Quercetin andisorhamnetin Prevent Endothelial Dysfunction, Superoxide Production, and Overexpression of p47phox Induced by Angiotensin II in Rat Aorta

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Abstract
The dietary flavonoid quercetin reduces blood pressure and improves endothelial function in several rat models of hypertension. We analyzed the effects of quercetin and its methylated metabolite isorhamnetin on the aortic endothelial dysfunction induced by incubation with angiotensin II (AngII) in vitro for 6 h. AngII diminished the relaxant responses to acetylcholine in phenylephrine-contracted aorta. Coincubation with quercetin or isorhamnetin, or addition of superoxide (O2−) dismutase or apocynin to the assay medium, prevented these inhibitory effects. At 6 h, AngII induced a marked increase in O2− production as measured by dihydroethidium fluorescence, which was prevented by quercetin and isorhamnetin. AngII also increased the expression of p47phox, a regulatory subunit of the membrane NADPH oxidase. Immunohistochemical analysis revealed that overexpression of p47phox occurred mainly in the medial layer. p47phox overexpression was also prevented by quercetin and isorhamnetin. Taken together, these results show for the first time, to our knowledge, that quercetin andisorhamnetin prevent AngII-induced endothelial dysfunction by inhibiting the overexpression of p47phox and the subsequent increased O2− production, resulting in increased nitric oxide bioavailability.

Introduction
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 (1,2). Quercetin relaxes vascular smooth muscle (3) and its chronic daily treatment reduces blood pressure and endothelial dysfunction in spontaneously hypertensive rats (SHR)4 (4,5), in rats made hypertensive by chronic inhibition of nitric oxide (NO) synthase (6), or in renovascular hypertensive rats (7). In all these experimental models of hypertension, characterized by an activation of the renin-angiotensin-system, the protective effects exerted by quercetin were associated with a reduction of systemic and vascular oxidative status. However, the mechanisms involved are unclear.

Angiotensin II (AngII) increases arterial pressure, impairs endothelial function, induces vascular smooth muscle hypertrophy, and induces the expression of multiple vasoactive and inflammatory substances, playing a key role in the pathophysiology of cardiovascular diseases, including hypertension, atherosclerosis, and heart failure (8). A significant body of evidence supports a role for the intracellular production of reactive oxygen species (ROS), such as superoxide anion (O2−) and hydrogen peroxide, in the signal transduction of AngII (9–12). The major source of intracellular ROS in vascular cells is NADPH oxidase, a multisubunit enzymatic complex that comprises 2 membrane-bound subunits, Nox (Nox-1, Nox-2, also referred to as gp91phox, Nox-4 or Nox-5) and p22phox, which are regulated by cytoplasmic subunits such as p47phox and other NADPH oxidase subunits (8,9,15).

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4 Abbreviations used: ACh, acetylcholine; AngII, Angiotensin II; AT1, AngII receptor type 1; DAPI, 4,6-diamidino,2-phenylindol; DHE, dihydroethidium; DMSO, dimethylsulfoxide; Emax, maximal relaxant effect; NO, nitric oxide; O2−, superoxide; pD2, half maximal relaxation; PEG-SOD, polyethyleneglycol superoxide dismutase; ROS, reactive oxygen species; SHR, spontaneously hypertensive rats; SOD, superoxide dismutase.
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In this study, we investigated the effects of quercetin and its methylated plasma metabolite isorhamnetin on the AngII-induced endothelial dysfunction in vitro and its relation with the production of $O_2^-$ and the expression of p47phox.

Materials and Methods

All of the procedures conform to the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85–23, revised 1996) and were approved by our Institutional Committee for the ethical care of animals. Male Wistar rats were obtained from Harlan Laboratories.

Tissue culture. The descending thoracic aortae were dissected and cut into rings. Rings were incubated in Krebs solution (composition in mmol/L: NaCl, 118; KCl, 4.75; NaHCO3, 25; MgSO4, 1.2; CaCl2, 2; KH2PO4, 1.2; and glucose, 11) containing an antibiotic-antimycotic mixture (penicillin, gentamycin, and anfotericin B) for 2, 4, 6, or 8 h in a cell culture incubator in the absence or presence of AngII (1 μmol/L) and isorhamnetin (0.1 μmol/L), quercetin (1 or 10 μmol/L), isorhamnetin (1 or 10 μmol/L), or losartan (10 μmol/L). Aortae were immediately used for contractile tension recording, frozen in liquid nitrogen, and stored at −80°C for Western blots or embedded in medium and then frozen in liquid nitrogen and stored at −80°C for immunohistochemistry or $O_2^-$ production analysis.

Contractile tension recording. Aortic rings, previously incubated as mentioned above, were mounted in organ chambers, as previously described (3,4). The chamber was filled with Krebs solution at 37°C and gassed with 95% O2 and 5% CO2. Rings were stretched to 2 g of tension and equilibrated for 90–120 min. The contraction was recorded using data acquisition hardware and software (REGXPC computer program from Cibercet). After equilibration, arteries were stimulated with 1 μmol/L phenylephrine and a concentration-response curve was constructed by cumulative addition of acetylcholine (ACh). In some experiments, polybrenylenglycol O2−-dismutase (PEG-SOD, 100 kU/L) or apocynin (100 μmol/L) were added to the organ chamber 60 min before the addition of phenylephrine. Endothelium-independent responses to sodium nitroprusside were also performed in the dark in rings precontracted with 1 μmol/L phenylephrine.

In situ detection of vascular $O_2^-$ production. Unfixed aortic rings were cryopreserved by incubation with PBS (0.1mol/L) 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) (16). Sections were incubated in a humidified chamber for 30 min in HEPES-buffered solution (in mmol/L: NaCl, 130; KCl, 5; MgCl2, 1.2; glucose, 10; and HEPES, 10, pH 7.3 with NaOH) at 37°C. Afterwards, the sections were further incubated for 30 min in HEPES solution containing dihydroethidium (DHE) (10 μmol/L) in the dark, counterstained with the nuclear stain 4',6-diamidino-2-phenylindol (DAPI), and mounted with a coverslip. Four sections of each preparation were examined on a fluorescence microscope (Leica DM IRB), photographed with a Leica DCM400F color digital camera, and images were saved for off-line analysis. Ethidium and DAPI fluorescence were quantified using ImageJ (version 1.32j, NIH, http://rsb.info.nih/g/). $O_2^-$ production was estimated from the ratio of ethidium/DAPI fluorescence (16). Negative controls were obtained in the absence of DHE.

Vascular p47phox expression. Aortae were frozen and homogenated in 200 μL of a buffer of the following composition (mmol/L): HEPES, 10 (pH 8); KCl, 10; EDTA, 1; EGTA, 1; dithiothreitol, 1; aprotinin, 0.006; leupeptin, 0.009; Nor-p-tosyl-L-lysine chloromethyl ketone, 0.011; NaF, 5; Na2MoO4, 10; NaVO4 and phenylmethanesulfonyl fluoride, 0.5 and centrifuged. Western blots were performed with 30 μg of protein of the supernatant per lane (5,17). Sodium dodecyl sulfate-polyacrylamide (10%) electrophoresis was performed in a mini-gel system (Bio-Rad Laboratories). The proteins were transferred to nitrocellulose membranes overnight and incubated with rabbit anti-p47phox polyclonal antibodies (1:200 dilution, Santa Cruz Biotechnology). The membranes were then washed 5 times for 10 min in Tris-buffered saline containing 0.1% Tween 20 and incubated with secondary peroxidase conjugated goat anti-rabbit antibody (1:2000, Santa Cruz Biotechnology). Antibody binding was detected by an enhanced chemiluminescence system (Amersham Pharmacia Biotech). 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 reposed for expression of α-actin. p47phox protein abundance/α-actin ratio was calculated and data are expressed as a percentage of the values in control aorta from the same gel.

Statistical analysis. Results are expressed as means ± SEM and n reflects the number of animals. Significant differences between groups were calculated by ANOVA followed by a Newman Keuls test. P < 0.05 was considered significant. Concentration-response curves were fitted to a logistic equation and from these plots the maximal relaxant effect (Emax) and the negative logarithm of the concentration producing half maximal relaxation (pD2) were calculated.

Results

Endothelial dysfunction. Incubation of the aortic rings for up to 8 h in the absence of AngII produced no significant changes in the contractile response to phenylephrine or in the relaxant response to ACh (Fig. 1; Table 1). Incubation of the aortic rings for 2, 4, 6, or 8 h with AngII produced no significant changes in the contractile response to phenylephrine but led to a progressive development of endothelial dysfunction, as indicated by the reduction in the Emax of ACh (Fig. 1; Table 1). The inhibition was maximal at 6 h and, therefore, this time point was chosen for further experiments. Coincubation with the AngII receptor type 1 (AT1) antagonist losartan (10 μmol/L) prevented AngII-induced endothelial dysfunction (Fig. 2). In AngII pretreated aorta, PEG-SOD (a membrane-permeable form of SOD, 100 kU/L added to the organ bath) and the NADPH oxidase inhibitor apocynin (100 μmol/L added to the organ bath) increased the relaxant response to ACh (Fig. 2).

Incubation with quercetin or isorhamnetin (1 or 10 μmol/L) for 6 h had no significant effect on the contractile response to phenylephrine in either control or AngII-treated arteries (Table 2). However, both flavonoids prevented AngII-induced endothelial dysfunction (Fig. 3; Table 2); i.e. ACh-induced relaxation was significantly increased by 1 and 10 μmol/L quercetin and by 10 μmol/L isorhamnetin. No differences were observed in the endothelium-independent relaxant responses to the endothelium-independent vasodilator sodium nitroprusside in arteries from vehicle-, AngII-, AngII-queretin-, and AngII-isorhamnetin-treated rings (pD2 = 7.78 ± 0.10, n = 5; 7.78 ± 0.13, n = 7;
Figure 1  Time course of AngII-induced endothelial dysfunction in rat aortic rings. Effects of incubation for 2, 4, 6, or 8 h with or without AngII (1 μmol/L) on endothelium-dependent relaxation to ACh in aortic rings precontracted with 1 μmol/L phenylephrine. Results are means ± SEM. Different from control rings at that concentration, * P < 0.05; ** P < 0.01. Calculated E\textsubscript{max} and pD\textsubscript{2} values and n in each set are shown in Table 1.

7.98 ± 0.12, n = 9; 7.97 ± 0.10, n = 9, respectively; and E\textsubscript{max} = 100 ± 1.0%; 99.0 ± 1.1%; 99.3 ± 0.3%; 99.9 ± 0.2%, respectively).

In situ localization of O\textsubscript{2}⁻ production. To characterize O\textsubscript{2}⁻ production and localize it within the vascular wall, endothium red fluorescence was analyzed in sections of aorta incubated with DHE that is converted into ethidium by O\textsubscript{2}⁻-induced oxidation. Positive red nuclei could be observed in adventitial, medial, and endothelial cells (Fig. 4A). Red fluorescence was quantified and the data were normalized by the blue fluorescence of the nuclear stain DAPI (Fig. 4B). At 6 h, Ang II induced an increase in O\textsubscript{2}⁻ production that was most evident in the medial layer of the vessel. Quercetin or isorhamnetin (10 μmol/L) did not modify O\textsubscript{2}⁻ production in control arteries but significantly prevented AngII-induced increase.

p47\textsuperscript{phox} Protein expression and localization. In aortic segments, incubation with AngII for 6 h increased the expression of p47\textsuperscript{phox} as measured by western blot (Fig. 5). Preliminary experiments showed that this increase was maximal at 6 or 8 h. Coincubation with quercetin or isorhamnetin had no effect by themselves but prevented AngII-induced increase. Immunohistochemical analysis revealed that overexpression of p47\textsuperscript{phox} occurred mainly in the medial layer and was also prevented by quercetin and isorhamnetin (10 μmol/L) (Fig. 6).

Role of PPAR\gamma. Incubation with the PPAR\gamma antagonist GW9662 (1 μmol/L) (18) did not modify ACh-induced relaxation in control arteries (pD\textsubscript{2} = 6.88 ± 0.13 and E\textsubscript{max} = 80 ± 3%, n = 8) and did not prevent AngII-induced endothelial dysfunction (pD\textsubscript{2} = 6.78 ± 0.09 and E\textsubscript{max} = 66.0 ± 6, n = 9).
Moreover, quercetin and isorhamnetin (10 μmol/L) improved AngII-induced impaired ACh relaxation were unaffected by GW9662 (pD2 = 7.12 ± 0.18, n = 10; 7.43 ± 0.18, n = 10; and Emax = 92.6 ± 3.9%; 89.2 ± 3.2% in rings coincubated with quercetin in the presence or absence of GW9662, respectively; and pD2 = 7.04 ± 0.17, n = 10; 7.08 ± 0.16, n = 10; and Emax = 85.6 ± 4.5%; 86.5 ± 3.1% in rings coincubated with isorhamnetin in the presence or absence of GW9662, respectively).

**Table 2** Contractile responses to phenylephrine (1 μmol/L) and pD2 and Emax values for ACh-induced relaxation in rat aortic rings incubated with AngII (1 μmol/L) for 6 h in the presence of vehicle (DMSO), quercetin, or isorhamnetin1,2

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<thead>
<tr>
<th>Treatment</th>
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<tr>
<td></td>
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1 Calculated from data in Figure 3.
2 Values are means ± SEM. *, **Different from AngII + DMSO-treated arteries, P < 0.05 and P < 0.01.

**Figure 3** Quercetin and isorhamnetin prevent AngII-induced endothelial dysfunction in rat aortic rings. Aortic rings were incubated with or without quercetin (Quer, 1 or 10 μmol/L) or isorhamnetin (Iso, 1 or 10 μmol/L) and with or without AngII (1 μmol/L) for 6 h. (A) Left pictures show arteries incubated in the presence of DHE, which produces a red fluorescence when oxidized to ethidium by O2- and right pictures show blue fluorescence of the nuclear stain DAPI. Both types of images are merged with green elastin autofluorescence. Negative controls were obtained in the absence of DHE. (B) Values of red ethidium fluorescence normalized to blue DAPI fluorescence. Results are means ± SEM of 6–10 sections analyzed. Means without a common letter differ, P < 0.05.

**Figure 4** Quercetin and isorhamnetin prevent AngII-induced O2- overproduction in rat aortic rings. Aortic rings were incubated with or without quercetin or isorhamnetin (10 μmol/L) and with or without AngII (1 μmol/L) for 6 h. (A) Left pictures show arteries incubated in the presence of DHE, which produces a red fluorescence when oxidized to ethidium by O2- and right pictures show blue fluorescence of the nuclear stain DAPI. Both types of images are merged with green elastin autofluorescence. Negative controls were obtained in the absence of DHE. (B) Values of red ethidium fluorescence normalized to blue DAPI fluorescence. Results are means ± SEM of 6–10 sections analyzed. Means without a common letter differ, P < 0.05.

**Discussion**

AngII is a well-known trigger for increased vascular oxidative stress and the subsequent O2- driven NO inactivation plays a major role in the genesis of clinical endothelial dysfunction and hypertension (9–12). In this study, we show that AngII-induced endothelial dysfunction in rat aortic rings in vitro can be prevented by the dietary flavonol quercetin and its methylated metabolite isorhamnetin. Moreover, the flavonols also prevented AngII-induced overexpression of p47phox in the vessel media and the increase in vascular O2- production.

Quercetin prevents angiotensin II-induced endothelial dysfunction 913

Quercetin and isorhamnetin prevent AngII-induced O2- overproduction in rat aortic rings. Aortic rings were incubated with or without quercetin or isorhamnetin (10 μmol/L) and with or without AngII (1 μmol/L) for 6 h. (A) Left pictures show arteries incubated in the presence of DHE, which produces a red fluorescence when oxidized to ethidium by O2- and right pictures show blue fluorescence of the nuclear stain DAPI. Both types of images are merged with green elastin autofluorescence. Negative controls were obtained in the absence of DHE. (B) Values of red ethidium fluorescence normalized to blue DAPI fluorescence. Results are means ± SEM of 6–10 sections analyzed. Means without a common letter differ, P < 0.05.
Endothelial dysfunction is present in animals made hypertensive by infusion of AngII as well as in mice that chronically overexpress renin and angiotensinogen (9,19). The fact that AngII can also induce endothelial dysfunction in vitro in the mouse carotid artery (20) and rat aorta (this study) indicates that these changes are due to direct effects of AngII on the vessel wall, independent of circulating hormones, neurogenic mechanisms, or changes in arterial pressure. This alteration was suppressed by coincubation with the AT1 receptor antagonist losartan, demonstrating that endothelial dysfunction is mediated by activation of AT1 receptors. Chronic oral administration of quercetin reduces blood pressure and restores endothelial function in several animal models of hypertension, including SHR, NO-deficient, deoxycorticosterone acetate-salt, and Goldblat hypertensive rats (4–7). However, it was unclear whether the effects on endothelial function were due to a direct effect on the vessel wall, secondary to the blood pressure-lowering effect, or driven by neurohumoral mechanisms of quercetin or its metabolites. Our results show that both quercetin and isorhamnetin are also effective in vitro, reducing endothelial dysfunction in aortic rings. Moreover, it should be noted that the effective concentration of quercetin was as low as 1 μmol/L, which is in the range achieved in plasma after a regular meal containing flavonoid (21), indicating that this effect appears to be physiologically relevant.

Excess of \( \text{O}_2^- \) generation is critically involved in the breakdown of NO associated to endothelial dysfunction in aortic rings from AngII-infused rats (9,22). In our experiments, the presence of SOD in the organ chamber restored the relaxant response induced by ACh in aortic rings exposed to AngII. Similarly, overexpression of SOD prevented, while downregulation of SOD potentiated, AngII-induced endothelial dysfunction (20). We also found that AngII increased intracellular \( \text{O}_2^- \) production, measured by ethidium red fluorescence, especially in the smooth muscle cells of the medial layer. Furthermore, quercetin and isorhamnetin diminished the increased \( \text{O}_2^- \) production in the smooth muscle cells. In addition, the endothelium-independent vasodilation induced by the soluble guanylyl cyclase activator nitroprusside was similar in control and AngII-treated rings and unaffected by the flavonoids, indicating that endothelial dysfunction is due to changes in endothelium-derived NO bioactivity rather than downstream effects on vascular smooth muscle.

The maximal effect of AngII was observed after 6–8 h of incubation even when AngII was absent during the challenge with ACh. Similarly, quercetin and isorhamnetin were present during the exposure to AngII but absent during the endothelial function test. These slow and persistent changes induced by AngII are consistent with the involvement of changes in gene expression. Several studies have shown that NADPH oxidase is critically involved in AngII-induced endothelial dysfunction (9,15). In fact, the NADPH oxidase inhibitor apocynin, which impedes the assembly of the \( p47^{phox} \) and \( p67^{phox} \) subunits within the membrane NADPH oxidase complex, diminished endothelial dysfunction in AngII-infused mice (23), SHR (5), and AngII-exposed aortic rings in vitro (our results). These results suggest that \( \text{O}_2^- \) generated by NADPH oxidase is also involved in the alteration of aortic endothelial function induced by in vitro incubation with AngII. The \( p47^{phox} \) subunit of NADPH oxidase plays a pivotal role of in the vascular oxidant stress and blood pressure response in AngII-dependent hypertension (24,25). Moreover, AngII-induced changes in \( p47^{phox} \) expression and phosphorylation have been widely analyzed in smooth muscle and endothelial cells in culture (12,13). In this study, in aortae incubated for 6 h with AngII, we found higher protein levels of this NADPH oxidase component, measured by western blot, than in control aortae. Immunohistochemical analysis revealed that overexpression of \( p47^{phox} \) occurred mainly in the medial layer. This increased \( p47^{phox} \) protein expression is consistent with the increased \( \text{O}_2^- \) production found in aortae stimulated by AngII. Coincubation with either quercetin or isorhamnetin decreased the levels of this protein in AngII-treated aortae and the immunohistochemical staining of \( p47^{phox} \) without a significant effect in control rings. These results suggest that quercetin and its methylated metabolite decreased \( \text{O}_2^- \) production stimulated by AngII by downregulating the expression of the \( p47^{phox} \) subunit of vascular NADPH oxidase. In a recent study, we demonstrated that in SHR, the improvement of endothelial function by chronic oral administration of quercetin is associated with a reduction in the NADPH oxidase activity and \( p47^{phox} \) expression that is abnormally high in these animals as compared with normotensive Wistar-Kyoto rats (5). These results suggest that decreased NADPH oxidase derived \( \text{O}_2^- \) and,
thus, diminished NO inactivation may be an important mechanism contributing to the prevention of endothelial dysfunction by quercetin, independently of its blood pressure-lowering properties.

Quercetin has been suggested to show agonistic effects on PPARγ (26). Because the PPARγ ligands reduce \( \text{O}_2^- \) generation stimulated by AngII in human coronary artery endothelial cells (27), we hypothesized that quercetin and isorhamnetin might also prevent endothelial dysfunction via activation of these receptors. However, the PPARγ antagonist GW9662 did not affect the effects induced by quercetin and isorhamnetin, suggesting that these protective effects are unrelated to PPARγ activation. Thus, 2 potential mechanisms might be involved in the effects of quercetin. First, ROS can activate its own production via increased expression of NADPH oxidase subunits (28). Thus, quercetin and isorhamnetin, via scavenging ROS, might inhibit this positive feedback mechanism. Additionally, several protein kinases (e.g. protein kinase C, mitogen-activated protein kinases, and Src) have been reported to be involved in AngII-induced activation of NADPH oxidase (29). Because quercetin and related flavonoids are broad protein kinase inhibitors (30), it might also be a possible role of protein kinase inhibition in preventing AngII-induced endothelial function.

Taken together, these results indicate that quercetin and isorhamnetin prevent AngII-induced endothelial dysfunction by inhibiting the overexpression of p47phox and the subsequent increased \( \text{O}_2^- \) production resulting in increased response to NO.

**Literature Cited**


