

## **Divergent effects of quercetin conjugates on angiogenesis**

Sandra Donnini, Federica Finetti, Lorenzo Lusini, Lucia Morbidelli, Veronique Cheynier, Denis Barron, Gary Williamson, Johannes Waltenberger, Marina Ziche

► **To cite this version:**

Sandra Donnini, Federica Finetti, Lorenzo Lusini, Lucia Morbidelli, Veronique Cheynier, et al.. Divergent effects of quercetin conjugates on angiogenesis. *British Journal of Nutrition*, Cambridge University Press (CUP), 2006, 95 (5), pp.1016-1023. 10.1079/BJN20061753 . hal-02668169

**HAL Id: hal-02668169**

**<https://hal.inrae.fr/hal-02668169>**

Submitted on 26 May 2021

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## Divergent effects of quercetin conjugates on angiogenesis

Sandra Donnini<sup>1</sup>, Federica Finetti<sup>1</sup>, Lorenzo Lusini<sup>1</sup>, Lucia Morbidelli<sup>1\*</sup>, Veronique Cheynier<sup>2</sup>, Denis Barron<sup>3</sup>, Gary Williamson<sup>4†</sup>, Johannes Waltenberger<sup>5</sup> and Marina Ziche<sup>1</sup>

<sup>1</sup>Section of Pharmacology, Department of Molecular Medicine, University of Siena, Via A. Moro 2, 53100 Siena, Italy

<sup>2</sup>Unité Mixte de Recherche Sciences pour l'œnologie, Equipe Polyphénols, INRA, 2, Place Viala, 34060 Montpellier Cedex, France

<sup>3</sup>Nestle Research Center, Vers-Chez-Les-Blanc, PO Box 44, CH-1000 Lausanne 26, Switzerland

<sup>4</sup>Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, UK

<sup>5</sup>Department of Cardiology, University Hospital and Cardiovascular Research Institute (CARIM), 6202 AZ Maastricht, The Netherlands

(Received 10 November 2005 – Revised 18 January 2006 – Accepted 20 January 2006)

The present study reports the activities of quercetin and its main circulating conjugates in man (quercetin-3'-sulphate (Q3'S) and quercetin-3-glucuronide (Q3G)) on *in vivo* angiogenesis induced by vascular endothelial growth factor (VEGF) and examines the effects of these molecules on cultured endothelial cells. We found opposing effects of quercetin and its metabolites on angiogenesis. While quercetin and Q3G inhibited VEGF-induced endothelial cell functions and angiogenesis, Q3'S *per se* promoted endothelial cell proliferation and angiogenesis. The inhibitory effect elicited by Q3G was linked to inhibition of extracellular signal-regulated kinases 1/2 (ERK1/2) phosphorylation elicited by VEGF. The activation of endothelial cells by Q3'S was associated to stimulation of VEGF receptor-2 and to downstream signalling activation (phosphatidylinositol-3 kinase/Akt and nitric oxide synthase pathways), ultimately responsible for ERK1/2 phosphorylation. These data indicate that the effects of circulating quercetin conjugates on angiogenesis are different depending on the nature of the conjugate. Q3G and Q3'S are the two major conjugates in plasma, but their ratio is dependent on several factors, so that inhibition or activation of angiogenesis could be subtly shifted as a result of metabolism *in vivo*.

**Angiogenesis: Quercetin: Quercetin conjugates: Microvascular endothelial cells: Proliferation: Mitogen-activated protein kinase**

Quercetin, like other natural polyphenols, has attracted attention for the variety of biological properties that it appears to possess. Predominant among these are the antioxidant and antitumour activities that have been extensively described. The antitumour effects of quercetin have been attributed to its ability to interfere with tumour vascularisation through inhibition of endothelial cell growth and migration (Igura *et al.* 2001; Tan *et al.* 2003). At the molecular level, quercetin reduces the expression and activity of matrix metalloproteinase-2, inhibits constitutive endothelial nitric oxide synthase (Chiesi and Schwaller, 1995; Tan *et al.* 2003), while other flavonoids have been demonstrated to inhibit vascular endothelial growth factor (VEGF) induced endothelial cell functions and signalling pathways (Kimura and Okuda, 2001; Hasebe *et al.* 2003; Lin *et al.* 2003; Bagli *et al.* 2004; Tseng *et al.* 2004).

Most, but not all, epidemiological studies on quercetin have shown that quercetin is protective against several different types of cancer (Hertog *et al.* 1993; Knekt *et al.* 1996,

2002; Garcia-Closas *et al.* 1998, 1999; Neuhaus, 2004; McCann *et al.* 2005). However, there are very few human intervention studies on the chemopreventive effects of quercetin, and so the effect in man *in vivo* is not proven. *In vitro*, many studies have used the aglycone of quercetin, which is not found in significant amounts *in vivo*, and so it is important to design *in vitro* experiments based on knowledge from *in vivo* metabolism (Williamson, 2003).

Quercetin is mainly absorbed after deglycosylation, and is rapidly metabolised to glucuronides and sulphates during and after intestinal absorption, which therefore constitute the major circulating forms of this polyphenol (Gee *et al.* 2000; Day *et al.* 2001; O'Leary *et al.* 2001). Thus, although much of the experimental work describes the pharmacodynamic activities of quercetin, there is substantial evidence that the molecule is very rapidly conjugated to form glucuronide and sulphate derivatives, which in fact predominate in human plasma after administration of quercetin-rich foods, while only traces of the parent molecule may be found (Day *et al.*

**Abbreviations:** BCS, bovine calf serum; CVEC, coronary venular endothelial cells; ERK1/2, extracellular signal-regulated kinases 1/2; PAEC, porcine aortic endothelial cells; PI3K, phosphatidylinositol-3 kinase; Q3'S, quercetin-3'-sulphate; Q3G, quercetin-3-glucuronide; VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor-2.

\* **Corresponding author:** Dr Lucia Morbidelli, fax +39 0577 234343, email morbidelli@unisi.it

† **Present address:** Nestle Research Center, Vers-Chez-Les-Blanc, PO Box 44, CH-1000 Lausanne 26, Switzerland.

2001; O'Leary *et al.* 2001). Two studies have clearly shown that the major quercetin conjugates are quercetin-3'-sulphate (Q3'S) and quercetin-3-glucuronide (Q3G), but the ratio in plasma in these studies was different. For one study, Q3'S > Q3G with a ratio of 3:1 after 90 min (de Pascual-Teresa *et al.* 2004), and for the other, Q3'S < Q3G with a ratio of 1:1.6 after 90 min (Day *et al.* 2001). Therefore, any differences in biological activity of quercetin conjugates would lead to an overall effect of quercetin depending on its metabolism. This metabolism would be directly affected by the ratio of the enzymes UDP-glucuronosyl transferase and sulphotransferase, which are both known to be inducible and to vary between individuals (Burchell & Coughtrie, 1997). The aim of the present study was to elucidate the activity of the two most abundant conjugates.

The present study reports the activities of quercetin and its circulating conjugates, Q3G derived from intestinal metabolism and Q3'S derived from liver metabolism, on angiogenesis induced by VEGF and examines the effects of these molecules on microvascular endothelial cells.

## Materials and methods

### *Angiogenesis in vivo: rabbit cornea assay*

Angiogenesis was studied in the cornea of albino rabbits and quantified by stereomicroscopic examination (Morbidelli *et al.* 2003; Bagli *et al.* 2004). Experiments have been performed in accordance with the guidelines of the European Economic Community for animal care and welfare (EEC Law no. 86/609). Test substances were incorporated in slow-release pellets prepared from a casting solution of ethynil-vinyl copolymer (Elvax-40; DuPont-De Nemours, Wilmington, Delaware, USA). Pellets were then implanted into micropockets surgically produced in the lower half of the eye of anaesthetised New Zealand white rabbits (fifteen animals; Charles River, Lecco, Italy). Compounds were delivered alone or in combination from one single micropocket. A concentration of 1 µg/pellet polyphenol metabolites corresponded to a concentration in the low micromolar range during the first 48 h after pellet implant. Observation and quantification of the angiogenic response were performed by a slit-lamp stereomicroscope. The potency of angiogenic activity was evaluated on the basis of the number and growth rate of newly formed capillaries, and an angiogenic score was calculated by the formula (vessel density × distance from limbus) as described in Morbidelli & Ziche (2004).

### *Cell culture*

Bovine post-capillary coronary venular endothelial cells (CVEC) were obtained and cultured as previously described (Ziche *et al.* 1997). Cells between passages 18 and 20 retaining endothelial markers were used in the experiments.

Porcine aortic endothelial cells (PAEC) overexpressing vascular endothelial growth factor receptor-2 (VEGFR-2; Waltenberger *et al.* 1994) were maintained with Ham's F-12 medium supplemented with 10% fetal calf serum (Hyclone, Inalco, Milan, Italy) and 500 µg/ml G418 sulphate antibiotic. PAEC-VEGFR-2 cells were split weekly 1:3.

### *Cell proliferation*

Cells resuspended in 10% serum ( $1.5 \times 10^3$ ) were seeded in each well of ninety-six multiwell plates. After adherence (5–6 h), the supernatant was replaced with medium containing 0.1% bovine calf serum (BCS; Hyclone) for 24 h to synchronise the cell cycle (Morbidelli *et al.* 2003). After 48 h incubation with test substances in medium containing 0.1% BCS, cells were fixed in 100% methanol and stained with Diff-Quik (Mertz-Dade; Biomap, Milan, Italy). Total cell number per well was counted at 100 × magnification.

To determine possible toxicity of quercetin metabolites on CVEC, the trypan blue exclusion test was used as previously reported (Morbidelli *et al.* 2003).

### *Cell migration*

Cell migration was assessed in forty-eight-well microchemotaxis chambers (NeuroProbe; Biomap) on a polycarbonate filter, 8 µm pore size, coated either with type I collagen or fibronectin as reported (Morbidelli *et al.* 2003). A cell suspension containing 12 500 cells exposed to 1 nM-quercetin metabolites was added to the upper chamber of each well and VEGF (20 ng/ml) to the lower one. Migrated cells were counted in ten random fields per well at 400 × magnification. Data are reported as number of migrated cells counted per well.

### *Immunoprecipitation and Western blotting*

Cells ( $3 \times 10^5$ ) were plated in 60 mm diameter dishes. After adhesion, cells were serum-starved overnight and exposed to VEGF (20 ng/ml) for 10 min (Cantara *et al.* 2004). In combination experiments polyphenols (10 nM) were administered 30 min before VEGF. Cells were then lysed. Supernatant (5 µl) from each lysate was used for protein assay. To evaluate extracellular signal-regulated kinases 1/2 (ERK1/2) and phosphatidylinositol-3 kinase (PI3K)/Akt activation, 30 µg proteins were mixed with 4 × reducing SDS-PAGE sample buffer and denatured at 100°C for 10 min. Electrophoresis was carried out in SDS-10% polyacrylamide gel. Proteins were then blotted on to activated nitrocellulose membranes, incubated overnight with the antibodies (anti-phospho-ERK1/2 or anti-phospho-Akt) and then detected by an enhanced chemiluminescence system (Amersham, Milan, Italy). Results were normalised to those obtained by using an antibody to total ERK1/2 or actin. For VEGFR-2 activation, PAEC-VEGFR-2 cells were treated as described earlier and lysed. VEGFR-2 was immunoprecipitated overnight by using anti-VEGFR-2 antibody. Immunocomplexes were bound to protein A-Sepharose and washed three times. Immunoprecipitates were resolved on SDS-PAGE (8%) and proteins were transferred to nitrocellulose membranes. Phosphorylated VEGFR-2 was evidenced by using anti-phospho-tyrosine antibody and the detection system as described earlier.

### *Measurement of cGMP levels*

cGMP levels were measured on cell extracts from confluent cell monolayers by enzyme immunoassay kit (Cayman; Cabru, Milan, Italy). Cell monolayers were treated

with 1 mM-3-isobutyl-5-methyl-xanthine for 15 min before stimulation (Cappelli *et al.* 2004). After stimulation, cells were rinsed with PBS and removed by scraping in ice-cold 60% ethanol (v/v). Following ethanol evaporation, cGMP levels were assayed in the supernatant according to the manufacturer's instructions, while proteins were measured in the pellet by Bradford's procedure. Data are expressed as pmol cGMP per mg protein.

#### Statistical analysis

Results are expressed as means with their standard errors. Statistical analysis was performed using Student's *t* test and ANOVA. When a significant difference was detected, multiple comparison analysis was performed using the Student–Newman–Keuls test. A value of  $P < 0.05$  was considered to denote statistical significance.

#### Reagents

Quercetin was purchased from Sigma-Aldrich (Milan, Italy). Q3'S was prepared as described by Day *et al.* (2001). Q3G was purified from grape skins (*V. vinifera* var. Muscat of Frontignan) using the following procedures. Skins were peeled and extracted with methanol containing 2% acetic acid during 4 h at room temperature. After filtration and concentration of the extract under reduced pressure, purification was achieved by chromatography on Fractogel TSK HW 50F. Elution was carried out with water (pH 7) and monitored by HPLC coupled to a diode array detector and an electrospray ionisation–MS detector. The fraction containing flavonol glucuronides was then concentrated and stored at  $-20^{\circ}\text{C}$  until freeze-dried. Final purification was achieved by chromatography on a polyamide column followed by HPLC on a C18 column, using a procedure up-scaled from Cheynier & Rigaud (1986).

Q3G was identified and its purity checked by HPLC–ESI–MS and NMR analysis. Mass signal at  $m/z$  477 in the negative ion mode;  $^1\text{H}$  NMR (500 MHz, dimethyl sulphoxide):  $\delta$  3.22–3.35 (2H, m H2-glcA, H3-glcA), 3.39 (1H, dd, 4-glcA), 3.58 (1H, d,  $J_{4,5}$  9.9, 5-glcA), 5.49 (1H, d,  $J_{1,2}$  7.6, 1-glcA), 6.41/6.21 (2H, d,  $J_{6,8}$  1.7, H6/H8), 6.84 (1H, d,  $J_{5',6'}$  8.3, H5'), 7.60 (1H, dd,  $J_{5',6'}$  8.3,  $J_{2',5'}$  1.6, H6'), 7.54 (1H, d,  $J_{2',5'}$  1.6, H2'). The position of the glucuronide was confirmed by heteronuclear multiple bond correlation from long-distance correlation between the carbon at C3 (133.3 ppm) and the H1-glcA (5.49 ppm).

A stock solution of 10 mM was prepared in dimethyl sulphoxide (free quercetin) or methanol (Q3'S and Q3G) and stored in aliquots at  $-80^{\circ}\text{C}$ . Free quercetin was dissolved in methanol for pellet preparation. VEGF was obtained from Peprotech (Inalco, Milan, Italy). The PI3K inhibitor LY294002, the mitogen-activated protein kinase kinase inhibitor U0126, the nitric oxide synthase inhibitor  $\text{N}^{\omega}$ -methyl-L-arginine and the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one were provided by Calbiochem (Inalco, Milan, Italy). Anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-Akt and anti-phospho-tyrosine antibodies were obtained from Cell Signalling (Celbio S.p.A., Milan, Italy). Anti-VEGFR-2 antibody was from Upstate Biotechnology (DBA, Milan, Italy). Anti- $\beta$ -actin was from Sigma-Aldrich.

## Results

### *Quercetin metabolites differentially affect angiogenesis in vivo*

We designed experiments to analyse the interaction between quercetin, its metabolites and VEGF on angiogenesis in the avascular rabbit cornea. The assay provides integrated information on the ability of molecules either to promote new capillary formation or to influence the response to angiogenic growth factors (Morbideilli & Ziche, 2004). VEGF (200 ng/pellet) elicited a florid growth of new capillaries, which developed during time, and gradually invaded the corneal stroma, giving an angiogenic score of 6 (Fig. 1(a)). In contrast, neither quercetin nor its metabolites (tested *per se* at 1  $\mu\text{g}$ /pellet) were capable of inducing formation of new capillaries (Fig. 1(a)). No inflammatory responses were observed in these implants.

When quercetin and its metabolites were delivered to the cornea simultaneously with VEGF, we observed a marked and significant reduction ( $P < 0.01$ ) of the angiogenic response to VEGF for free quercetin and its glucuronide metabolite Q3G (Fig. 1(b–d)). On the contrary, Q3'S exerted no inhibition, with a slight not significant potentiation of VEGF response (Fig. 1(b, c, e)).

### *Quercetin metabolites exert a dual role on endothelial cell proliferation and migration*

The influence of polyphenols on VEGF-induced formation of capillaries prompted us to study their effects on CVEC, as the activation of their basic functions, i.e. proliferation and migration, which are the prerequisite for the expression of the angiogenic phenotype. In quiescent endothelial cells (0.1% BCS) Q3'S induced cell growth (Fig. 2(a)). When expressed in terms of the response attainable in these cells with 0.1% BCS medium, Q3'S increased proliferation by 40% at 1 nM ( $P < 0.01$ ) and 25% at 1 pM (Fig. 2(a)), being the proliferative effect of VEGF of 43 (SEM 2)% over basal response. Both quercetin and its glucuronide exerted no effect on proliferation at sub-nanomolar concentrations (Fig. 2(a)).

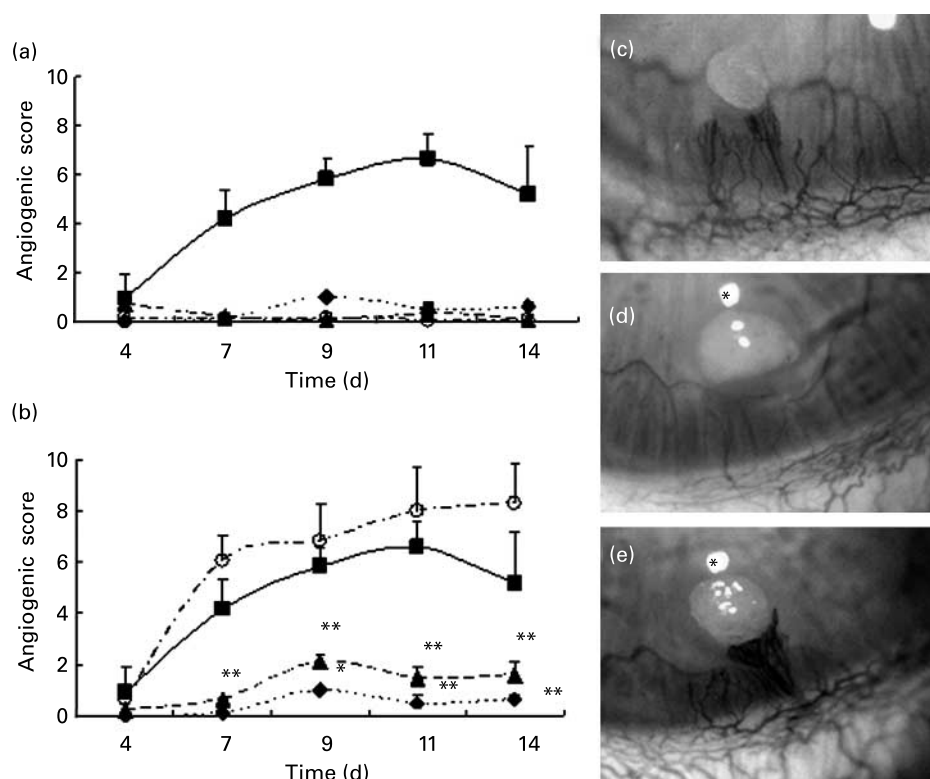
When cells were induced to grow by exposure to VEGF, addition of Q3'S (1 nM to 1  $\mu\text{M}$  range) did not change proliferation (Fig. 2(b)), while quercetin and its glucuronide conjugate inhibited the VEGF response. Quercetin inhibited cell proliferation by 40% at 1 nM, this concentration being the most effective. The inhibitory effect elicited by Q3G was significant in the range 10 nM to 1  $\mu\text{M}$  (Fig. 2(b)).

Quercetin metabolites were then tested on cell migration towards the VEGF gradient. Again quercetin and Q3G, but not Q3'S, significantly inhibited VEGF-induced chemotaxis (Table 1).

The inhibitory effect of Q3G on endothelial functions was not due to cytotoxic effects, since neither quercetin metabolites nor the corresponding solvents were toxic at the concentrations used in the experiments (data not shown).

Thus, quercetin metabolites appeared to exert divergent actions on cell proliferation and migration in the absence and in the presence of exogenous VEGF. The sulphate conjugate clearly induced replication of unstimulated cells. Under VEGF stimulation, quercetin and the glucuronide metabolite, but not the sulphate conjugate, exhibited an inhibitory effect on cell migration and proliferation.





**Fig. 1.** Quercetin metabolites differentially affect *in vivo* angiogenesis. The rabbit cornea assay was used to assess the angiogenic effect of quercetin-free aglycone ( $\cdots\blacktriangle\cdots$ ) and conjugates quercetin-3-glucuronide (Q3G;  $\cdots\blacklozenge\cdots$ ) and quercetin-3'-sulphate (Q3'S;  $\cdots\circ\cdots$ ) *per se* ( $\blacksquare$ ), vascular endothelial growth factor (VEGF) (a) and on VEGF-induced (tested at 200 ng/pellet) neovascularisation ( $\blacksquare$ , VEGF;  $\cdots\blacktriangle\cdots$ , VEGF + quercetin;  $\cdots\blacklozenge\cdots$ , VEGF + Q3G;  $\cdots\circ\cdots$ , VEGF + Q3'S) (b). For details of procedures, see p. 1017. Values, reported as angiogenic score measured daily, are means (of at least four implants) with their standard errors depicted by vertical bars. Mean values were significantly different from those of VEGF alone: \*\* $P < 0.01$ . (c, d, e) Representative pictures of corneal neovascularization (day 14) induced by VEGF alone (c) and in the presence of Q3G (d) and Q3'S (e) (1  $\mu$ g each). Original magnification 18  $\times$   $\blacksquare$ , \*Flash artefacts.

### Opposing effects of quercetin metabolites on cell signalling

In light of the results obtained in cultured cells and on angiogenesis, we investigated signals known to be involved in the expression of the angiogenic phenotype. ERK1/2 phosphorylation, a reliable marker of cell activation and proliferation, increased as a result of VEGF (20 ng/ml) application (Fig. 3). Quercetin and Q3G (tested at 10 nM) had no effect on basal ERK1/2 activation. Interestingly, Q3'S increased the levels of phospho-ERK1/2 by 50% (Fig. 3(a)) and produced a further not significant increase in VEGF-induced mitogen-activated protein kinase phosphorylation (Fig. 3(b)). In contrast, quercetin and Q3G inhibited VEGF-induced ERK1/2 phosphorylation (Fig. 3(b)).

### Molecular mechanisms for the activity of quercetin-3'-sulphate on endothelial cells

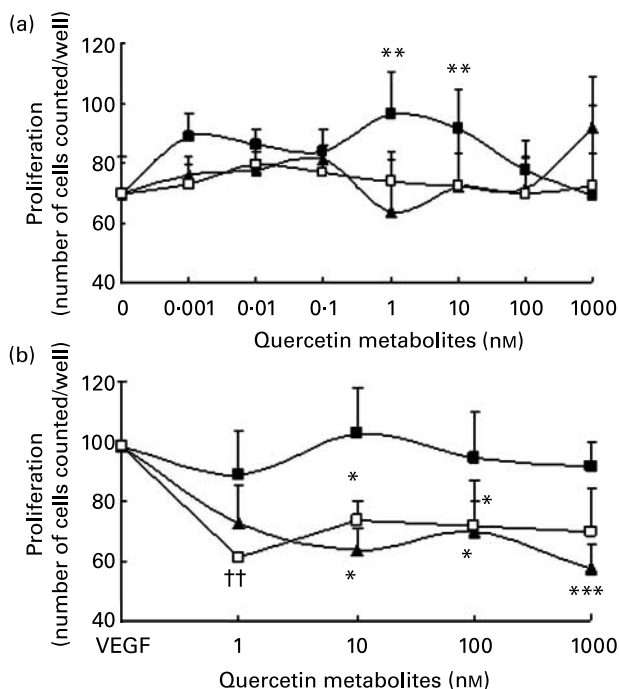
Since Q3'S enhanced ERK1/2 phosphorylation and stimulated endothelial cell activation both in the quiescent state or when activated by VEGF, we examined whether this metabolite would interact with VEGF receptor (VEGFR-2) signalling (Zachary, 2003). Measurement of CVEC proliferation following challenge with Q3'S (or quercetin for comparison) in the presence of the PI3K inhibitor LY294002, the nitric oxide synthase inhibitor *N*<sup>ω</sup>-methyl-L-arginine,

the guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-*a*]-quinoxalin-1-one or mitogen-activated protein kinase kinase inhibitor U0126 showed a distinct pattern of growth inhibition. As illustrated in Fig. 4, exposure to Q3'S elicited a significant proliferation of CVEC above control ( $P < 0.01$ ). Inhibitors of the VEGFR-2 signalling cascade invariably reduced the effect of Q3'S on cell growth, leaving control cells or those treated with quercetin (not shown) unaffected (Fig. 4).

Further analysis of the interaction between Q3'S and the VEGF signalling was performed in PAEC in which the VEGFR-2 is stably transfected (PAEC-VEGFR-2) by monitoring the phospho-tyrosine residues linked to the receptor. As expected, VEGF enhanced phosphorylation signalling threefold, while concomitant exposure to VEGF and Q3'S enhanced phospho-VEGFR-2 fivefold, indicating a positive interaction of the metabolite with VEGF signalling (Fig. 5(a)).

Signals downstream of the VEGFR-2, such as PI3K/Akt and cGMP, were also enhanced in endothelial cells exposed simultaneously to VEGF and Q3'S, and to Q3'S alone, respectively (Fig. 5(b, c)), reflecting the increased tyrosine phosphorylation observed earlier.

Collectively, the present findings demonstrate that Q3'S, but not quercetin or Q3G, interacts positively with VEGF receptor signalling, leading to activation of extracellular signal-regulated kinase and promoting cell growth.



**Fig. 2.** Proliferation of capillary endothelial cells is differentially affected by quercetin metabolites. (a), The effect of increasing concentrations (1 pM to 1  $\mu$ M) of quercetin ( $\square$ ), quercetin-3'-sulphate ( $\blacksquare$ ) and quercetin-3-glucuronide ( $\blacktriangle$ ). (b), The effect of increasing concentrations (1 nM to 1  $\mu$ M) of quercetin metabolites on vascular endothelial growth factor-induced (VEGF; 20 ng/ml) proliferation. For details of procedures, see p. 1017. Values are means (of four experiments run in triplicate) with their standard errors depicted by vertical bars. Mean values were significantly different from those of the basal response: \*\* $P < 0.01$ . Mean values were significantly different from those of VEGF alone: \* $P < 0.05$ ; †† $P < 0.01$ ; \*\*\* $P < 0.001$ .

## Discussion

A number of studies have elucidated the biological profile of quercetin, demonstrating the antioxidant and anti-angiogenic properties of this flavonol in a variety of experimental systems (Igura *et al.* 2001; Fan *et al.* 2003; Tan *et al.* 2003). Quercetin was found to inhibit several steps of angiogenesis, including

**Table 1.** Effect of quercetin metabolites on endothelial cell migration $\ddagger$  (Mean values with their standard deviations for three experiments run in triplicate)

Compound (10 nm)	No. of migrated cells counted per well			
	Control		VEGF	
	Mean	SEM	Mean	SEM
None	110	5	138	4*
Quercetin	109	3	122	2†
Q3'S	112	1	138	8
Q3G	100	1	100	12†

Q3'S, quercetin-3'-sulphate; Q3G, quercetin-3-glucuronide; VEGF, vascular endothelial growth factor.

Mean values were significantly different from those of the basal condition: \* $P < 0.05$ .

Mean values were significantly different from those of VEGF alone: † $P < 0.05$ .

$\ddagger$  Coronary venular endothelial cells exposed to 10 nM-quercetin metabolites were challenged for migration toward VEGF (20 ng/ml). Cell migration was monitored after 4 h incubation. For details of procedures, see p. 1017.

proliferation, migration and differentiation of endothelial cells. The molecular mechanisms described to be inhibited in endothelial cells by the free aglycone are mitogen-activated protein kinase, c-Jun NH<sub>2</sub>-terminal kinase and focal adhesion kinases (Kobuchi *et al.* 1999; Kaneider *et al.* 2004), and metalloproteinase-2 expression and activity (Tan *et al.* 2003).

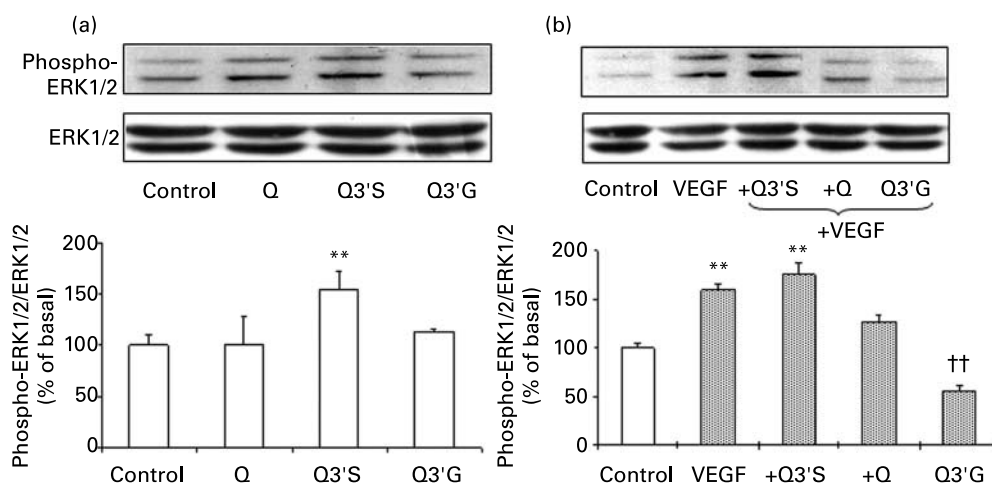
However, following administration of quercetin-rich foods, the glucuronide and sulphate derivatives of quercetin predominate. The ratio of these appears to vary according to as yet unknown factors, but it is clear that only traces of the free aglycone are found in human plasma (Gee *et al.* 2000; Day *et al.* 2001; O'Leary *et al.* 2001). This is due to the rapid and extensive conjugation of quercetin, so that it is assumed that aglycone is less important in the biological effects observed *in vivo*.

In the present study, we found that quercetin conjugates exhibit divergent effects on the endothelium and on angiogenesis. On one hand, Q3G and quercetin itself have no effect, at least at the concentration examined in the present study, on quiescent endothelium, while they inhibit endothelial functions and *in vivo* angiogenesis elicited by VEGF. Consistently, these compounds reduce mitogen-activated protein kinase activation, the intracellular signal underlying endothelial activation. On the other hand, Q3'S increases significantly the growth of quiescent endothelial cells reaching maximal activity at 1  $\mu$ M, while it has no effect on cell proliferation stimulated by VEGF. Its actions on *in vivo* angiogenesis consist of a small, non-significant enhancement of the VEGF response. The observed increase of ERK1/2 phosphorylation by Q3'S is entirely in line with the findings given earlier.

Exploring further the molecular mechanism for the pro-proliferative effect of Q3'S and its partial potentiating effect on VEGF-induced angiogenesis, we observed that the sulphate metabolite is able to facilitate the activation of the VEGF receptor by its ligand and to ignite the intracellular signalling pathways characteristic of VEGFR-2 activation, including in the order, the PI3K/Akt pathway and the nitric oxide synthase/cGMP cascade (Kroll and Waltenberger, 1998; Parenti *et al.* 1998, Zachary, 2003). These signals ultimately converge on mitogen-activated protein kinase activation and support the synergistic effect of Q3'S and VEGF in inducing an angiogenic phenotype.

Differences in the biological action between quercetin conjugates have been noted also in other cells as well as at the biochemical level. For example, in lymphoblastoid cells, Q3'S prevented H<sub>2</sub>O<sub>2</sub>-induced chromosomal damage, while quercetin did not (Saito *et al.* 2004). This preventive effect toward oxidative injury fits with the pro-proliferative action on the endothelium observed in the present study. Differences between quercetin conjugates have also been reported in their ability to inhibit LDL oxidation and albumin binding, with Q3G a more efficient antioxidant than Q3'S (Janisch *et al.* 2004).

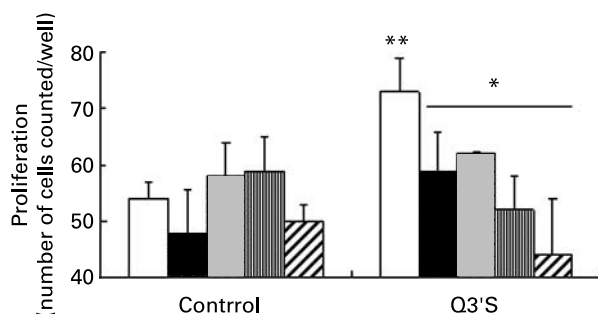
Opposing properties of natural compounds contained in the same plant have been recently reported. Sengupta *et al.* (2004) have described opposing activities of two ginsenosides contained in ginseng, which exert totally different effects on the angiogenic phenotype. These authors have rationalised their findings by postulating the existence of a yin–yang paradigm in this plant.



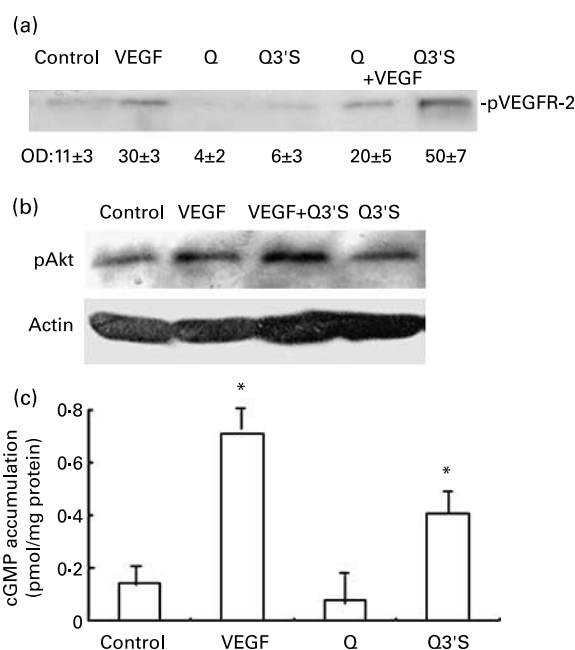
**Fig. 3.** Effect of quercetin metabolites on extracellular signal-regulated kinases 1/2 (ERK1/2) activation in basal conditions and upon vascular endothelial growth factor (VEGF) stimulation. (a), Basal ERK1/2 phosphorylation measured after 10 min of incubation with 10 nM of the compounds. (b), VEGF-induced (20 ng/ml) ERK1/2 phosphorylation in the presence of quercetin metabolites (10 nM, 30 min preincubation). For details of procedures, see p. 1017. A representative gel of phospho-ERK1/2 (upper bands) and total ERK1/2 (lower bands) out of three with similar results is reported. Data have been normalised on total ERK1/2 bands; histogram values are means (of three experiments) with their standard errors depicted by vertical bars. Mean values were significantly different from those of the control: \*\* $P < 0.01$ . Mean values were significantly different from those of VEGF alone: †† $P < 0.01$ . Q, quercetin; Q3'S, quercetin-3'-sulphate; Q3'G, quercetin-3'-glucuronide.

The changes of either endothelial cell proliferation in response to VEGF or angiogenic response observed in the present study occurs at low concentrations of quercetin metabolites, generally in the upper nanomolar range. This is similar to a range of quercetin metabolites found at peak in plasma (0.02–4  $\mu\text{M}$ ) following administration of high-quercetin onions to man (de Pascual-Teresa *et al.* 2004). Moreover, as shown in the present study, high micromolar concentrations of these compounds produce fairly severe cell toxicity. This casts doubt upon the relevance of reported effects by quercetin on angiogenesis or other systems at high micromolar concentrations (usually above 100  $\mu\text{M}$ ).

In summary, the present study demonstrates the anti-angiogenic activities of quercetin and its conjugate Q3G on VEGF-activated endothelium, and the pro-angiogenic property of Q3'S. Thus, the balance of circulating quercetin conjugates rather than quercetin itself may determine the final effect of



**Fig. 4.** Vascular endothelial growth factor receptor-2 and its intracellular signalling are involved in quercetin-3'-sulphate-induced (Q3'S) proliferation. Cells were pretreated with 50  $\mu\text{M}$  Ly294002 (■), 100  $\mu\text{M}$  N<sup>G</sup>-methyl-L-arginine (▨), 10  $\mu\text{M}$  1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (▧) or 10  $\mu\text{M}$  U0126 (▩) for 30 min before the addition of Q3'S (10 nM). (□), No pretreatment (0.1% bovine calf serum). For details of procedures, see p. 1017. Values are means (of three experiments) with their standard errors depicted by vertical bars. Mean values were significantly different from those of Q3'S alone: \* $P < 0.05$ . Mean values were significantly different from those of the control: \*\* $P < 0.01$ .



**Fig. 5.** Biochemical characterisation of quercetin-3'-sulphate (Q3'S) pro-angiogenic effect. (a), Serum-starved semi-confluent porcine aortic endothelial cells overexpressing vascular endothelial growth factor receptor-2 (VEGFR-2) were stimulated for 5 min with 20 ng/ml vascular endothelial growth factor (VEGF) in the absence and in the presence of quercetin (Q) and Q3'S (10 nM, 30 min pretreatment). Lysates were immunoprecipitated with anti-VEGFR-2 antibody. The immunoprecipitated proteins were collected, resolved on SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with an anti-phospho-tyrosine antibody (-pVEGFR-2). A representative blot is shown. Optical density (OD) values are means (of three gels) with their standard errors. (b) Phospho-Akt (pAkt) was evaluated by Western blotting of coronary venular endothelial cells (CVEC) lysates. Blots are representative of four experiments with similar results. Membranes were re-probed with anti-actin antibody for protein level. (c), cGMP accumulation following exposure of CVEC to VEGF or quercetin metabolites for 10 min was measured by enzyme immunoassay kit. Values are means (of three experiments) with their standard errors depicted by vertical bars. For details of procedures, see p. 1017. Mean values were significantly different from those of the control: \* $P < 0.05$ .



dietary quercetin *in vivo*. This knowledge, together with information on individual variability in the metabolism of quercetin, may be useful in interpreting the results of clinical studies.

### Acknowledgements

Sandra Donnini and Federica Finetti contributed equally to the work. The study was funded by the EU project POLYBIND (QLK1-1999-00505) and the Italian Ministry for Research (MIUR, PRIN project no. 2004065317\_001) (M.Z.). This research was also supported by EC FP6 funding (LSHM-CT-2004-0050333). The technical support of Jean-Marc Souquet and Frederic Veran for Q3G purification, and Emmanuelle Meudec and Christine Le Guernevé for MS and NMR analysis, is acknowledged. We are also grateful to Dr Antonio Giachetti (Lifetech, Florence, Italy) for helpful discussion. S. D. was supported by funds from NuGO (FOOD-CT-2004-506360). F. F. was a recipient of a Fondazione Callerio (Trieste, Italy) fellowship.

### References

- Bagli E, Stefanidou M, Morbidelli L, Ziche M, Psillas K, Murphy C & Fotsis T (2004) Luteolin inhibits vascular endothelial growth factor-induced angiogenesis; inhibition of endothelial cell survival and proliferation by targeting phosphatidylinositol 3'-kinase activity. *Cancer Res* **64**, 7936–7946.
- Burchell B & Coughtrie MWH (1997) Genetic and environmental factors associated with variation of human xenobiotic glucuronidation and sulfation. *Environ Health Perspect* **105**, 739–747.
- Cantara S, Donnini S, Morbidelli L, Giachetti A, Schulz R, Memo M & Ziche M (2004) Physiological levels of amyloid peptides stimulate the angiogenic response through FGF-2. *FASEB J* **18**, 1943–1945.
- Cappelli A, Giuliani G, La Pericot Mohr G, *et al.* (2004) A nonpeptide NK<sub>1</sub> receptor agonist showing subpicomolar affinity. *J Med Chem* **47**, 1315–1318.
- Cheyrier V & Rigaud J (1986) HPLC separation and characterization of flavonols in the skins of *Vitis vinifera* var. Cinsault. *Am J Enol Vitic* **37**, 248–252.
- Chiesi M & Schwaller R (1995) Inhibition of constitutive endothelial NO-synthase activity by tannin and quercetin. *Biochem Pharmacol* **49**, 495–501.
- Day AJ, Mellon F, Barron D, Sarrazin G, Morgan MR & Williamson G (2001) Human metabolism of dietary flavonoids: identification of plasma metabolites of quercetin. *Free Radic Res* **35**, 941–952.
- de Pascual-Teresa S, Johnston KL, Dupont MS, O'Leary KA, Needs PW, Morgan LM, Clifford MN, Bao Y & Williamson G (2004) Quercetin metabolites downregulate cyclooxygenase-2 transcription in human lymphocytes *ex vivo* but not *in vivo*. *J Nutr* **134**, 552–557.
- Fan PS, Gu ZL, Sheng R, Liang ZQ, Wang XX & Zhu Y (2003) Inhibitory effect of quercetin on proliferation of human microvascular endothelial cells *in vitro*. *Acta Pharmacol Sin* **24**, 1231–1234.
- Garcia-Closas R, Agudo A, Gonzalez CA & Riboli E (1998) Intake of specific carotenoids and flavonoids and the risk of lung cancer in women in Barcelona, Spain. *Nutr Cancer* **32**, 154–158.
- Garcia-Closas R, Gonzalez CA, Agudo A & Riboli E (1999) Intake of specific carotenoids and flavonoids and the risk of gastric cancer in Spain. *Cancer Causes Control* **10**, 71–75.
- Gee JM, Dupont MS, Day AJ, Plumb GW, Williamson G & Johnson IT (2000) Intestinal transport of quercetin glycosides in rats involves both deglycosylation and interaction with the hexose transport pathway. *J Nutr* **130**, 2765–2771.
- Hasebe Y, Egawa K, Yamazaki Y, Kunimoto S, Hirai Y, Ida Y & Nose K (2003) Specific inhibition of hypoxia-inducible factor (HIF)-1 alpha activation and of vascular endothelial growth factor (VEGF) production by flavonoids. *Biol Pharm Bull* **26**, 1379–1383.
- Hertog MG, Feskens EJ, Hollman PC, Katan MB & Kromhout D (1993) Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet* **342**, 1007–1011.
- Igura K, Ohta T, Kuroda Y & Kaji K (2001) Resveratrol and quercetin inhibit angiogenesis *in vitro*. *Cancer Lett* **171**, 11–16.
- Janisch KM, Williamson G, Needs P & Plumb GW (2004) Properties of quercetin conjugates: modulation of LDL oxidation and binding to human serum albumin. *Free Radic Res* **38**, 877–884.
- Kaneider NC, Mosheimer B, Reinisch N, Patsch JR & Wiedermann CJ (2004) Inhibition of thrombin-induced signaling by resveratrol and quercetin: effects on adenosine nucleotide metabolism in endothelial cells and platelet-neutrophil interactions. *Thromb Res* **114**, 185–194.
- Kimura Y & Okuda H (2001) Resveratrol isolated from *Polygonum cuspidatum* root prevents tumor growth and metastasis to lung and tumor-induced neovascularization in Lewis lung carcinoma-bearing mice. *J Nutr* **131**, 1844–1849.
- Knekt P, Jarvinen R, Reunanen A & Maatela J (1996) Flavonoid intake and coronary mortality in Finland: a cohort study. *Br Med J* **312**, 478–481.
- Knekt P, Kumpulainen J, Jarvinen R, Rissanen H, Heliovaara M, Reunanen A, Hakulinen T & Aromaa A (2002) Flavonoid intake and risk of chronic diseases. *Am J Clin Nutr* **76**, 560–568.
- Kobuchi H, Roy S, Sen CK, Nguyen HG & Packer L (1999) Quercetin inhibits inducible ICAM-1 expression in human endothelial cells through the JNK pathway. *Am J Physiol* **277**, C403–C411.
- Kroll J & Waltenberger J (1998) VEGF-A induces expression of eNOS and iNOS in endothelial cells via VEGF receptor-2 (KDR). *Biochem Biophys Res Commun* **252**, 743–746.
- Lin MT, Yen ML, Lin CY & Kuo ML (2003) Inhibition of vascular endothelial growth factor-induced angiogenesis by resveratrol through interruption of Src-dependent vascular endothelial cadherin tyrosine phosphorylation. *Mol Pharmacol* **64**, 1029–1036.
- McCann SE, Ambrosone CB, Moysich KB, Brasure J, Marshall JR, Freudenheim JL, Wilkinson GS & Graham S (2005) Intakes of selected nutrients, foods, and phytochemicals and prostate cancer risk in Western New York. *Nutr Cancer* **53**, 33–41.
- Morbidelli L, Donnini S, Chillemi F, Giachetti A & Ziche M (2003) Angiosuppressive and angiostimulatory effects exerted by synthetic partial sequences of endostatin. *Clin Cancer Res* **9**, 5358–5369.
- Morbidelli L & Ziche M (2004) The rabbit corneal pocket assay for the study of angiogenesis. *Cancer Treat Rev* **117**, 147–151.
- Neuhouser ML (2004) Dietary flavonoids and cancer risk: evidence from human population studies. *Nutr Cancer* **50**, 1–7.
- O'Leary KA, Day AJ, Needs PW, Sly WS, O'Brien NM & Williamson G (2001) Flavonoid glucuronides are substrates for human liver beta-glucuronidase. *FEBS Lett* **503**, 103–106.
- Parenti A, Morbidelli L, Cui XL, Douglas JG, Hood J, Granger HJ, Ledda F & Ziche M (1998) Nitric oxide is an upstream signal for vascular endothelial growth factor-induced extracellular signal-regulated kinases1/2 activation in postcapillary endothelium. *J Biol Chem* **273**, 4220–4226.
- Saito A, Sugisawa A, Umegaki K & Sunagawa H (2004) Protective effects of quercetin and its metabolites on H<sub>2</sub>O<sub>2</sub>-induced chromosomal damage to WIL2-NS cells. *Biosci Biotechnol Biochem* **68**, 271–276.



- Sengupta S, Toh SA, Sellers LA, Skepper JN, Koolwijk P, Leung HW, Yeung HW, Wong RN, Sasisekharan R & Fan TP (2004) Modulating angiogenesis: the yin and the yang in ginseng. *Circulation* **110**, 1219–1225.
- Tan WF, Lin LP, Li MH, Zhang YX, Tong YG, Xiao D & Ding J (2003) Quercetin, a dietary-derived flavonoid, possesses antiangiogenic potential. *Eur J Pharmacol* **459**, 255–262.
- Tseng SH, Lin SM, Chen JC, Su YH, Huang HY, Chen CK, Lin PY & Chen Y (2004) Resveratrol suppresses the angiogenesis and tumor growth of gliomas in rats. *Clin Cancer Res* **10**, 2190–2202.
- Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M & Heldin CH (1994) Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J Biol Chem* **269**, 26988–26995.
- Williamson G (2003) The use of flavonoid aglycones in *in vitro* systems to test biological activities: based on bioavailability data, is this a valid approach? *Phytochem Rev* **1**, 215–222.
- Zachary I (2003) VEGF signalling: integration and multi-tasking in endothelial cell biology. *Biochem Soc Trans* **31**, 1171–1177.
- Ziche M, Morbidelli L, Choudhuri R, Zhang HT, Donnini S, Granger HJ & Bicknell R (1997) Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis. *J Clin Invest* **99**, 2625–2634.