The flavonoid quercetin induces apoptosis and inhibits JNK activation in intimal vascular smooth muscle cells

Francisco Perez-Vizcaino a,b,*, David Bishop-Bailley a, Federica Lodi b, Juan Duarte c, Angel Cogolludo b, Laura Moreno b, Lisardo Bosca d, Jane A. Mitchell e, Timothy D. Warner a

a Cardiac, Vascular and Inflammation Research, William Harvey Research Institute, Queen Mary University of London, UK
b Department of Pharmacology, School of Medicine, Universidade Complutense de Madrid, Spain
c Department of Pharmacology, School of Pharmacy, Universidad de Granada, Spain
d Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain
e National Heart and Lung Institute, Imperial College School of Medicine, London, UK

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Abstract

Quercetin, the most abundant dietary flavonol, exerts vasodilator, anti-hypertensive, and anti-atherogenic effects and reduces the vascular remodelling associated with elevated blood pressure. Here, we have compared the effects of quercetin in intimal- and medial-type rat vascular smooth muscle cells (VSMC) in culture. After 48 h, quercetin reduced the viability of a polyclonal intimal-type cell line derived from neonatal aorta but not of a medial-type cell line derived from adult aorta. These differential effects were similar in both proliferating and quiescent VSMC. Quercetin also preferentially reduced the viability of intimal-type over medial-type VSMC in primary cultures derived from balloon-injured carotid arteries. The effects of quercetin on cell viability were mainly dependent upon induction of apoptosis, as demonstrated by nuclear condensation and fragmentation, and were unrelated to PPARγ, pro-oxidant effects or nitric oxide. The expression of MAPKs (ERK, p38, and JNK) and ERK phosphorylation were not different between intimal- and medial-type VSMC. p38 phosphorylation was negligible in both cell types. Medial-type showed a weak JNK phosphorylation while this was markedly increased in intimal-type cells. Quercetin reduced JNK phosphorylation but had no consistent effect on ERK phosphorylation. In conclusion, quercetin preferentially produced apoptosis in intimal-type compared to medial-type VSMC. This might play a role in the anti-atherogenic and anti-hypertensive effects of quercetin.

Keywords: Flavonoid; Intimal; JNK; Quercetin; Vascular smooth muscle

Vascular smooth muscle growth and remodelling play a major role in the genesis and development of hypertension and atherosclerosis [1]. Vascular smooth muscle cells (VSMC) exist in different phenotypes with different morphologies, functions, and patterns of protein expression [2–4]. Elongated spindle-shaped cells predominate in the medial layer of healthy adult arteries and have contractile functions. Intimal (also referred as epithelid or p) cells are present in neonatal arteries but are re-expressed in injured vessels [4]. These cells proliferate in the intima where they form the neointima during the progression of atherosclerosis and in the restenosis that follows angioplasty. Intimal cells have lower levels of contractile proteins but express a large number of proteins which are normally scarce in spindle contractile cells including cyclooxygenase-2, plasminogen activator, PDGF-B, CYP1AI, elastin, osteopontin, matrix metalloproteinases-2, cellular retinal binding protein-1, cytokeratin, PPARγ, and retinoid receptor [3,5,6]. Due to their pathological role intimal VSMC may represent a therapeutic target in cardiovascular diseases.
Large epidemiological studies have shown that the intake of flavonols and flavones (two main classes of flavonoids) from vegetables and fruit is inversely associated with mortality from cardiovascular disease [7,8]. Quercetin, the most abundant dietary flavonol [7], exerts vasodilator and anti-hypertensive effects and reduces the vascular remodelling associated with elevated blood pressure in spontaneously hypertensive rats [9,10]. Several studies have also shown that quercetin and its glucuronized metabolite (quercetin 3-O-β-D-glucuronide) inhibit proliferation, hypertrophy, and migration of cultured VSMC in vitro when stimulated by serum or growth factors [11–14]. These inhibitory effects have been related to the interaction of quercetin with extracellular signal-regulated kinases (ERK1/2) [14] as well as c-Jun NH2-terminal kinase (JNK) [11,12] and p38 [13], members of the mitogen-activated protein kinase (MAPK) family. These kinases are activated in response to inflammatory and atherogenic stimuli, such as tumour necrosis factor α (TNF-α), interleukin-1β, angiotensin-II, phorbol esters, oxidative stress, hypertension, balloon injury, and shear stress, and stimulate the expression of several inducible proteins [15].

Interestingly, VSMC phenotypes show a differential sensitivity to growth and apoptosis induced by drugs such as nitric oxide donors and PPARγ ligands [5,16]. We hypothesized that quercetin might also induce different responses in VSMC of different phenotypes. Therefore, we have compared the effects of quercetin in intimal- and medial-type VSMC in culture.

Materials and methods

Chemicals and cell culture. All drugs, chemicals, and cell culture media were purchased from Sigma (Poole, UK) except rosiglitazone and GW80072 which were generous gifts from Glaxo SmithKline (Harlow, UK). Antibodies against ERK1/2, phospho-ERK1/2, p38, phospho-p38, JNK, and phospho-JNK were from Cell Signalling Technology. Polyclonal rat aortic VSMC lines of intimal-type WKY2s (WKY12-11, WKY12-12, and WKY12-18) and medial-type WKY3m (WKY3m-1, WKY3m-2, and WKY3m-3) derived from neonatal rat aorta and of intimal-type WKY3m (WKY3m-1, WKY3m-2, and WKY3m-3) derived from adult rat aorta were used between passage 18 and 22 [5]. Primary spindle and intimal rat carotid VSMC were grown from explants of intact and balloon-injured carotid arteries, respectively, from male Wistar rats. The phenotype was characterized morphologically and used between passage 4 and 6. All cells were propagated in DMEM containing 10% fetal bovine serum and supplemented with antibiotic/mycotic mix. Nitrites were measured in the incubation media using the Griess reaction.

Cell viability, proliferation, and apoptosis. In some experiments, VSMC (approximately 50% confluence) were grown arrested by an exposure for 24 h in serum-free medium, and then exposed to quercetin or vehicle (0.1% DMSO) in DMEM containing 10% FBS for 48 h. In another set of experiments, cells (approximately 80–90% confluence) were growth arrested for 24 h and then exposed to quercetin or vehicle in serum-free DMEM. Serum-free medium was supplemented with 0.1% bovine serum albumin. Medium in all experiments using quercetin or vehicle was supplemented with ascorbic acid (30 μM) to minimize quercetin oxidation. Cell viability was measured after 48 h of drug treatment using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay [5] and by determination of the levels of lactate dehydrogenase (LDH) in the incubation media using a commercially available kit (Roche Applied Science). Nuclear morphology was assessed after 48 h of treatment by incubating the cells with Hoechst 33258 (4 μg/ml) and propidium iodide (20 μg/ml) for 40 min at 37 °C followed by observation using a fluorescence microscope. In preliminary experiments, the maximal effect of quercetin on cell viability was observed after 48 h.

Western blot. Intimal and medial VSMC were incubated in DMEM with quercetin (30 μM) or vehicle for 90 min, washed once in cold PBS, and scrapped in lysis buffer [5]. The lysate was centrifuged and the supernatant was frozen and stored. Protein content was analyzed using the Bradford assay (Bio-Rad). Western blot analysis using specific antibodies for ERK1/2, JNK, p38MAPK, and for their phosphorylated forms (p-ERK1/2, p-JNK, and p-p38MAPK) was performed with 20 μg of the lysates as previously described [17]. Briefly, lysates were subjected to SDS-polyacrylamide (10%) gel electrophoresis and proteins were transferred to PVDF membranes. Membranes were first probed with a specific anti-phospho antibody, then stripped and probed with the corresponding specific antibody against total protein. Bands were visualized using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) and quantified by densitometry using QuantityOne software (Bio-Rad). Results obtained with anti-phosphorylated antibodies are expressed as a percentage of the corresponding full protein.

Statistical analysis. Values are expressed as means ± SEM. Comparisons of parameters between two groups were made with unpaired Student’s t test. A value of P < 0.05 was considered statistically significant.

Results and discussion

Effects of quercetin on cell viability

WKY2s and WKY3m cells are intimal and medial phenotype stable cell lines derived from rat aorta from animals with identical genetic backgrounds [5]. After 24 h of serum starvation, intimal-type (WKY2s cells) (at ~50% confluence) rapidly grew when incubated with 10% FCS (MTT values at 12, 24, and 48 h were 173 ± 15%, 186 ± 38%, and 226 ± 65% of the values at time 0, respectively; n = 3 in triplicate). In contrast, no change in cell number was observed when intimal-type cells were incubated in 0% FCS (100 ± 10% at 48 h). Quercetin produced a concentration-dependent decrease in the viability of proliferating intimal-type VSMC at 48 h but had no significant effect on proliferating medial-type cells (WKY3m cell lines) (Fig. 1).

In order to test the effects of quercetin in quiescent VSMC, cells were grown to ~80–90% confluence before the removal of FCS. Viability was then analyzed after a further 48 h (Fig. 2). As before the viability of intimal-type (WKY2s) cells was concentration-dependently reduced by quercetin whereas medial-type (WKY3m) cells were unaffected (Fig. 2A). Quercetin also increased the levels of LDH in the incubation media of intimal-type cells that paralleled the reduction in MTT values, but did not affect LDH release from medial-type VSMC (Fig. 2B). Therefore, we show for the first time that quercetin-induced decrease in VSMC viability was dependent on cell phenotype but independent on the rate of cell growth.

In medial- and intimal-type VSMC derived from balloon-injured carotid arteries the decrease in viability induced by quercetin was also phenotype-dependent (Fig. 3). Thus, quercetin preferentially reduced cell viability of intimal-type primary cells, as demonstrated by a reduction in the MTT test and a parallel increase in the levels of LDH in the incubation media. At the highest concentration...
tested quercetin did inhibit medial-type cell viability and increase LDH release. This result is in agreement with a previous study showing that quercetin reduces the viability of aortic VSMC (medial-type) in primary culture at concentrations \( \geq 100 \mu M \) [18].

Nuclear morphology

Two types of cell death, necrosis and apoptosis, have been discriminated based on cell morphology [19]. Apoptosis is characterized by alterations of nucleus morphology (chromatin condensation and nucleus fragmentation), organelle relocation, and cell fragmentation whereas necrosis is characterized by cellular swelling, organelles alterations, and leakage of the cellular components. The effects of quercetin on nuclear morphology in serum deprived intimal-type cells (WKY2s) were analyzed using the membrane impermeable nuclear dye propidium iodide which stains only membrane disrupted cells and the membrane permeable nuclear dye Hoechst 33258 which stains both healthy and membrane disrupted cells. Less than 5% cells incubated in the presence of vehicle were positive to propidium iodide but this was markedly increased in the presence of quercetin at 48 h (Fig. 4). In quercetin-treated wells, apoptotic cells clearly showing nuclear condensation and fragmentation could be observed (Fig. 4D), indicating that quercetin reduced cell viability mainly via induction of apoptosis. Apoptosis with DNA fragmentation induced by quercetin has also been demonstrated in a number of tumour and nontumour cell types [20–22].

Lack of involvement of PPAR\(\gamma\), generation of reactive oxygen species or nitric oxide

Quercetin has been suggested to show agonistic effects on peroxisome proliferator activated receptors (PPAR\(\gamma\)) [23]. Because the PPAR\(\gamma\) ligands induce preferential apoptosis in intimal-type VSMC [5], we hypothesized that quercetin might also induce apoptosis via activation of these receptors. Thus, we analyzed the effects of the PPAR\(\gamma\) agonist rosiglitazone and the PPAR\(\gamma\) partial agonist GW0072 on quercetin-induced decrease in intimal-type VSMC viability (Table 1). Rosiglitazone induced a concentration-dependent reduction in viability, which at 30 \( \mu M \) was additive with the effect of quercetin. GW0072, at a concentration of

Fig. 1. Effects of quercetin on viability in proliferating medial- (WKY3m) and intimal-type (WKY2s) VSMC. Cells were grown in 10% FCS until 50–60% confluent, then were serum-starved (0% FCS) for 24 h before being returned to 10% FCS for 48 h in the presence of vehicle (0.1% DMSO) or quercetin. Viability was measured by the MTT test. Results are means ± SEM for three different cell types, each one performed in triplicate. \(*P < 0.05\) vs medial-type.

Fig. 2. Effects of quercetin on viability in quiescent medial- (WKY3m) and intimal-type (WKY2s) VSMC. Cells were grown until 80–90% confluence and then were serum-starved (0% FCS) for 24 h before being incubated in 0% FCS for a further 48 h in the presence of vehicle (0.1% DMSO) or quercetin. (A) Viability measured by the MTT test. (B) LDH in the incubation media. Results are means ± SEM of three cell types, each one performed in triplicate. \(*P < 0.05\) vs medial-type.
1 μM, at which it behaves as a PPARγ antagonist and inhibits rosiglitazone-induced apoptosis [5], did not affect viability or responses to quercetin. These results suggest that the effects of quercetin on VMSC viability are not related to PPARγ.

Besides the well-known antioxidant effect of quercetin [24], under certain conditions it may also behave as a pro-oxidant, generating reactive oxygen species (ROS) [25,26]. In fact, the pro-apoptotic effects of high concentrations (200 μM) of quercetin in aortic VMSC have been
attributed to generation of ROS [18]. However, the antioxidant N-acetylcysteine (NAC) had no significant effect per se and did not modify the effects of quercetin (Table 1).

It should be also noted that all the experiments were performed in the presence of ascorbic acid. This is necessary to prevent the auto-oxidation of quercetin, e.g., in the absence of ascorbic acid quercetin is fully degraded in less than 24 h [26]. Therefore, it seems unlikely that under our experimental conditions, quercetin-induced decrease in viability is due to generation of ROS.

Because nitric oxide donors have also been shown to induce preferential apoptosis in intimal- vs medial-type VSMC [16], we measured the concentration of nitrites in the incubation media of intimal-type VSMC as an indicator of nitric oxide production. However, nitrites were not detected by the Griess method in either the presence or absence of quercetin for 48 h (not shown), indicating that quercetin does not decrease viability via production of nitric oxide.

**MAPK expression and phosphorylation**

MAPKs (i.e., ERK, JNK, and p38) play a central role in the regulation of VSMC proliferation, apoptosis, migration, and gene expression [15]. However, particular MAPKs have different and often contradictory functions in the control of the proliferation/apoptosis ratio [27]. While ERK activity is usually involved in proliferation, p38 and JNK may induce either apoptosis or cell survival in VSMC in culture [15,28,29]. Previous studies on the effects of quercetin on VSMC viability, migration, and proliferation have related their findings to interactions with MAPKs [11–14,18].

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**Table 1**

<table>
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<th>Treatment</th>
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<th>GW0072 1 μM</th>
<th>Rosiglitazone 30 μM</th>
<th>Rosiglitazone 100 μM</th>
<th>NAC 1 mM</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>100 ± 6</td>
<td>89 ± 2</td>
<td>67 ± 6 *</td>
<td>31 ± 23 *</td>
<td>79 ± 9</td>
</tr>
<tr>
<td>Quercetin</td>
<td>42 ± 8</td>
<td>47 ± 8</td>
<td>28 ± 4 *</td>
<td>20 ± 10</td>
<td>51 ± 8</td>
</tr>
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</table>

Results are means ± SEM of three wells from a single cell type expressed as a percent of vehicle (untreated).

* $P < 0.05$ vs vehicle.

# $P < 0.05$ vs no treatment.

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Fig. 5. Expression and phosphorylation of the MAP kinases (ERK1/2, p38, and JNK) in medial-type (med, WKY3m cell line) and intimal-type (int, WKY2s) VSMC. Cells were incubated in the presence of vehicle (C) or quercetin (Q, 30 μM) for 90 min. Representative Western blots are shown in (A). (B) MAPK expression expressed as a percent of the data in intimal-type cells. (C) Phosphorylated MAPK relative to total MAPK expressed as a percent of the data in intimal-type cells. Results are means ± SEM of two (medial) or three (intimal) cell types performed in duplicate. * $P < 0.05$ medial vs intimal type, # $P < 0.05$ quercetin vs vehicle.
et al. [14] found that quercetin inhibited ERK1/2 phosphorylation in response to TNFα in human aortic VSMC but did not analyze other MAPKs. Alcocer et al. [13] found inhibition of p38 phosphorylation and again did not analyze other MAPKs. Yoshizumi et al. [11] found that quercetin specifically inhibited angiotensin II-induced JNK activation, but not ERK and p38 activation in rat aortic VSMC. Interestingly, Yoshizumi et al. [12] later found that quercetin-3-O-β-p-glucuronide, the main circulating plasma form of quercetin, also potently inhibited JNK activation. On the contrary, Shih et al. [18] found increased JNK activation at high quercetin concentrations, which was related to its pro-oxidant effects.

Therefore, we compared the patterns of MAPKs expression and phosphorylation in intimal- and medial-type VSMC and the effects of quercetin. We observed a high ERK expression in some medial-type cells (e.g., Fig. 5A) but, on average, differences did not reach statistical significance when comparing medial- and intimal-type cells (Fig. 5B). The expression of p38 and JNK was similar in the two cell types. As expected, after 90 min incubation with quercetin there were no changes in the expression of MAPKs. ERK phosphorylation was also variable within medial-type cells, but on average there were no differences between cell types and they were similarly unaffected by quercetin treatment. p38 phosphorylation was negligible in either cell type. Interestingly, medial-type cells also showed a weak JNK phosphorylation, but this was markedly more prominent in intimal-type cells (Fig. 5A and C). In addition, JNK was up-regulated in unstable human carotid plaque regions [29] and in balloon-injured mouse carotid arteries [30,31]. Furthermore, dominant negative mutants of JNK [32] or JNK inhibition [33] reduced intimal hyperplasia in balloon-injured rat artery rats. Taken together, these results suggest that JNK activation plays an important role in intimal hyperplasia and that JNK may represent a therapeutic target for intimal proliferation. In agreement with the data of Yoshizumi et al. [11,12], we found that quercetin reduced JNK phosphorylation in both cell types while having no consistent effect on ERK phosphorylation. Thus, our data are consistent with the view that JNK represents a pro-survival signal in intimal-type VSMC and that its inhibition by quercetin leads to apoptosis.

Conclusion

Quercetin produced a preferential decrease in cell viability in intimal-type compared to medial-type VSMC in both established aortic cell lines and primary cultures from rat balloonod carotid arteries. The pattern of nuclear condensation and fragmentation indicated that reduced viability was mainly due to apoptosis. Intimal-type VSMC showed increased JNK activation which was inhibited by quercetin. These effects might play a role in the anti-atherogenic and anti-hypertensive effects of quercetin.

Acknowledgments

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References


