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ORIGINAL ARTICLE

Quercetin and catechin synergistically inhibit angiotensin II-induced redox-dependent signalling pathways in vascular smooth muscle cells from hypertensive rats

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Abstract

Dietary flavonoids, present in different amount in foods, are associated with the prevention of hypertension, but little is known about the interactions between them. The aim of this study was to explore the effect of quercetin (Q), catechin (C) and the mixture, on Angiotensin II (AngII)-induced redox-dependent signalling pathways and cell behaviour. Mesenteric smooth muscle cells (MesSMC) from spontaneously hypertensive rats (SHR) were incubated with AngII (0.1 μmol/L) alone, or with the mixture of low concentrations of Q and C. AngII-increased ROS production was reduced by the mixture of separately ineffective low concentration of Q (15 μmol/L) plus C (20 μmol/L). This mixture reduced AngII-stimulated NAD(P)H oxidase activation and p47phox translocation to the cell membrane, without affecting Nox2 expression. Co-incubation of Q + C significantly inhibited AngII-induced migration and proliferation, and these effects were independent of p-ERK1/2 and related with reduced p38MAPK phosphorylation. These findings demonstrated that low concentrations of singly non-effective flavonoids when are combined exert a synergistic effect in inhibiting AngII-induced redox-sensitive signalling pathways.

Keywords: *quercetin, catechin, angiotensin II, oxidative stress, smooth muscle cells, hypertension*

Introduction

In recent years, it has become important to study the beneficial effects of consumption of natural antioxidants contained in food. Antioxidants and agents that interrupt the production of reactive oxygen species (ROS), reverse vascular remodelling, improve endothelial function [1], reduce inflammation [2] and decrease blood pressure in experimental models of hypertension [3]. It has been shown through epidemiological and basic studies that these substances may promote endothelium-dependent vasodilatation in aorta and human coronary arteries [4,5].

Among the components with antioxidant capacity, dietary flavonoids compounds such as quercetin (Q) and catechin (C) are considered to have beneficial effect on human health [2,6]. Flavonoids have ubiquitous distribution and are commonly present and/or are consumed together in food. However, the interactions between them are not yet completely established.

Hypertension is associated with vascular changes characterized by endothelial dysfunction, altered vascular tone, vascular remodelling and mechanical alterations [7,8]. Vascular smooth muscle cells (VSMCs), due to its dynamic characteristics and multifunctional plasticity, are involved in these

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processes [9]. Angiotensin II (AngII) plays an important role in endothelial dysfunction, vascular damage [10] and ROS generation [11], through the activation of NAD(P)H oxidase complex expressed by endothelial cells, VSMCs and adventitial fibroblasts. ROS regulate vascular function by modulating cell growth, apoptosis, migration, inflammation, secretion and extracellular matrix production [12,13].

The aim of this study was to determine if pure dietary flavonoids Q and C may regulate the proliferative and migrant response of VSMCs to AngII, through the modification of the activity and/or expression of NAD(P)H oxidase. We also assessed whether the signalling pathways activated by AngII, MAPK ERK1/2 and p38MAPK, are modified by the action of the flavonoids. The study of biological interactions of Q and C is important to demonstrate that these products could prevent and/or attenuate some vascular changes that occur in hypertension.

Material and methods

Materials

Antibodies were purchased from Santa Cruz Biotechnology: p-ERK1/2 (sc-7383, reactive with Tyr-204-phosphorylated ERK1 and ERK2), and ERK2 (sc-154, reactive with ERK2 and, to a lesser extent, ERK1), p47 (sc-14015), Nox2 (54.1; sc-1305) and Actin (sc-3225). p-p38MAPK (4511) was purchased from Cell Signalling. 5-(and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) was purchased from Molecular Probes, Inc.Eugene, Oregon. AngII and apocynin were purchased from Calbiochem (Merck Darmstadt, Germany). Q and C were provided by Sigma (St. Louis, MO, USA).

Cell cultures

Vascular SMCs derived from mesenteric arcade (MesSMC) obtained from 12-week-old male, spontaneously hypertensive rats (SHR) were isolated according to a technique previously described [14]. Briefly, the mesenteric arcades were digested with 2 mg/mL collagenase, 0.15 mg/mL elastase, 2 mg/mL bovine serum albumin and 0.35 mg/mL soybean trypsin inhibitor in Ham's F-12 medium. MesSMCs were cultured in DMEM/F12 containing 10% foetal calf serum (FCS), L-glutamine and antibiotic-antimicotic mixture at 37°C under humid and 5% CO_2-air conditions. Early passage cells were used. MesSMCs at 80–90% confluence were rendered quiescent by serum deprivation (0.1% FCS) for 24 hour prior to experimentation.

Measurement of ROS in MesSMCs

Intracellular ROS levels were measured with the fluoroprobe CM-H_2DCFDA in unstimulated cells and in cells exposed to AngII (0.1 μmol/L) in the absence or 1-hour pre-treatment of Q (15, 30, 60 μmol/L), C (20, 50, 100 μmol/L) or the combination Q (15 μmol/L) + C (20 μmol/L) (i.e. Q15 + C20). Cells were loaded with CM-H_2DCFDA (6 μmol/L), dissolved in DMSO and incubated 45 minutes at room temperature. Fluorescence was measured continuously for 15 minutes on a microplate fluorometer (Fluoroskan Ascent, Labsystems) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

Measurement of NAD(P)H oxidase activity

MesSMCs were exposed to AngII (0.1 μmol/L) in the absence and presence of Q (15 μmol/L), C (20 μmol/L) or the combination Q15 + C20 for 1-hour pre-treatment. MesSMC were lysed in a buffer containing 20 mmol/L monobasic potassium phosphate (pH 7.0), 1 mmol/L ethylene glycol tetraacetic acid (EGTA), Protease Inhibitor Cocktail (Calbiochem), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 10 mmol/L NaF, 1 mmol/L VO_4Na_3 and 1 mmol/L dithiothreitol (DTT). Cell lysates were centrifuged at 21 000 g for 20 minutes at 4°C. Plasma membrane fractions were measured in by lucigenin chemiluminescence assay using 5 μmol/L lucigenin (Sigma) and 1 mmol/L β-nicotinamide adenine dinucleotide phosphate-oxidase (β-NAD(P)H) (Sigma) as described previously [12]. Chemiluminescence as relative light units was measured in a microtiter luminometer (Fluoroskan Ascent, Labsystems) as an indicator of enzyme activity.

Cell migration assay

MesSMC migration was assessed by the scrape-wound migration assay described by Pukac [15] with some modifications. Cells were cultured in 24-well tissue culture plates until confluence and then rendered quiescent for 24 hour before the experiments. All the experiments were performed in the presence of hydroxyurea to prevent cell proliferation. Before stimulation, a gap was made in confluent cultured MesSMC in 24-well plates, using a sterile tip. Under inverted microscopy, video images of selected fields were obtained at the beginning of the assay (0 hour) and after 24 hours of incubation with AngII (0.1 μmol/L) alone or AngII plus Q (15, 30, 60 μmol/L), C (20, 50, 100 μmol/L) or the combination Q15 + C20. In order to determine if ROS derived from NAD(P)H oxidase were involved in AngII-stimulated migration, Apocynin (50 μmol/L), an inhibitor of NAD(P)H oxidase, was used. Dulbecco's Modified Eagle Medium: Nutrient Mixture F–12 (DMEM/F–12) with 0.1% FCS was used as control. Migration was determined by the difference between the cell-free area at 0 and 24 hours at four marked fields on each well.

Cell proliferation

Cell proliferation was measured quantitatively by using CellTiter non-radioactive cell proliferation Assay (Promega). Briefly, cells were seeded in 96-well plates (1×10^4 cells per well), and left to adhere to the plastic plates overnight before being exposed 48 hour to 0.1% FCS/DMEM/F12 to synchronize the culture. Cells were stimulated with AngII (0.1 μmol/L) alone or plus Q (15 μmol/L), C (20 μmol/L) or the combination Q15 + C20. DMEM/F12 with 0.1% FCS was used as control. After 24 hour exposure, 50 μL of 3-[4,5-dimethylthiazol-2—yl]-2,5-diphenyl tetrazolium bromide (MTT) solution (5 mmol/L) was added to each well, and the cells were incubated in dark at 37°C for 4 hours. Thereafter, MTT was removed; the formazan crystals were dissolved in 200 μL of Phosphate Buffered Saline (PBS). The absorbance was measured at 570 nm using a microplate reader (Molecular Devices, Spectra MAX 250). The results are expressed as relative cell proliferation, established as 1 the value obtained with 0.1% FBS.

Western blot analysis

Western blot analysis for pERK1-2 and p-p38 MAPK were performed in the cytosolic fractions and p47phox and Nox2 were performed on both the plasma membrane and cytosolic fractions. The fractions were prepared as described previously but with modifications [16]. Briefly, MesSMCs were lysed in buffer containing 20 mmol/L Tris–HCl, 10 mmol/L EGTA, 2 mmol/L EDTA, 2 mmol/L DTT, 1 mmol/L PMSF and Protease Inhibitor Cocktail (Calbiochem). Cell lysates were subjected to three cycles of freezing and thawing, and then centrifuged at 21 000 g for 20 minutes at 4°C. The supernatant was collected and designated cytosolic fraction. The pellets were resuspended in lysis buffer and designated membrane fraction. Immunoblot analyses of NOX2 (expressed in membrane but not in cytosol) were used to check the purity of the membrane and cytosolic extracts obtained.

Statistical analyses

Data are presented as mean ± SEM of (n) independent experiments. The statistical significance was assessed by one-way analysis of variance (ANOVA) and Bonferroni's post-hoc test using Prism-4 software. P value < 0.05 was considered significant.

Results

Effect of Q and C on oxygen reactive species production stimulated by AngII

AngII significantly increased CMH_2-DCFDA fluorescence and responses were sustained for up 15 minutes (Figure 1) as described previously [17]. Apocynin, an inhibitor of NAD(P)H oxidase; completely inhibited AngII-stimulated responses, indicating that flavin-containing enzyme, such NADH/NADPH oxidase, is the source of intracellular ROS. Pre-exposure of MesSMC to high concentrations of Q (30 and 60 μmol/L; Figure 1A) and C (100 μmol/L; Figure 1B), significantly reduced AngII-mediated ROS generation as far as apocynin did. Co-incubation of low concentration of Q plus C, which have no effect separately, significantly inhibited AngII-induced ROS production (Figure 1C), coinciding with a synergic effect.

Effect of Q and C on AngII-mediated activation of NAD(P)H oxidase

Knowing the inhibitory effect of antioxidants on ROS production, we were interested to know whether these antioxidants altered the activation of NAD(P)H oxidase complex. Figure 2 shows that treatment with AngII (0.1 μmol/L) significantly increased the activity of NAD(P)H oxidase compared to control. Q15 and C20 treatments showed separately no significant difference on AngII-induced NAD(P)H oxidase activation, whereas treatment with the combination Q15 + C20, significantly abolished the activity of NAD(P)H oxidase stimulated by AngII.

Then, we sought to investigate the protein levels of NAD(P)H oxidase membrane component Nox2 and the translocation of the cytosol component p47phox protein to the membrane after AngII stimulation alone or plus Q15 and C20. Nox2 expression was not affected by any of the treatments used. AngII induced the translocation of p47phox to the membrane and only the co-incubation with Q15 + C20 prevented this effect (Figure 3).

Effect of Q and C on AngII-stimulated; Cell migration

AngII mediates many of its pleiotropic vascular effects through NAD(P)H oxidase-derived reactive oxygen species (ROS), so we addressed the effect of flavonoids on AngII-mediated cell behaviour. Treatment with AngII (0.1 μmol/L) induced a significant increase of cell migration compared to control (Figure 4). Different concentrations of Q (15, 30, 60 μmol/L) caused a significant decrease in cell migration induced by AngII. Only the highest concentration of C (100 μmol/L) produced a significant reduction on AngII-stimulated cell migration. We tested the effect of combined Q15 + C20 and we found that the mixture produced a significant decrease on AngII-induced cell–migration, compared with Q15 alone, indicating that the inhibitory effect is greater with the combination of both antioxidants. AngII-stimulated migration was inhibited by apocynin (50 μmol/L), the NADPH oxidase inhibitor, implicating NAD(P)H oxidase-derived ROS in this response.

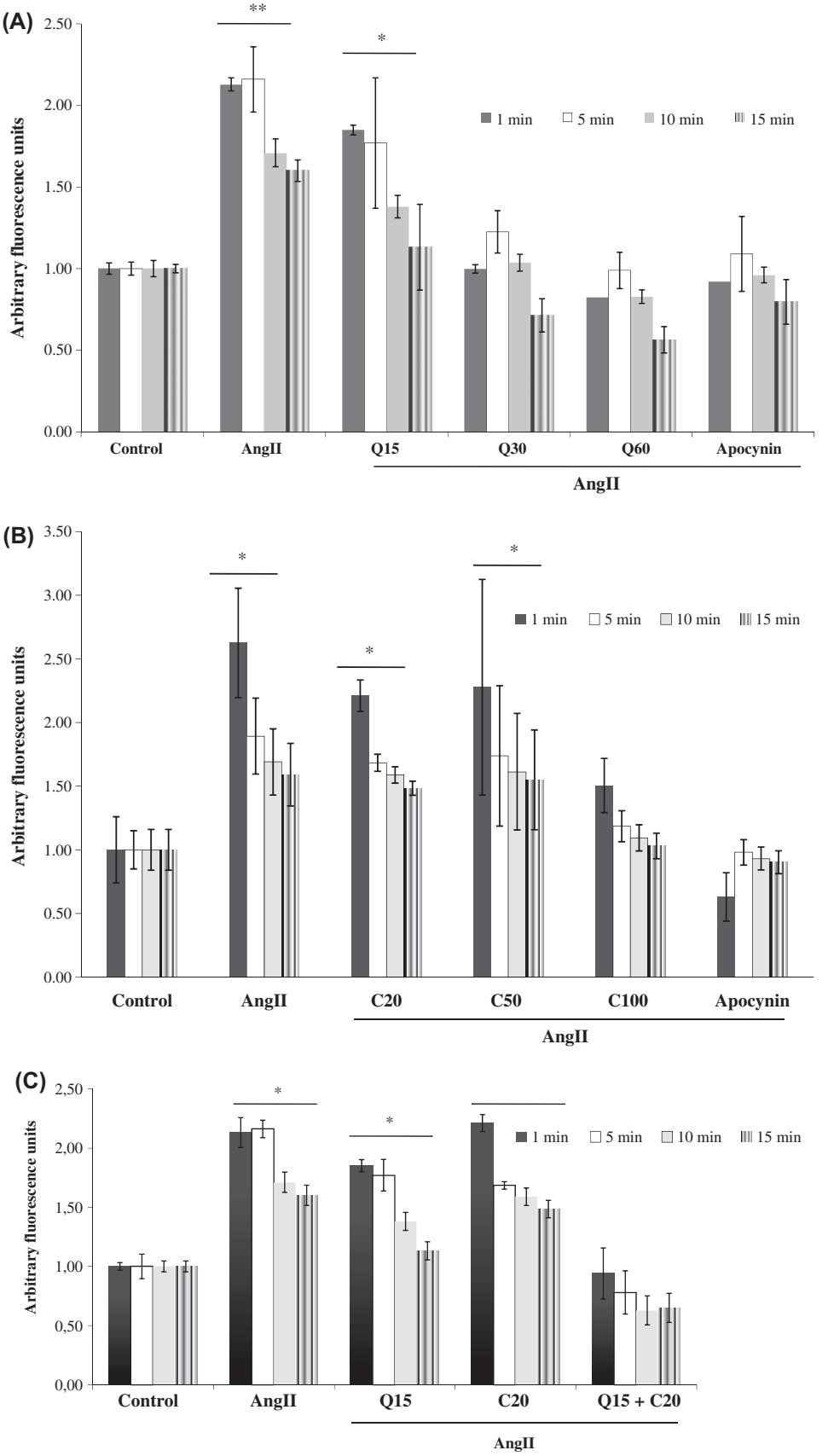

Figure 1. Quercetin (Q) and catechin (C) inhibit AngII-induced ROS generation. MesSMC were exposed to **(A)** quercetin (15, 30 and 60 µmol/L), **(B)** catechin (20, 50 and 100 µmol/L), or **(C)** the mixture Q (15 µmol/L) + C (20 µmol/L) for 1 hour before the addition of AngII (0.1 µmol/L).The generation of intracellular ROS was assessed during 15 minutes (5 minutes intervals) using CM-H2DCFDA and expressed in arbitrary units as a fold increase of the signal obtained with control cells. Apocynin, an inhibitor of NAD(P)H oxidase was used. Results are shown as Mean (± SEM) of three different experiments performed in triplicate. $^{*}p < 0.01$; $^{**}p < 0.001$ vs control at each time.

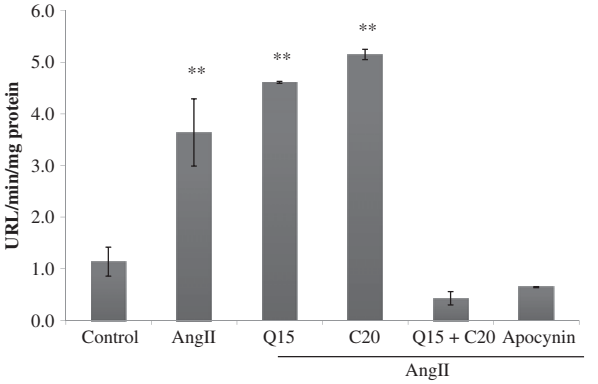

Figure 2. Effect of quercetin (Q) and catechin (C) mixture on AngII-mediated activation of NAD(P)H oxidase. Cells were treated with quercetin (Q; 15 µmol/L), catechin (C 20 µmol/L), or the mixture for 1 hour before AngII (0.1 µmol/L) addition. NAD(P)H (1 mmol/L) was added to 5 µmol/L lucigenin-treated cell homogenates and chemiluminescence was measured. Enzyme activity is expressed as luminescence relative units (URL)/min/mg protein). Bar graphs are mean ± SEM of four experiments. $^{**}p < 0.001$ vs control.

Effect of Q and C on AngII-induced MesSMC proliferation

AngII induced cell proliferation after 24 hour treatment as compared with control group (1.39 ± 0.04 vs 1.00 ± 0.09; $p < 0.01$; Figure 5). Q15 and C20 did not affect cell proliferation when used separately, but the combination significantly inhibited AngII-induced cell growth (1.39 ± 0.04 vs 1.06 ± 0.16; $p < 0.05$); suggesting an interaction between these flavonoids.

Effect of Q and C on AngII-induced intracellular signalling transduction pathways

AngII stimulates cell migration and proliferation via signalling pathways dependent on MAP kinases (mitogen-activated protein kinases). Among them, ERK1/2 and p38MAPK pathways have been involved in vascular remodelling in hypertension. Therefore, we assessed the effect of Q and C on MAPK pathways activated by AngII.

ERK1/2 phosphorylation was induced by AngII, but neither Q nor C alone nor the mixture, affected this response to AngII (Figure 6 upper panel). Instead AngII-stimulated p38MAPK activation was significantly reduced by the combination of Q15 + C20 (Figure 6, lower panel), suggesting that these antioxidants in combination inhibit AngII effects through a redox-sensitive pathway such as p38MAPK.

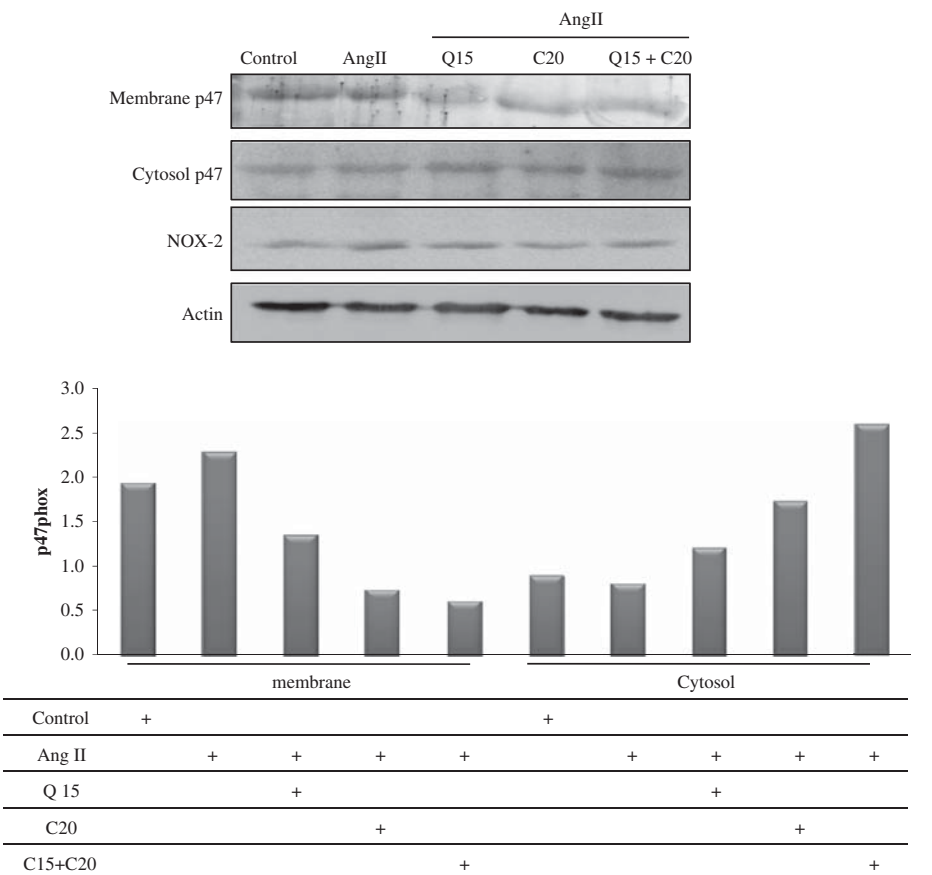

Figure 3. Quercetin (Q) and catechin (C) mixture impaired the AngII-mediated translocation of p47phox to the cell membrane without affecting Nox2 expression. Western blot analysis was performed of p47phox (in membrane and cytosol fractions), Nox2 in membrane fraction of MesSMCs following 4-hour AngII stimulation alone or with quercetin (Q; 15 µmol/L), catechin (C 20 µmol/L), or the mixture. Data from a representative experiment was quantified by densitometry analysis and represents the expression of p47 in the membrane and in the cytosol.

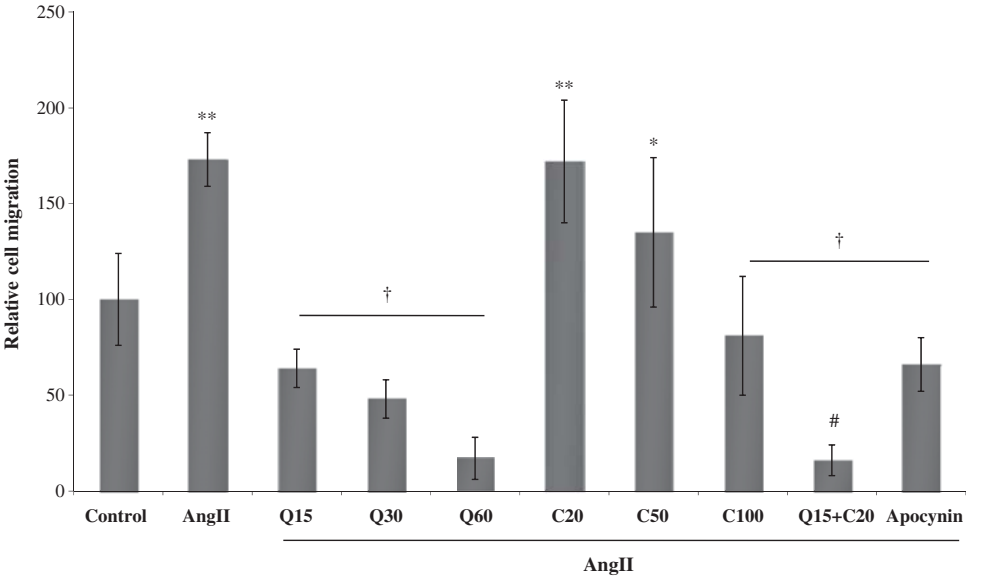

Figure 4. Quercetin (Q) and catechin (C) inhibit AngII-stimulated cell migration. MesSMCs were incubated in the presence of hydroxyurea with AngII alone, or with increasing concentrations of Q or C, and the mixture Q (15 μmol/L) + C (20 μmol/L) for 24 hour. Apocynin, a NAD(P)H oxidase inhibitor was also added. Cell migration was measured as described in Methods. Data, expressed as the mean ± SEM (n = 8), are presented as percentage of area relative to control conditions (0.1% FCS). $^{*}p < 0.05$; $^{**}p < 0.001$ vs control; $^{\dagger}p < 0.001$ vs AngII alone ; $^{\#}p < 0.01$ vs Q15 μmol/L.

Discussion

Flavonoids from different classes are commonly present together in foods and/or are consumed in combinations. In fact, prototypical flavonoid rich foods contain a large number of different flavonoids in variable amounts [18].

Q and C are the most abundant and are widely distributed flavonoids in food. The average daily intake in the occidental diet of flavonoids is estimated to be approximately 20–25 mg, with Q contributing 60–75% of the total. The average intake of C is within a similar range (approximately, 18–31 mg), with C and epicatechin being the most abundant [18].

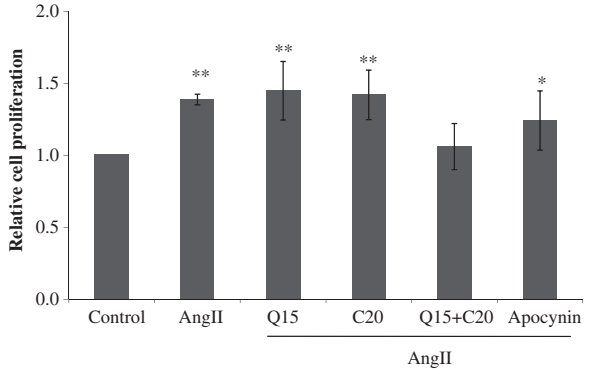

Figure 5. The combination of quercetin (Q) and catechin (C) inhibits AngII-induce cell proliferation. MesSMC were incubated with AngII containing 0, Q (15 μmol/L), C (20 μmol/L) or the mixture for 24 hours. Cell proliferation was measured by MTT assay. Data, mean ± SEM, from three different experiments $^{*}p < 0.05$, $^{**}p < 0.01$ vs control.

However, little is known about the interactions between them. We studied the effect of combined pure low concentrations of antioxidant Q and C, on the actions mediated by AngII.

In hypertension, an enhanced ROS generation occurs, which is not counterbalanced by the endogenous antioxidant mechanisms, leading to a state of oxidative stress [19]. In our study, we used smooth muscle cells obtain from mesenteric resistance arteries from spontaneously hypertensive rats. These cells contain NAD(P)H oxidase that produces ROS via activation of AngII receptor 1 [20]. It is known that inhibition of ROS generation by NAD(P)H oxidase activity by flavonoids is mainly a result of scavenging of superoxide by these compounds [21]. We demonstrated that the enhanced ROS generation in MesSMC on AngII stimulation was prevented by high concentration of Q or C. Interestingly, the combination of low concentrations of Q plus C, which separately has no effect, produced a synergistic inhibitory effect on AngII-induced ROS production. When these flavonoids were used in absence of AngII, we found no effect in ROS production and cell viability (data not shown).

NAD(P)H oxidase is composed by membrane-bound gp91phox (Nox2 homolog), catalytic subunit p22phox and regulatory subunits p47phox, p40phox, p67phox and Rac1 [22]. Smooth muscle cells from human resistance arteries expressed Nox2 and the activation of NAD(P)H oxidase, depends on the translocation of the cytosolic subunits p47phox and p67phox to the membrane [23]. In the present study, we evaluated the effect

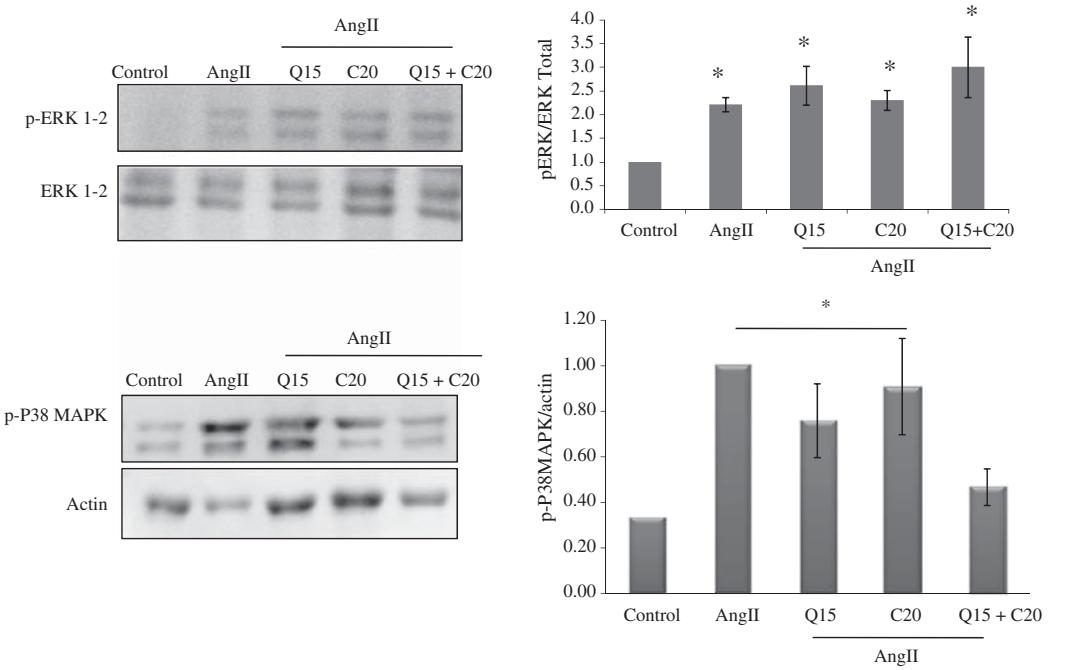

Figure 6. Effect of quercetin (Q) and catechin (C) on AngII-induced MAP Kinases signalling pathways. MesSMCs were pre-exposed 1 hour to Q (15 μmol/L), C (20 μmol/L), the mixture or vehicle and then treated with AngII for 10 minutes incubation. The levels of ERK1/2 phosphorylated (upper panel), and phospho-p38 MAPK (bottom panel) were measured using phospho-specific antibodies, and quantified by densitometry analysis. Data represents means of three independent experiments. $^{*}p < 0.05$ vs control.

of Q and C alone or combined, on the activation of NAD(P)H oxidase complex. We first found that a low concentration of Q and C separately had no effect on AngII-induced NAD(P)H oxidase activation, but together they were more effective in inhibiting the AngII-increased activity of NAD(P)H oxidase. We then determined the expression of Nox2 and the AngII-mediated translocation of p47phox to the plasma membrane. We found evidence that the mixture Q plus C inhibited the translocation of p47 to the membrane, suggesting that the observed suppression of ROS accumulation and NAD(P)H oxidase activity by these flavonoids likely involved a direct inhibition of AngII-induced NAD(P)H oxidase activation in smooth muscle cell from resistance arteries.

A large body of evidence from experimental and clinical studies unequivocally demonstrated that AngII exerts several pleiotropic deleterious vascular effects including functional and structural changes through NAD(P)H oxidase-derived ROS generation [8,19,24]. ROS act as second messengers in numerous signalling pathways. In the vascular wall, ROS are involved in the remodelling process by modulating vascular cell growth, migration and extracellular matrix production [25,26]. In this study, we investigated if Q and C together had a beneficial and cooperative effect on preventing AngII-induced vascular damage. Clear differences were detected in the protective role of both flavonoids. Our data showed that different concentrations of Q inhibited AngII-induced migration and only a high concentration of C significantly retarded AngII-stimulated migration in MesSMC. The mixture of low concentrations of Q and C exhibited a potent inhibitor effect on AngII-induced migration suggesting a synergistic effect between these two flavonoids. Similar results were found when we evaluated the effect of the mixture on AngII-induced MesSMC proliferation.

There are direct evidences that separately Q [27] and C [28] can effectively suppress the proliferation and migration of VSMCs. Previous studies showed that flavonoids such as Q and C act synergistically to inhibit platelet adhesion to collagen and collagen-induced platelet aggregation [29], but there were no synergistic interactions between Q and C in concentration-dependent relaxant effect on pulmonary smooth muscle cells [30]. Interestingly, our data show a synergistic interaction between low concentrations of Q and C, ineffective separately, in preventing AngII-mediated effects on cell migration and growth.

To study the molecular mechanisms involved in these effects, we analysed the action of Q and C in some of the signalling pathways activated by AngII, ERK 1/2 and p38MAPK. We noted that neither Q nor C separately, or its mixture, affected the phosphorylation of ERKs stimulated by AngII, instead, ineffective low concentrations of Q and C decreased AngII-induced p38MAPK phosphorylation, suggesting that in the studied conditions, the combination of these flavonoids inhibit cell migration and proliferation by a mechanism independent of ERK1/2 phosphorylation and dependent of a redox-sensitive pathway such as p38MAPK.

Polyphenolic compounds induced the redox-sensitive activation of the PI3-kinase/Akt pathway in endothelial cells [31], so it is possible that other redox-mediated signalling pathways could be involved in our model.

Our findings are in good agreement with previous evidences showing that red wine polyphenols inhibited platelet-derived growth factor beta (PDGF–BB) induced activation of p38 MAPK without affecting ERK1/2 phosphorylation [32].

The combination of C and Q elicited more profound effects on AngII-mediated actions, which was almost completely suppressed when MesSMC were treated with two flavonoids. So the question arises, why the mixture of low concentrations of Q plus C is more effective than high concentrations of flavonoids separately? It is suggested that high concentration of flavonoids such as Q have a pro-oxidant effect [33], and individually, various concentrations of C and Q produced both antioxidant and pro-oxidant effects depending on enrichment concentrations of the polyphenolic compounds [34]. In our study, when the mixture of low concentrations of phenolic compounds was used, significant antioxidant capacity was demonstrated, suggesting that these antioxidants are correlated in an interdependent or mutually supportive manner while protecting against the AngII-mediated actions.

In summary, individually-ineffective low concentrations of Q and C when are combined, act in a synergistic manner avoiding vascular damage induced by AngII. These experimental evidences could lead to consider new therapies targeted against deleterious action of AngII on vascular wall, which may be useful in minimizing organ damage in hypertension.

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Declaration of interest

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