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Quercetin inhibits vascular superoxide production induced by endothelin-1: Role of NADPH oxidase, uncoupled eNOS and PKC

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Abstract

Chronic administration of the most abundant dietary flavonoid quercetin exerts antihypertensive effects and improves endothelial function. We have investigated the effects of quercetin and its methylated metabolite isorhamnetin (1–10 μM) on endothelial dysfunction and superoxide ($O_2^{\bullet-}$) production induced by endothelin-1 (ET-1, 10 nM). ET-1 increased the contractile response induced by phenylephrine and reduced the relaxant responses to acetylcholine in phenylephrine contracted intact aorta, and these effects were prevented by co-incubation with quercetin, isorhamnetin or chelerythrine (protein kinase C (PKC) inhibitor). This endothelial dysfunction was also improved by superoxide dismutase (SOD), apocynin (NADPH oxidase inhibitor) and sepiapterin (tetrahydrobiopterin synthesis substrate). Furthermore, ET-1 increased intracellular $O_2^{\bullet-}$ production in all layers of the vessel, protein expression of NADPH oxidase subunit $p47^{phox}$ without affecting $p22^{phox}$ expression and lucigenin-enhanced chemiluminescence signal stimulated by calcium ionophore A23187. All these changes were prevented by both quercetin and isorhamnetin. Moreover, apocynin, endothelium denudation and N^G-nitro-L-arginine methylester (L-NAME, nitric oxide synthase inhibitor) suppressed the ET-1-induced increase in A23187-stimulated $O_2^{\bullet-}$ generation. Moreover, quercetin but not isorhamnetin, inhibited the increased PKC activity induced by ET-1. Taken together these results indicate that ET-1-induced NADPH oxidase up-regulation and eNOS uncoupling via PKC leading to endothelial dysfunction and these effects were prevented by quercetin and isorhamnetin.

Keywords: Endothelial dysfunction; Endothelin-1; NADPH oxidase; Protein kinase C; Quercetin

1. Introduction

Endothelin-1 (ET-1) is a potent endothelial-derived vasoconstrictor peptide playing a crucial role in cardiovascular physiology and its alterations have been associated with most forms of cardiovascular disease. Experimental models of hypertension such as deoxicorticosterone acetate (DOCA)-salt or angiotensin II-infused rats, atherosclerosis and diabetes display high levels of circulating ET-1 and are associated with endothelial dysfunction [1–4]. A significant body of evidence indicates that ET-1, through ET_A/ET_B receptor activation, increases reactive oxygen species (ROS) production in the vasculature (e.g. superoxide anion, $O_2^{\bullet-}$), resulting in endothelial dysfunction [5–7].

Abbreviations: ACh, acetylcholine; BH_4, tetrahydrobiopterin; DAPI, 4′,6-diamidin-2-phenylindol dichlorohydrate; DHE, dihydroethidium; DMSO, dimethylsulfoxide; DOCA, deoxycorticosterone acetate; DPI, diphenylene iodonium; eNOS, endothelial nitric oxide synthase; ET-1, endothelin-1; L-NAME, N^G-nitro-L-arginine methylester; NADPH, nicotinamide adenine dinucleotide phosphate; NIH, National Institutes of Health; NO, nitric oxide; $O_2^{\bullet-}$, superoxide anion; OCT, optimum cutting temperature; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RLU, relative luminescence units; ROS, reactive oxygen species; RT-PCR, Reverse Transcriptase-Polymerase Chain Reaction; SOD, superoxide dismutase; SHRs, spontaneously hypertensive rats.

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The major source of intracellular ROS in vascular cells is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a multi-subunit enzymatic complex which comprises two membrane-bound subunits, Nox (Nox-1, Nox-2 or gp91^{phox}, Nox-4 or Nox-5) and p22^{phox} which are regulated by cytoplasmic subunits such as p47^{phox}, p67^{phox} and a low-molecular-weight G protein (rac 1 or rac 2) [8]. The mechanisms involved in angiotensin II-induced vascular $O_2^{\bullet -}$ production are well characterized. ROS production by angiotensin II can be separated into an acute phase involving protein kinase C (PKC), c-Src, growth factor receptors transactivation and translocation of cytosolic p47^{phox} to the membrane [9,10], and a sustained phase involving up-regulation of NADPH oxidase subunits [11] which also involves PKC activation [12,13]. In contrast, the underlying mechanisms implicated in ET-1-induced $O_2^{\bullet -}$ production are controversial. ET-1 does not produce acute increases in ROS [14] and NADPH oxidase [5–7,15], uncoupled endothelial nitric oxide synthase (eNOS) [7] and the mitochondria [16,17] have been reported as the sources of ROS in its long-term effects.

The flavonol quercetin is one of the most abundant polyphenolic compounds found in the human diet. Large epidemiological studies have shown an inverse association between dietary flavonol intake and mortality from coronary heart disease and/or risk of stroke [18,19]. We have reported that quercetin relaxes vascular smooth muscle [20] and its chronic daily treatment reduced blood pressure and endothelial dysfunction in experimental models of hypertension, characterized by an activation of the renin–angiotensin system, such as in spontaneously hypertensive rats (SHRs) [21–23], in rats made hypertensive by chronic inhibition of nitric oxide (NO) synthase [24] or in renovascular hypertensive rats [25]. Furthermore, we recently found that quercetin prevents endothelial dysfunction, $O_2^{\bullet -}$ production, and overexpression of p47^{phox} induced by angiotensin II in rat aorta [26]. Moreover, chronic quercetin also reduces blood pressure and improves endothelial function in DOCA-salt rats, a low renin model of hypertension in which ET-1 plays a predominant role. The effects of quercetin were associated with a reduction in systemic and vascular oxidative stress and restoration of aortic Cu/Zn SOD expression [27,28]. Since the sustained $O_2^{\bullet -}$ production by angiotensin II could be an effect derived from the increased ET-1 synthesis [14] it is possible that inhibition in angiotensin II-mediated $O_2^{\bullet -}$ production induced by both quercetin and isorhamnetin [26] are a result of decreased ET-synthesis and/or interference with ET-1 signaling pathways. In fact, quercetin decreased ET-1 synthesis in cultured bovine aortic endothelial cells [29] and vascular smooth muscle cells [30].

Given the key role of ET-1 and oxidative stress in cardiovascular disease, we have investigated the mechanisms involved in ET-1-induced vascular oxidative response and endothelial dysfunction and the effects of the dietary flavonoid quercetin and its methylated plasma metabolite isorhamnetin.

2. Materials and methods

All of the procedures conform to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health (NIH) publication no. 85-23, revised 1996) and approved by our Institutional Committee for the ethical care of animals.

2.1. Isolation and incubation of rat aortic rings

Experiments were conducted in male Wistar rats obtained from Harlan Laboratories (Barcelona, Spain) weighing 200–250 g. The descending thoracic aortae were dissected and cut into rings. Rings were incubated in 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) containing an antibiotic–antimycotic mixture (penicillin, gentamicin and anfotericin B) for 2 h in a cell culture incubator in the absence or presence of ET-1 (10 nM) and in the presence of vehicle (dimethylsulfoxide, DMSO 0.1%), quercetin (1 or 10 μM), isorhamnetin (1 or 10 μM) or the PKC inhibitor chelerythrine (1 μM). In preliminary experiments, ET-1-induced endothelial dysfunction only after 2 h of incubation. The concentrations of bioflavonoids used are in the physiological range found in human circulating blood after ingestion of a single dose of quercetin provided as pure compound, plant extract, or whole food/beverage [31]. Aortae were immediately used for $O_2^{\bullet -}$ production or contractile tension recording or frozen in liquid nitrogen and stored at −80 °C for Western blotting or included in optimum cutting temperature (OCT) compound and then frozen in liquid nitrogen and stored at −80 °C for intracellular $O_2^{\bullet -}$ production analysis.

2.2. Contractile tension recording

Aortic rings, previously incubated as mentioned above, were mounted in organ chambers 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 coupled to a signal amplifier (Dynamometer UF1, Cibertec, Madrid) and connected to a computer via an A/D interface. Contractile tension was recorded by a PowerLab 800 (AD Instruments, Cibertec, Madrid), as previously described [26]. The chamber was filled with Krebs solution at 37 °C and gassed with 95% O_2 and 5% CO_2. Rings were stretched to 2 g of tension and equilibrated for 90–120 min. During this period, Krebs solution was changed every 30 min and rings were re-stretched as needed to maintain a final tension of 2 g. After equilibration, arteries were stimulated with phenylephrine (1 μM or 0.1 μM, in control or ET-1-treated aortic rings, respectively, to obtain similar level of precontraction) and a concentration–response curve was constructed by cumulative addition of acetylcholine (ACh). In some experiments, SOD (100 U/ml) or apocynin (100 μM) were added to the organ chamber 30 min before the addition of phenylephrine. To evaluate the participation of endothelial NO on endothelial dysfunction induced by ET-1, several aortic rings were incubated with the inhibitor L-NAME (100 μM) or sepiapterin (100 μM) in organ chambers for 30 min before the addition of phenylephrine (10^{-9} to 10^{-5} M).

2.3. *In situ detection of vascular $O_2^{\bullet-}$ production*

Dihydroethidium (DHE) was used to localize $O_2^{\bullet-}$ in vessel sections in situ. DHE enters the cells and is oxidized by $O_2^{\bullet-}$ to yield ethidium, which binds to DNA to produce bright red fluorescence. Aortic rings, previously incubated as mentioned above, were cryopreserved by incubation with phosphate buffered solution (0.1 M) containing 30% sucrose for 1–2 h, included in OCT, frozen, and 10 μm cross sections were obtained in a cryostat (Microm international Model HM500 OM). Sections were incubated in a humidified chamber for 30 min in Hepes buffered solution (in mM: NaCl 130, KCl 5, $MgCl_2$ 1.2, glucose 10, and HEPES 10, pH 7.3 with NaOH) at 37 °C. Then, the sections were further incubated for 30 min in Hepes solution containing DHE (10 μM) in the dark. Then preparations were counterstained with the nuclear stain 4′,6-diamidino,2-phenylindol dichlorohydrate (DAPI, 0.3 μM) for 5 min at 37 °C in the dark, washed and mounted with a coverslip. Some sections from control rings were incubated for 30 min with the $O_2^{\bullet-}$ intracellular scavenger tiron (10 μM) before DHE. Four sections of each preparation were examined on a fluorescence microscope (Leica DM IRB, Wetzlar, Germany) using a 40× objective with the following pairs of excitation/emission filters: 340-380 nm/425 nm long pass to visualize DAPI blue fluorescence, BP450-490 nm/ 515 nm long pass to visualize the green autofluorescence of elastin and 545-30 nm/610-75 nm long pass to visualize ethidium red fluorescence. Sections were photographed with a Leica DC300F color digital camera and images were saved for off-line analysis. Microscope and camera settings were kept constant for all preparations. Ethidium and DAPI fluorescence were quantified using ImageJ (Version 1.32j, NIH, http://rsb.info.nih/ij/). $O_2^{\bullet-}$ production was estimated from the ratio of ethidium /DAPI fluorescence [32].

2.4. *Measurement of ET-1 mediated $O_2^{\bullet-}$ production with lucigenin-enhanced chemiluminescence*

To characterize the involvement of uncoupled eNOS on ET-1-stimulated aortic $O_2^{\bullet-}$ generation, some endothelium-intact aortic rings were also coincubated for 2 h with ET-1 plus L-NAME (100 μM), apocynin (300 μM), sepiapterin (100 μM), quercetin (10 μM) or isorhamnetin (10 μM) or were endothelium-denuded. At the end of incubation period, aortic rings were then incubated for 30 min at 37 °C in HEPES-containing physiological salt solution (pH 7.4) of the following composition (in mM): NaCl 119, HEPES 20, KCl 4.6, $MgSO_4$ 1, Na_2HPO_4 0.15, KH_2PO_4 0.4, $NaHCO_3$ 1, $CaCl_2$ 1.2 and glucose 5.5. Rings were then placed in tubes containing physiological salt solution, stimulated with the calcium ionophore A23187 (10 μM) and lucigenin was injected automatically at a final concentration of 5 μM. Changes in $O_2^{\bullet-}$ release were determined by measuring luminescence over 200 s in a luminometer (Lumat LB 9507, Berthold, Germany) in 5-s intervals. Vessels were then dried, and dry weight was determined. $O_2^{\bullet-}$ release is expressed as relative luminescence units (RLU)/min/mg dry aortic tissue. The luminescence signal was almost suppressed by superoxide dismutase (SOD, 100 U/ml), showing the involvement of $O_2^{\bullet-}$.

In order to analyze the effects of flavonoids in the PKC-mediated $O_2^{\bullet-}$ production, some aortic rings were exposed to phorbol 12-myristate 13-acetate (PMA, 1 μM) for 30 min in the presence or absence of quercetin (10 μM), isorhamnetin (10 μM) or chelerythrine (1 μM), before lucigenin injection. Aortic production of $O_2^{\bullet-}$ was measured in the presence of NADPH (100 μM).

2.5. *Vascular p22^{phox} and p47^{phox} gene and protein expression*

For Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) analysis, total RNA was extracted from aorta by homogenization and converted to cDNA by standard methods. Polymerase chain reaction was performed with a Techne Techgene thermocycler (Techne, Cambridge, UK). Initial denaturation was done at 95 °C for 3 min and followed by 25–32 cycles of amplification. Each cycle consisted of 1 min of denaturation at 94 °C, 45 s of annealing at 60 °C for p47^{phox} or 55 °C for p22^{phox}, and 1 min for enzymatic primer extension at 72 °C. After the final cycle, the temperature was held at 72 °C for 10 min to allow reannealing of amplified products. RT-PCR products were then size-fractionated through a 1.5% agarose gel, and the bands were visualized with ethidium bromide and quantified by densitometric analysis performed on the scanned images using Scion Image-Release Beta 4.02 software (http://www.scioncorp.com). The sequences for primers for p47^{phox} and p22^{phox} were selected according to the published sequences in GenBank and were as follows: p47^{phox} (191 base pairs) sense, 5′-CCCAGCGACAGATTAGAAGC-3′ and antisense, 5′-TGGATTGTCCTTTGAGTCAGG-3′; p22^{phox} (220 base pairs) sense, 5′-GCGGTGTGGACAGAAGTACC-3′ and antisense, 5′-CTTGGGTTTAGGCTCAATGG-3′). GADPH was used as an internal control for the coamplification. The signals were expressed relative to the intensity of GADPH in each sample.

For Western blotting experiments, aortae were frozen, homogenated and centrifuged. Western blottings were performed with 30 or 50 μg of protein for p22^{phox} or p47^{phox}, respectively, of the supernatant per lane [23,26]. Sodium dodecyl sulphate-polyacrilamide (10%) electrophoresis was performed in a mini-gel system (Bio-Rad Laboratories). The proteins were transferred to polyvinylidene difluoride membranes for 1 h and incubated with primary goat polyclonal antibody for p22^{phox} or with rabbit anti-p47^{phox} polyclonal antibody (1:1000 dilution, Santa Cruz Biotechnology) overnight. The membranes were then washed five times for 10 min in Tris-buffered saline–0.1% Tween 20 and incubated with secondary peroxidase conjugated goat anti-goat or goat anti-rabbit antibodies (1:2000, 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 ECL 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). Samples were reprobed for expression of α-actin. $p22^{phox}$ or $p47^{phox}$ protein abundance/α-actin ratio was calculated and data is expressed as a percentage of the values in control aorta from the same gel.

2.6. Determination of vascular PKC activity

Aortae were incubated for 30 min with vehicle, ET-1 or PMA in the presence or absence of quercetin (10 μM), isorhamnetin (10 μM) or chelerythrine (1 μM). Then, aortae were homogenized in a glass homogenizer on ice. Homogenates were sonicated three times for 10 s and centrifuged for 10 min at 2000 × *g*. PKC activity was measured using an ELISA kit from Calbiochem. Briefly, 6 μg of protein was mixed with a reaction buffer containing (in mM) 25 Tris–HCl, pH 7.0, 3 $MgCl_2$, 0.2 ATP, 2 $CaCl_2$, and 50 μg/ml phosphatidylserine. For the negative control, $CaCl_2$ was omitted from the buffer and 20 mM EGTA was added in place of phosphatidylserine. Samples were transferred into a 96-well plate coated with pseudosubstrate. PKC present in the sample catalyzed the phosphorylation of pseudosubstrate on serine. A biotinylated antibody was added, which bound to the phosphorylated pseudosubstrate. This was followed by a peroxidase-catalyzed reaction of which the OD was measured at 492 nm. Data is expressed as a percentage of the values in control aorta.

2.7. Drugs

All drugs and reagents were from Sigma, except DAPI from Calbiochem and isorhamnetin from Extrasynthese. Quercetin, isorhamnetin, PMA and chelerythrine were initially dissolved in DMSO and all other drugs in distilled water.

2.8. Statistical analysis

Results are expressed as means ± standard error of mean and *n* reflects the number of animals. Statistically significant differences between groups were calculated by Student's *t*-test for unpaired observations or for multiple comparisons by an ANOVA followed by a Newman Keuls test. $P < 0.05$ was considered statistically significant. Concentration–response curves were fitted to a logistic equation.

3. Results

3.1. Effects on endothelial dysfunction

Incubation of the aortic rings for 2 h in the absence of ET-1 produced no significant changes in the contractile response to phenylephrine or in the relaxant response to ACh (data not shown). ET-1 led to the development of endothelial dysfunction as indicated by the reduction in the maximal relaxant effect of ACh (Fig. 1A, B, D and E) and by the increased contractile response to phenylephrine (Fig. 1C and F). Incubation with quercetin or isorhamnetin (1 or 10 μM) for 2 h prevented ET-1-induced endothelial dysfunction, i.e. both flavonoids increased the relaxant response to ACh (Fig. 1A and B, respectively) and decreased the contractile response to phenylephrine (Fig. 1C) in ET-1-treated arteries. The impaired relaxant response to ACh induced by ET-1 was partially restored by SOD (Fig. 1D) and fully restored by the NADPH oxidase inhibition with apocynin (Fig. 1E). This drug also reduced the hyper-responsiveness to phenylephrine in aortic rings incubated with ET-1 (Fig. 1F). The BH_4 synthesis substrate sepiapterin decreased the contractile response to phenylephrine (Fig. 1F) but the impaired vasodilatation to ACh was only partially and not significantly improved (Fig. 1E). The relaxant response to ACh was suppressed by NO synthase inhibition with L-NAME, in both control (data not shown) or ET-1-treated aortic rings (Fig. 1E).

In order to determine if stretch could modify the effects of ET-1 on endothelial dysfunction, in another set of experiments, rings were first mounted in the organ chambers and then incubated with or without ET-1 for 2 h. Afterwards, after removing ET-1, the relaxant response to ACh was analyzed in phenylephrine precontracted rings. Under these more physiological experimental conditions, ET-1 incubation also impaired the vasodilatation induced by ACh as compared with control rings (maximal effects (%): 45.1 ± 3.7, $n = 9$; 61.6 ± 3.9, $n = 9$, respectively, $P < 0.05$).

3.2. Effects on $O_2^{\bullet -}$ production

In order to characterize $O_2^{\bullet -}$ production and localize it within the vascular wall, ethidium red fluorescence was analyzed in sections of aorta incubated with DHE which is converted into ethidium by $O_2^{\bullet -}$-induced oxidation. The nuclear staining was almost suppressed by the $O_2^{\bullet -}$ intracellular scavenger tiron. Positive red nuclei could be observed in adventitial, medial and endothelial cells (Fig. 2A). Red fluorescence was quantified and the data was normalized by the blue fluorescence of the nuclear stain DAPI (Fig. 2B). At 2 h, ET-1 induced an increase in $O_2^{\bullet -}$ production which was evident in all layers of the vessel. Quercetin or isorhamnetin (1 and 10 μM) prevented ET-1-induced increase in DHE staining (Fig. 2A and B).

ET-1 also increased aortic $O_2^{\bullet -}$ generation, measured by lucigenin-enhanced chemiluminescence, in rings stimulated with the calcium ionophore A23187. This increased $O_2^{\bullet -}$

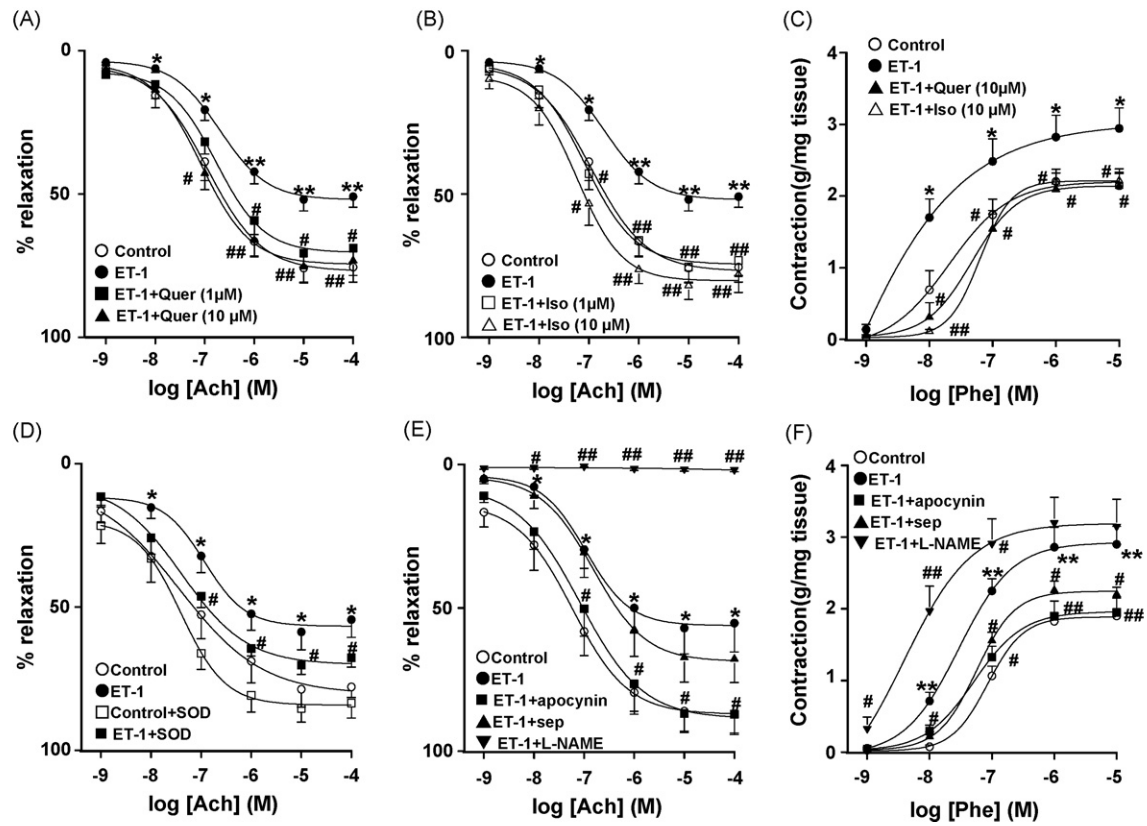

Fig. 1. Effects of quercetin (Quer) and isorhamnetin (Iso) on ET-1-induced endothelial dysfunction. Endothelium-intact aortic rings were incubated for 2 h with vehicle or the flavonoids and with or without ET-1. (A and B) Acethylcholine (ACh)-induced relaxant responses in phenylephrine (Phe) precontracted vessels. (C) Phe-induced contractile response. In some experiments, superoxide dismutase (SOD, 100 U/ml) (D), apocynin (100 μM), N^G-nitro-L-arginine methylester (L-NAME, 100 μM) or sepiapterin (sep, 100 μM) (E and F) were added to the organ chamber 30 min before the addition of Phe. Results are means ± S.E.M., $n = 5$–9, * and ** indicate $P < 0.05$ and $P < 0.01$, respectively, *vs.* control rings, # and ## indicate $P < 0.05$ and $P < 0.01$, respectively, *vs.* ET-1-treated rings.

production was suppressed by denudation of endothelium and by co-incubation with L-NAME, apocynin or sepiapterin (Fig. 3A) or the bioflavonoids quercetin and isorhamnetin (10 μM) (Fig. 3B).

3.3. Effects on p47^{phox} and p22^{phox} gene and protein expression

In aortic segments, incubation with ET-1 for 2 h increased both gene and protein expression of p47^{phox} (Fig. 4A and C) without affecting p22^{phox} expression (Fig. 4B and D), as measured by RT-PCR and Western blot, respectively. Co-incubation with quercetin or isorhamnetin (1 and 10 μM) prevented ET-1-induced overexpression of p47^{phox}.

3.4. Role of PKC

ET-1-induced impaired relaxant response to ACh and increased expression of p47^{phox} protein were suppressed by co-incubation with the PKC inhibitor chelerythrine (Fig. 5A and B, respectively). The PKC activator PMA also increased aortic $O_2^{\bullet -}$ generation in control rings. This effect was inhibited by quercetin, isorhamnetin and chelerythrine (Fig. 5C).

After 30 min ET-1 incubation of aortic rings an increase in vascular PKC activity was detected as compared with control rings. Similarly, PMA also increased PKC activity. Both ET-1 and PMA induced increase in PKC activity was prevented by chelerythrine. Co-incubation with quercetin, but not with isorhamnetin, significantly decreased the PKC activity increase stimulated by ET-1 (Fig. 5D).

4. Discussion

ET-1 is a well-known trigger for increased vascular oxidative stress and the subsequent $O_2^{\bullet -}$ driven NO inactivation plays a major role in the genesis of clinical endothelial dysfunction in a variety of pathophysiological conditions, including atherosclerosis, hypertension and diabetes [33]. The present study supports the hypothesis that ET-1 mediates vascular $O_2^{\bullet -}$ production via activation of NADPH oxidase. The oxidative pathway is amplified by $O_2^{\bullet -}$-induced eNOS uncoupling leading to further ROS generation which ultimately leads to endothelial dysfunction in a mechanism similar to that previously described [7,34]. Moreover, the new findings of this study are that: (1) ET-1 activates NADPH oxidase through up-regulation of p47^{phox} but

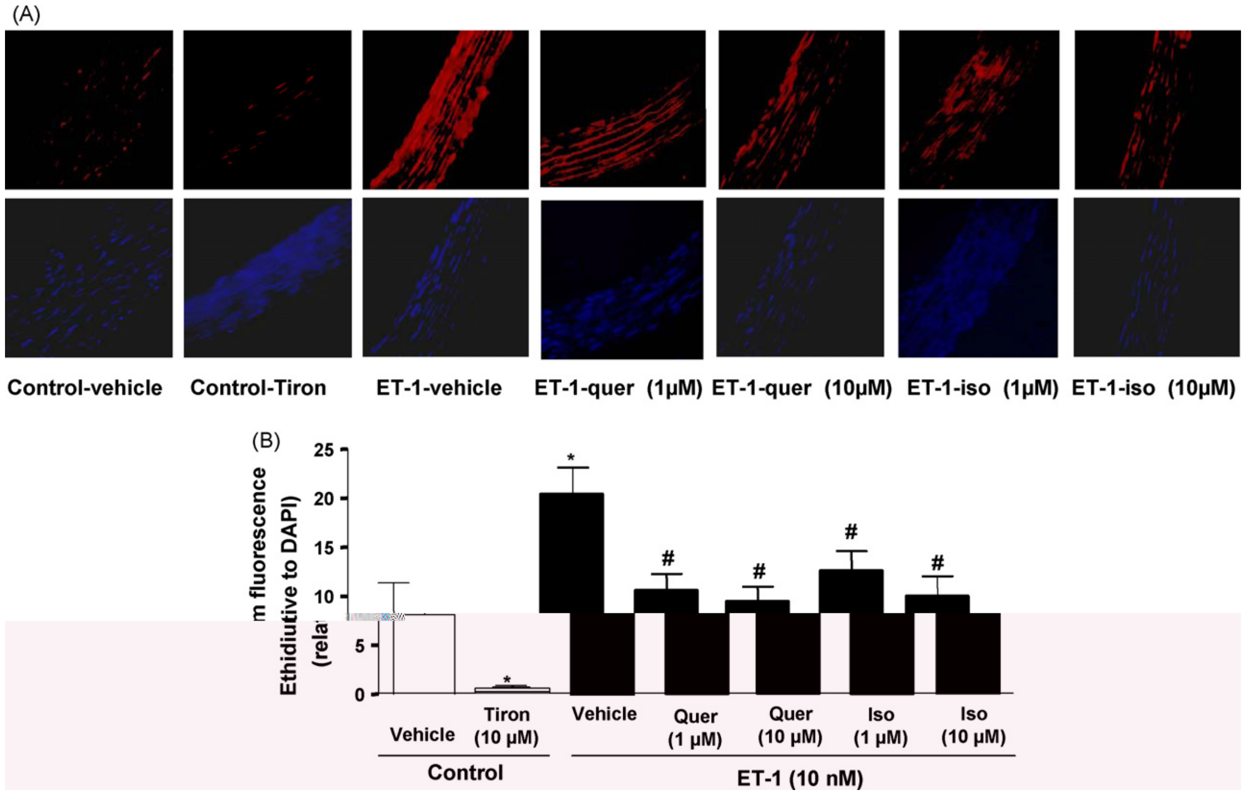

Fig. 2. Effects of quercetin (Quer) and isorhamnetin (Iso) on ET-1-induced vascular $O_2^{\bullet-}$ production measured by dihidroethidium (DHE) fluorescence. (A) Upper pictures show arteries incubated in the presence of DHE which produces a red fluorescence when oxidized to ethidium by $O_2^{\bullet-}$ and lower pictures show blue fluorescence of the nuclear stain DAPI. (B) Values of red ethidium fluorescence normalized to blue DAPI fluorescence. Vehicle was DMSO (0.1%). Results are means ± S.E.M. of four sections obtained from each ring ($n = 4$). $^*P < 0.05$, *vs.* control rings, $^{\#}P < 0.05$, *vs.* ET-1-treated rings.

not p22[phox] in a PKC-dependent manner and (2) the two sources of $O_2^{\bullet-}$, uncoupled eNOS and NADPH oxidase contribute to ET-1-induced endothelial dysfunction. In this study, we also show for the first time that ET-1-induced endothelial dysfunction can be prevented by the dietary flavonol quercetin and its metabolite isorhamnetin, independently of their possible interference with ET-1 production. Moreover, these flavonoids also prevented the ET-1-induced increase in vascular $O_2^{\bullet-}$ production from uncoupled eNOS and NADPH oxidase through down-regulation of p47[phox] expression. Quercetin also prevented ET-1-induced PKC activation.

Fig. 3. Effects of ET-1 on $O_2^{\bullet-}$ production stimulated by the calcium ionophore A23187 (10 μM) as measured by lucigenin-enhanced chemiluminescence. (A) Role of the endothelium, eNOS and NADPH oxidase. Some aortic rings were endothelium denuded (-E) before incubation with ET-1 for 2 h, or endothelium-intact vessels were coincubated with N^G-nitro-L-arginine methylester (L-NAME), apocynin or sepiapterin (sep). (B) Effects of quercetin (Quer) or isorhamnetin (Iso) coincubated with ET-1 for 2 h prior A23187 addition. Vehicle was DMSO (0.1%). Results are means ± S.E.M., $n = 5$–6, $^*P < 0.05$, *vs.* control rings, $^{\#}P < 0.05$, *vs.* ET-1-treated rings. PSS indicates HEPES-containing physiological salt solution.