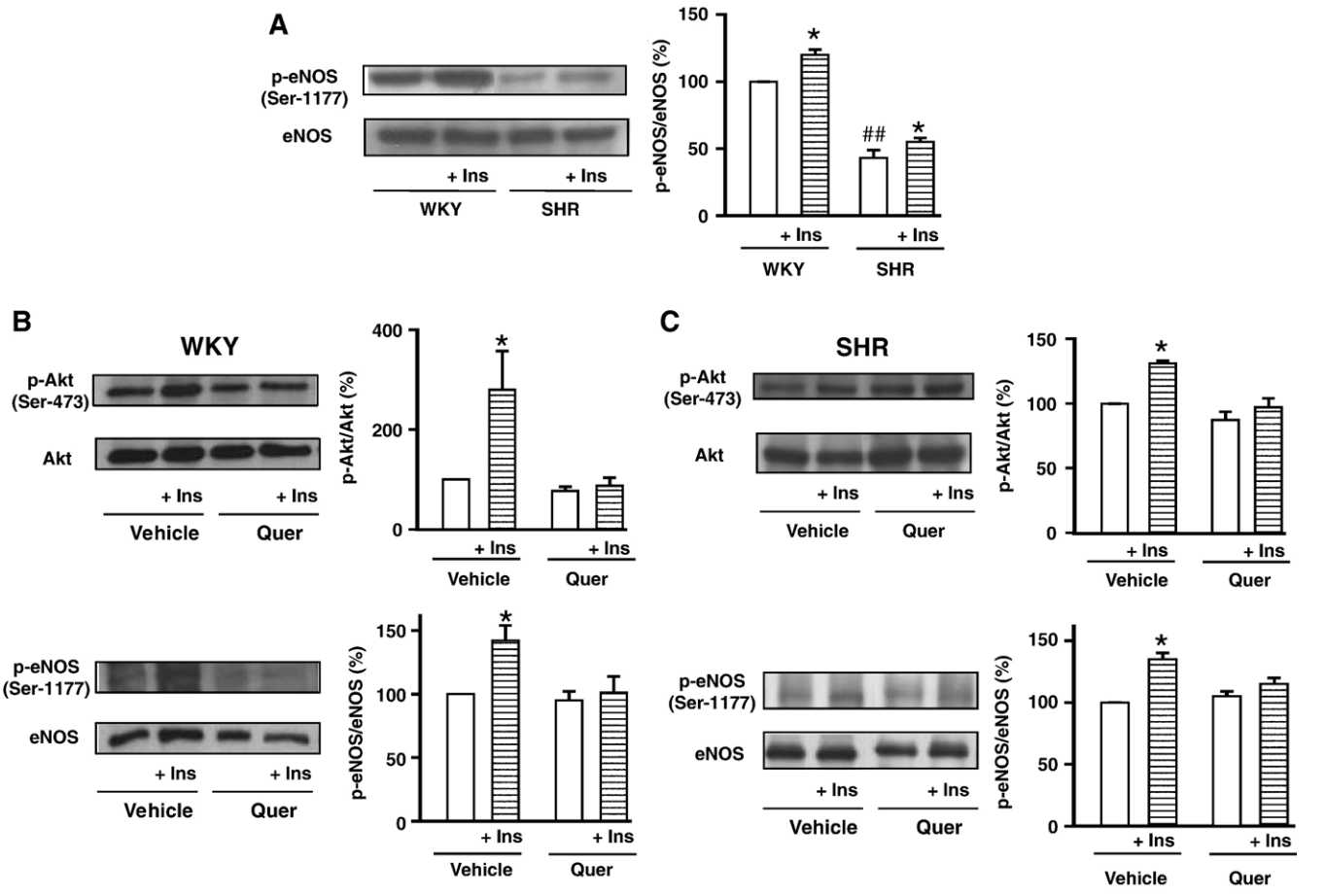

Fig. 6. (A) Immunoblots showing the effects of insulin (Ins, 10^{-7} M) incubation for 5 min on eNOS phosphorylation in aortic rings from WKY rats and SHR. The histograms showed the relative ratio of the phosphorylated and non-phosphorylated form of eNOS. Immunoblots showing the effects of chronic quercetin on Akt and eNOS phosphorylation induced in aortic rings from the WKY vehicle and WKY quercetin (B), and from the SHR vehicle and SHR quercetin groups (C) after Ins (10^{-7} M) incubation for 5 min. The histograms showed the relative ratio of the phosphorylated and non-phosphorylated form of both Akt and eNOS. Values are expressed as means ± S.E.M. $^{\#\#}P<0.01$ *vs* WKY; $^{*}P<0.05$ *vs* non incubated rings with Ins.

had no effects on the reduced endothelium-dependent relaxation to insulin in SHR and even inhibited insulin-evoked relaxant responses in WKY rats. Moreover, quercetin treatment inhibited the signalling of insulin, e.g. Akt- and eNOS-phosphorylation, in both SHR and WKY rats.

Hypertension and insulin resistance are disorders of hemodynamic and metabolic homeostasis, respectively, that are interrelated and that both have strong genetic components (Lake et al., 2003). In this study, we used the SHR as a genetic model of essential hypertension with insulin resistance to characterize defects in endothelial insulin action that may contribute to hypertension (Potenza et al., 2005). For example, male SHR demonstrate hyperinsulinemia and elevated blood glucose concentrations during oral glucose tolerance test compared with age-matched WKY rats (present results, Swislocki and Tsuzuki, 1993). Antihypertensive agents differ in their impact on glucose and lipid homeostasis. Human studies are not unanimous, but in general thiazide diuretics and β-adrenergic antagonists have slight adverse effects, results with calcium channel blockers are controversial, and α1-antagonists and inhibitors of the renin–angiotensin system have positive effects (Sarafidis et al., 2007).

Several theories have been advanced to account for the metabolic effects of various classes of antihypertensives. A prevalent theory that could be called the "hemodynamic hypothesis" postulates that both glucose and insulin deliveries to target tissues is a major limitation on glucose disposal in hypertension (Julius et al., 1992). Thus, antihypertensives with direct or indirect vasodilating actions will improve glucose disposal, whereas agents such as β-blockers that reduce cardiac output and increase vascular resistance will worsen glucose disposal. The vasorelaxant effects of quercetin and related metabolites have been widely assessed *in vitro* (Perez-Vizcaíno et al., 2002). Moreover, this hypothesis might collaborate based in two experimental findings: i) chronic quercetin reduces the media/lumen ratio of mesenteric arteries from SHR (Duarte et al., 2001), and ii) improves endothelium-dependent vasorelaxation induced by acetylcholine (present results; Duarte et al., 2001). However, we recently showed that the major metabolites of quercetin in human plasma, quercetin-3-glucuronide, quercetin-3′-sulfate and isorhamnetin-3-glucuronide have no acute vasorelaxant effect in isolated rat aorta at physiological concentrations in either endothelium-intact or denuded arterial rings (Lodi et al., 2009). The lack of vasorelaxant effects of quercetin metabolites suggests that the antihypertensive effect observed in our present results is unrelated to an acute vasorelaxant effect, and possibly might explain the absence of beneficial metabolic effects of quercetin in SHR.

Many animals and human studies have demonstrated that treatment with antioxidants improves insulin sensitivity, preventing the alterations in intracellular signalling pathway inducing insulin resistance (Ceriello, 2000). The SHR model is characterized by an increased oxidative stress (Suzuki et al., 1995). Plasma levels of MDA have been proposed to be reliable markers of lipid peroxidation and oxidative stress (Kitts et al., 1998) and in the present study, this parameter was reduced in quercetin-treated SHR as compared to vehicle-treated SHR, indicating that chronic oral quercetin treatment reduced the oxidative stress in the SHR, without affecting WKY rats. Moreover, chronic quercetin up-regulates paraoxonase 1 gene expression, an antioxidative enzyme of LDL, with

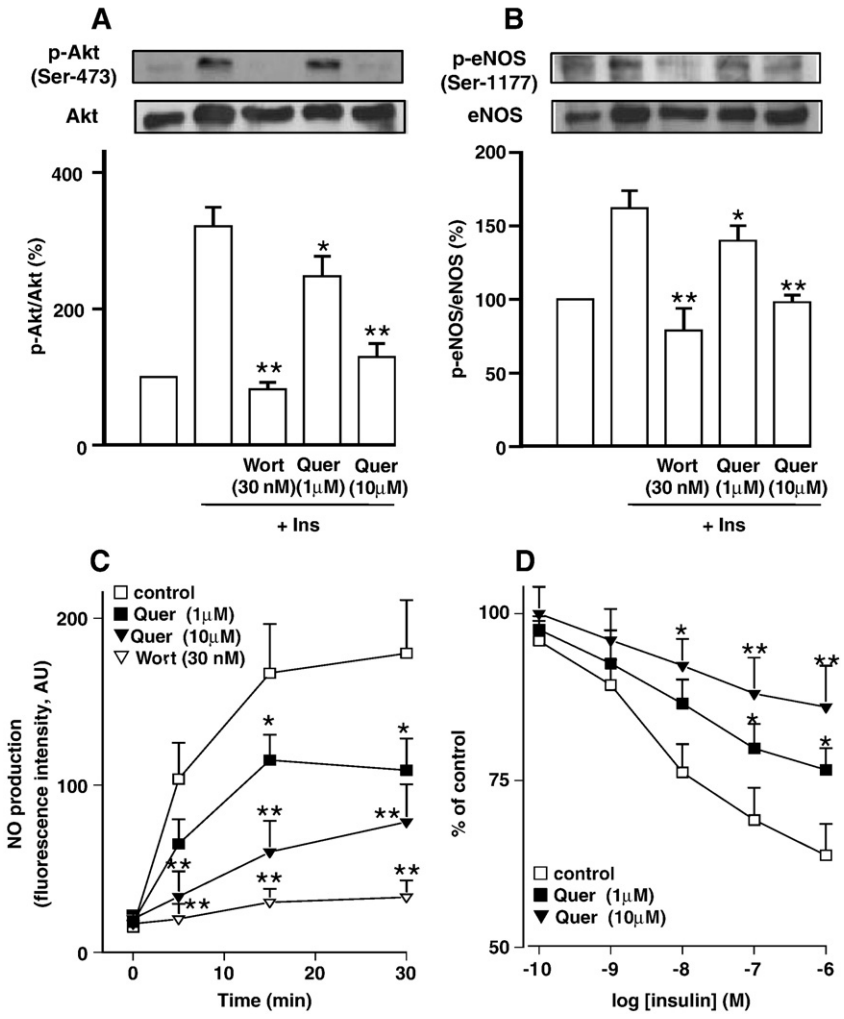

Fig. 7. In vitro effects of quercetin in vascular Akt/eNOS insulin-stimulated pathway. Immunoblots showing the effects of insulin (Ins, 10^{-7} M) incubation for 5 min on Akt (A) and eNOS phosphorylation (B) in aortic rings from WKY rats pre-incubated for 30 min with wortmannin (wort), or quercetin (quer). The histograms showed the relative ratio of the phosphorylated and non-phosphorylated forms, expressed as percentage of unstimulated control rings. Values are expressed as means ± S.E.M. *P<0.05, **P<0.01 *vs* control rings stimulated with Ins. (C) DAF-2-detected NO released from HUVEC after treatment with insulin (10^{-7} M) incubated for 30 min in the absence or in the presence of quer or wort. The difference between fluorescence signal without and with L-NAME was considered NO production. All data are mean ± S.E.M. (n = 8). *P<0.05, **P<0.01 *vs* control HUVEC (D) Relaxations induced by insulin in endothelium-intact aortic rings from WKY rats pre-contracted by 10^{-7} M phenylephrine, with or without previous incubation for 30 min with quer (n = 6–8). Data are presented as percentage of control contraction and values are expressed as means ± S.E.M. *P<0.05 and **P<0.01 *vs* control rings.

concomitant protection against LDL oxidation (Gong et al., 2009). However, in the present study we found that insulin resistance in SHR, measured by homeostatic model assessment of insulin resistance, was unaffected by quercetin, despite its antioxidant activity. This unexpected result could be related to previous data found in intact rat adipocytes, showing that *in vitro* quercetin inhibits the effects of insulin. Thus, quercetin inhibited the receptor tyrosine kinase-catalyzed phosphorylation of exogenous substrate and inhibited insulin-stimulating effects on glucose transport, oxidation, and its incorporation into lipids (Shisheva and Shechter, 1992).

Previous studies have demonstrated that the metabolic and the vasodilator effects of insulin share similar signalling pathways (Montagnani et al., 2001). In SHR, a genetic model of hypertension with features of the metabolic syndrome, insulin resistance is associated with impaired insulin-stimulated NO-dependent vasodilatation (present results; Potenza et al., 2005). Pharmacological interventions in animals and humans support the existence of reciprocal relationships between insulin resistance and endothelial dysfunction. For example, ACE inhibitors and angiotensin II type 1 receptor blockers that improve endothelial function (resulting in lower peripheral vascular resistance and blood pressure in hypertensive subjects) also improve insulin sensitivity (Koh et al., 2007). Likewise, thiazolidinediones, ligands for peroxisome proliferator-activated receptor that improve the metabolic actions of insulin in skeletal muscle, also improve endothelial function and reduce blood pressure in subjects with insulin resistance or diabetes (Raji et al., 2003).

In the present study we found that quercetin improved the endothelium-dependent vasodilatation to acetylcholine which is impaired in SHR as compared to WKY rats. This protective effect of the endothelium-dependent relaxation might result from an enhanced eNOS activity and a decreased NADPH oxidase mediated-O_2^- generation (Sánchez et al., 2006), preventing the O_2^--induced NO degradation and thus prolonging its half-life. In fact, we showed that quercetin reduced aortic O_2^- generation, measured by the DHE probe. However, endothelium-dependent relaxations to insulin which are also impaired in SHR were unaffected after chronic treatment with quercetin. The different profile of quercetin against the relaxations induced by these two endothelial NO releasing agents, despite its protective effect on O_2^--driven NO inactivation, might be related to the different pathways of acetylcholine and insulin to activate eNOS. Acetylcholine is a classic cholinergic agonist that activates eNOS by a calcium-dependent mechanism (Ungvari et al., 2001). However, insulin has calcium-independent vasodilator actions

that are mediated by a phosphatidylinositol 3-kinase (PI3-K) dependent mechanism involving phosphorylation of eNOS by Akt (present results; Montagnani et al., 2001). Likewise, we found that the PI3-K inhibitor wortmannin fully abolished insulin-dependent relaxation. However, acetylcholine-induced relaxation is either unaffected (Florian et al., 2004) or only partially affected (present results) by wortmannin. Interestingly, in our study, the vasorelaxation induced by insulin was accompanied by an increase in both Akt and eNOS phosphorylation and endothelial NO production, which were abolished by wortmannin. Similarly, these insulin-stimulated phosphorylations were reduced in aortic rings from SHR and WKY rats treated with quercetin. This inhibitory effect on the insulin signalling pathway leading to eNOS activation explains the lack of beneficial effect of quercetin both on insulin-induced vasodilation and on insulin resistance despite its antioxidant activity in SHR. This effect might be related to a direct inhibitory effect of quercetin on PI3-K (Yoshizumi et al., 2001).

In conclusion, these data confirm that chronic treatment with this antioxidant bioflavonoid reduces blood pressure, left ventricular and renal hypertrophy and systemic and vascular oxidative status. In contrast, the beneficial metabolic effects of quercetin on lipid profile, oral glucose tolerance, insulin resistance and vascular insulin action were not evident. These latter results seem to be related to the interference of quercetin with the insulin signalling resulting in reduced Akt-mediated eNOS phosphorylation.

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