The flavonoid quercetin induces acute vasodilator effects in healthy volunteers: Correlation with beta-glucuronidase activity

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A B S T R A C T

Quercetin exerts vasodilator, antiplatelet and antiproliferative effects and reduces blood pressure, oxidative status and end-organ damage in hypertensive humans and animal models. We hypothesized that oral quercetin might induce vasodilator effects in humans and that they might be related to the deconjugation of quercetin-3-O-glucuronide (Q3GA). Design: double blind, randomized, placebo-controlled trial. Fifteen healthy volunteers (26 ± 5 years, 6 female) were given a capsule containing placebo, 200 or 400 mg of quercetin in random order in three consecutive weeks. At 2 h a dose-dependent increase in Q3GA was observed in plasma (~0.4 and 1 μM for 200 and 400 mg, respectively) with minor levels of quercetin and isorhamnetin. No changes were observed in blood pressure. At 5 h quercetin induced and increase in brachial arterial diameter that correlated with the product of the levels of Q3GA by the plasma glucuronidase activity. There was an increase in urinary levels of glutathione but there was no increase in nitrites plus nitrates. Quercetin and isorhamnetin also relaxed human umbilical arteries in vitro while Q2GA was without effect. In conclusions, quercetin exerts acute vasodilator effects in vivo in normotensive, normocholesterolemic human subjects. These results are consistent with the effects being due to the deconjugation of the metabolite Q3GA.

Introduction

Flavonoids constitute a large class of polyphenols found in plant-derived foodstuffs which includes several subclasses; such as flavonols; flavones; flavanones; flavanols; anthocyanidins; isoflavones; dihydroflavonols and chalcones [1]. Among them, the flavonol quercetin is one of the most abundant and widely distributed. Epidemiological studies have found an inverse association between dietary flavonol and flavone intake and the risk of several diseases; including cancer; cardiovascular disease; and neurodegenerative disorders [2–4] which may account; at least partly; for the health effects of fruits and vegetables. The beneficial effect of flavonols on cardiovascular disease is supported by continuously growing evidence based on animal studies and short term clinical trials [5,6]. One of the main mechanisms by which flavonols are thought to lower cardiovascular risk is via its vasodilator and antihypertensive effect [7–11]. This might have a large impact on global mortality and morbidity since in 2010 elevated blood pressure was estimated to be the most important risk factor while a diet low in fruits is in the top four for disease burden globally [12]. In rats and humans quercetin lowers blood pressure in hypertensive but not in normotensive subjects; an effect which seems to be independent of endothelial function or angiotensin-converting enzyme.

Abbreviations: CE, collision energy; DAD, diode array detector; DP, declustering potential; EP, entrance potential; FMD, flow-mediated dilatation; iso-PCF20s, 8-isoprostaglandin F2α; MS, mass spectrometer; NO, nitric oxide; NO2, NO3 and NOx; Q3GA, quercetin-3-O-glucuronide.

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Table 1
Baseline characteristics.

<table>
<thead>
<tr>
<th>N</th>
<th>16</th>
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<tr>
<td>Age, years</td>
<td>25.8 ± 5.2</td>
</tr>
<tr>
<td>Male (%)</td>
<td>10(62.5)</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>24.0 ± 4.7</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>117.1 ± 3.6</td>
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<tr>
<td>Diastolic blood pressure, mm</td>
<td>69.3 ± 2.9</td>
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<tr>
<td>Total cholesterol, mg/dL</td>
<td>169.1 ± 30.6</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>92.8 ± 7.0</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>57.1 ± 2.3</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>93.1 ± 44.8</td>
</tr>
<tr>
<td>Fasting glucose, mg/dL</td>
<td>84.2 ± 5.8</td>
</tr>
<tr>
<td>Creatinine, mg/dL</td>
<td>0.87 ± 0.20</td>
</tr>
</tbody>
</table>

Values are mean SD unless indicated otherwise.

activity [13,11]. Quercetin induces arterial vasodilation in isolated arteries from several animal species [7,14] but to our knowledge its effects on human arterial tone have not been tested so far in vitro or in vivo.

Quercetin may be administered orally as its glycosylated forms as they are regularly present in foods or as an aglycone in food supplements [15]. The major metabolites of quercetin in human plasma are quercetin-3-O-glucuronide (Q3GA), quercetin-3-O-sulfate and isorhamnetin-3-O-glucuronide (3’-methyl quercetin-3-O-glucuronide) while minimal concentrations of free aglycone are found in plasma or urine [16]. However, the time-course of the biological effects of quercetin does not correlate with the presence in plasma of these metabolites. In fact, these metabolites are usually less active than the parent aglycones and often totally inactive in vitro when tested for short periods of time [17]. However, the glucuronono-conjugated metabolites of quercetin can be hydrolyzed by β-glucuronidase in several tissues, releasing the parent aglycone which accumulates intracelullary [18–22]. Therefore, at time periods longer than 1–2 h, Q3GA induces vasorelaxation in vitro [21] and reduces blood pressure in vivo [23] and both effects can be prevented by inhibition of β-glucuronidase. Thus conjugation is a reversible process and, at least regarding the vasodilator and antihypertensive effects in laboratory animals, the conjugation-deconjugation cycle appears to be an absolute requirement [24]. We hypothesized that oral quercetin might induce vasodilator effects in humans and that they might be related to the deconjugation of Q3GA.

Materials and methods

Subjects and experimental design

Seventeen healthy volunteers participated in the study. All subjects were healthy, had no evidence of chronic disease, had not taken any medication or vitamin supplement and were advised not to take flavonoid rich foods from a list provided 48 h before each intervention. The baseline characteristics are shown in Table 1. All subjects had normal values of blood pressure, electrolytes, hepatic enzymes, urea, uric acid, homocysteine and blood cells.

The acute effects of two doses of quercetin aglycone (200 and 400 mg) were assessed in a randomized double-blind placebo-controlled crossover study. The doses were chosen as they represent a maximal amount of flavonoids which could be achieved by eating flavonoid-rich foods but lower than those present in most food supplements (1 g/day is generally used). During the initial visit subjects were interviewed, gave informed written consent, received the instructions and blood samples were taken for routine biochemical and hematological analysis. The treatments consisted in a single administration of a capsule containing 200 and 400 mg of quercetin or placebo (capsule with rice starch indistinguishable from active treatments). The subjects received each of the three treatments in random order 1 week apart on the same day of the week and at the same time of the day. On the treatment day, a blood sample was collected at time 0 (baseline) and 2 h and a urinary sample at time 0 and 5 h after oral administration of the capsules. We choose 2 h to collect plasma samples because after oral ingestion of quercetin aglycone, the median plasma concentrations of total quercetin peaked at 60–360 min in healthy volunteers [25–27,11]. One subject was excluded because only received one treatment, other subject only received placebo and 200 mg quercetin. No adverse effects after quercetin or placebo intake were found.

Brachial artery diameter measurements

Participants rested for 15 min in the supine position. A blood pressure cuff was placed on the upper right arm proximal to the elbow but distal to the placement of an ultrasound Doppler probe on the brachial artery. All measurements were performed by the same trained ultrasound operator. The brachial artery was assessed 2–3 cm above the elbow using external B-mode ultrasound imaging and arterial diameter was analyzed off-line using automatic edge-detection (Sonosite, Titan, 7.0 MHz linear transducer). Angle-corrected and intensity-weighted mean velocities (Vmean) were determined using Logic 7 software. Brachial artery blood flow was calculated using the formula blood flow = Vmean×arterial diameter/2×60. Three blood pressure measurements were performed in the left arm using an automatic sphygmomanometer (Omron, Spain).

Analysis of quercetin and metabolites in plasma

Plasma samples (300 µL) supplemented with ascorbic acid (30 µL of 10 mM aqueous solution) and apigenin 7-O-glucoside as internal standard (40 µL of 10 µM solution in 50% acetonitrile in water), were extracted and analyzed as described [23]. Briefly, samples were treated with acetonitrile/0.5 M acetic acid (80:20, v/v) and centrifuged. The pellet was submitted to a similar process twice with acetonitrile and the combined supernatants were dried. Extracts were dissolved in acetonitrile/water (30:70, v/v) and analyzed in a Hewlett-Packard 1100 chromatograph (Agilent Technologies, Waldbronn, Germany) with a quaternary pump and a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station as described. An Ascentis TM RP-Amide 3 µm (2.1 × 150 mm) column was used. The solvents used were: (A) 0.1% formic acid, and (B) acetonitrile. An elution gradient was established from 15% to 50% B over 15 min, isocratic 50% B for 10 min, from 50% to 75% B over 3 min, isocratic 75% B for 10 min, and re-equilibration of the column, at a flow rate of 0.2 mL/min. Double online detection was carried out in the DAD using 370 nm as a preferred wavelength and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet. MS detection was performed in a API 3200 Q Trap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer that was controlled by the Analyst 5.1 software. Zero grade air served as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 C, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). Both quadrupoles were set at unit resolution. The ion spray voltage was set at –4500 V in the negative mode. Method settings used were: declustering potential (DP) –40 V, entrance potential (EP) –10 V, collision energy (CE) –50 V, and cell exit potential –3 V. In order to obtain the fragmentation pattern of the parent ion(s), enhanced product ion mode was also performed using the following parameters: DP –50 V, EP –6 V, CE –25 V, and collision energy spread 0 V. Quantitative determination of the assayed quercetin and conjugated metabolites was performed from their chromatographic peaks at 370 nm by comparison with calibration curves of standard compounds either commercial
(quercetin and isorhamnetin) or obtained and characterized in the laboratory (conjugated metabolites) [28]. MS analysis was used to confirm the identity of the detected quercetin metabolites.

**Determination of β-glucuronidase activity**

β-Glucuronidase activity was measured by a colorimetric analysis using phenolphthalein mono-β-glucuronide as the substrate [29]. Briefly, plasma (50 μL) was incubated with 1 mM phenolphthalein glucuronic acid in 0.1 mM sodium phosphate buffer at pH 4.5 for 4 h at 37 °C. At 4 h, the reaction was stopped by adding 0.1 M sodium phosphate buffer pH 11. A spectrophotometer (model 680XR, BIORAD laboratories, USA) was used to monitor the intensity of the resulting yellow color, which is proportional to β-glucuronidase activity. The enzyme activity was from a standard curve and expressed as μg phenolphthalein released/mL serum/h at 37 °C.

**Measurements of glutathione, nitrite and nitrate and isoprostanes**

Total 8-iso-prostaglandin (iso-PC)F2αs determination was analyzed in 50 μL of urine by competitive enzyme immunoassay kit (Cayman Chemical), and the results were expressed as pg/mg creatinine. Urine NO2− and NO3− (NO3) concentrations were measured using nitrate reductase and Griess reaction [30], and the results were expressed as μmol/mg creatinine. Total plasma GSH was determined with Cayman’s GSH assay kit (Cayman Chemical, Ann Arbor, MI).

**In vitro vascular contractility**

Umbilical cords were obtained from normal term pregnancies after vaginal delivery, with the consent of the donor mothers, and placed in a cold Krebs solution. All the procedures carried out using the umbilical cords were approved by the Ethics Committee of Clinic Hospital of Granada. Umbilical arteries were dissected from the cords and cut into rings (2–3 mm) which were suspended in an organ bath containing Krebs solution bubbled with 95% O2 5% CO2 at 37 °C for isometric tension recording. The tissues were stretched to a resting tension of 2 g which was determined in preliminary experiments to be optimal based on the maximal response to 40 mM KCl [31]. After equilibration, rings were stimulated with 0.1 mM U46619, in the absence or in the presence of Nω-nitro-L-arginine methyl ester (l-NAME, 100 μM), added 30 min before the addition of U46619, until they reached a steady-state contractile response and the flavonoids or vehicle (dimethylsulfoxide) were added to the chamber in a cumulative fashion. In some rings, the endothelium was removed by gently rubbing the intimal surface with a fine forceps. Removal of the endothelium was confirmed in each preparation by the absence of relaxation to the endothelium-dependent vasodilator acetylcholine (10−6 M).

**Statistical analysis**

The main end-point of the study was an increase by quercetin in arterial diameter. Sample size was calculated using a one tail test with the following parameters: statistical significance (α) of 0.05, a power (1 − β) of 0.8, a clinically important difference of 20% change in arterial diameter, a standard deviation of 20% and a drop-out rate of 20%. Normality of data was assessed with the Shapiro–Wilk test. Because most datasets could not be assumed to be normally distributed, differences between groups were assessed by a Wilcoxon signed rank test or for multiple comparisons by a Friedman test followed, in case of significance, by a Dunn’s test for post hoc pairwise comparisons. The in vitro experiments in isolated arteries are expressed as mean ± SEM and the potency of the flavonoids, i.e.
the concentration producing 50% relaxation or IC$_{50}$, was calculated from the concentration–response curve by nonlinear regression analysis.

**Ethical approval**

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Human Ethics Committee of the Virgen de las Nieves Hospital in Granada. Written informed consent was obtained from all subjects.

**Results**

**Plasma quercetin metabolites profile after a single oral dose**

Quercetin or its metabolites were undetectable in plasma in most participants at baseline (Fig. 1). At 2 h post ingestion of 200 and 400 mg quercetin there was a dose-dependent increase in plasma Q3GA, which was the major metabolite [0.35 μM (CI 95% 0.22–0.48) and 0.95 μM (0.57–1.71), respectively]. There was a lower increase in quercetin aglycone [0.043 μM (0.026–0.060) and 0.031 μM (0.007–0.054), respectively] and isorhamnetin aglycone [0.008 μM (0–0.019) and 0.035 μM (0.001–0.069), respectively] while glucuronides of isorhamnetin were not detected (Fig. 1).

**Effects of quercetin in blood pressure and brachial artery blood flow and diameter**

No changes in systolic and diastolic blood pressure were observed after 2 and 5 h post ingestion of 200 and 400 mg quercetin (Fig. 2). However, a time-dependent increase in brachial artery diameter was observed after 400 mg quercetin (Fig. 2). A trend for an increase in arterial flow was also observed which did not reach significance. However, we found a good correlation between the changes in flow and diameter ($p$ = 0.003, $r^2$ = 0.48 for 400 mg, not shown).

Neither plasma flavonoids concentration, nor plasma β-glucuronidase activity were significantly correlated with the increase in artery diameter at 5 h post ingestion (Fig. 3). However, the change in diameter correlated with the product of the levels of Q3GA by the plasma glucuronidase activity (Fig. 3).

**Effects of quercetin in plasma total glutathione, and urinary isoprostanes and NO$_x$ levels**

Only quercetin ingestion of 400 mg significantly increased the plasma levels of glutathione at 2 h (Fig. 4). The levels of urinary isoprostanes decreased in the three experimental groups after 5 h. This decrease tended to be higher after 400 mg quercetin but the changes were not significantly different among groups (Fig. 4).
A significant reduction of urinary NOx levels was found at 5 h post ingestion of 400 mg of quercetin (Fig. 4).

**Effects of quercetin, isorhamnetin and Q3GA on human umbilical arteries in vitro**

Exposure of isolated umbilical arteries to the U46619 (0.1 μM) resulted in sustained contraction. Addition of quercetin or its methylated metabolite isorhamnetin produced a concentration-dependent relaxation in endothelium intact vessels, which was significant at concentrations ≥10 nM while vehicle (dimethylsulfoxide) or Q3GA had no effect (Fig. 5). The potency of the flavonoids, calculated as the IC50, was 1.4 and 5 μM, respectively. Inhibition of endothelial nitric oxide synthase (eNOS) with L-NAME or endothelium denudation partially inhibited the relaxation induced by low concentrations of quercetin and isorhamnetin.
Discussion

This study provides the first evidence that acute administration of quercetin aglycone exerted a vasodilator effect in human arteries both in vitro and in vivo in a dose- and time-dependent manner. This functional change was associated with an increase in the plasma levels of the antioxidant glutathione but no increase in NOx. The increase in arterial diameter found after 5 h post ingestion correlated with plasma concentrations of Q3GA at 2 h and plasma β-glucuronidase activity.

Blood pressure is mainly regulated by changes in peripheral vascular resistance generated by functional narrowing of the resistance arteries and arterioles. As age advances, structural damage and disease in larger conduit arteries become more important determinants of blood pressure [32]. In young healthy humans the diameter increase induced by quercetin in a large conduit artery, such as brachial artery was not associated with changes in blood pressure. These data are in agreement with previous evidences showing that quercetin is effective in humans [10,27,11] and rats [8,33] when blood pressure is elevated but either acute [11] or chronic [10] quercetin ingestion does not affect blood pressure in normotensives. Consistently, we also noticed a parallel trend for an increase in arterial flow which may explain the lack of change in blood pressure. However, the larger variability in flow measurements precluded the finding of significant differences for this parameter.

Quercetin induces vasodilator effects on animal [34,9] and human (present study) arteries in vitro. Some studies indicate that the effects of quercetin in healthy vessels are independent of endothelium and nitric oxide (NO) production while other show NO-dependent effects [6]. We found that inhibition of eNOS or endothelium denudation partially inhibited the vasodilator response of quercetin in human placental vessels in vitro, showing the partial involvement of endothelial NO. Loke et al. [35] speculated that the quercetin-induced increase in nitrites and nitrates (the stable metabolites of NO) in normotensive subjects might improve endothelial function, but no direct measurements of endothelial function or blood pressure were performed in that study. Larson et al. [11] reported that flow-mediated dilatation, the standard technique to measure endothelial function in humans, and serum nitrites were unaffected by a single large dose of quercetin (1095 mg) in hypertensive subjects. In our study, we showed that quercetin did not to increase urine NOx levels which further support the variable dependence on NO of the responses of quercetin. However, we found that quercetin increased plasma glutathione levels, an antioxidant biomarker. It also decreased urinary isoprostanes levels, a biomarker of oxidative stress, although the change was not different compared to the placebo effect.

In previous studies administering pure quercetin to humans, total flavonoids in plasma were analyzed in samples hydrolyzed with glucuronidase and sulfatase [25,27,11]. To our knowledge this is the first paper reporting the conjugated metabolites found
in human plasma after pure quercetin. A single oral dose of quercetin increased circulating Q3GA concentrations (~0.4 and 1 μM for 200 and 400 mg, respectively) with smaller changes in the aglycones in plasma (always below 0.1 μM) while other metabolites were not detected. Quantitatively, these levels are consistent with those reported previously for total flavonoids after hydrolysis (0.2 for 100 mg [25], 0.43 for 150 mg [27] and 1–2.2 μM for 1095 mg [111]). Interestingly, the metabolic profile after pure quercetin reported herein seems to be different from that after quercetin rich foods in humans [16,26,36] which includes quercetin-3’-O-sulfate, quercetin-3-O-glucuronide, isorhamnetin-3-O-glucuronide, quercetin diglucuronide and quercetin glucuronide sulfate while the aglycones are undetected. Similarly, different metabolites of quercetin were found in rat plasma after a single intragastric administration compared to those after free access mixed with food [37]. Thus, quercetin administration, as a capsule to humans or as an intragastrical suspension to rats, results mainly in an increase of Q3GA with minor increases in other metabolites including the aglycones. Quercetin rich foods in humans or quercetin added to the diet results in a more complex metabolic profile in which methylated and sulfated derivatives are more important and aglycones are absent.

Despite oral flavonoids exert biologically demonstrable systemic effects, the concentration of aglycones in plasma are very low and their circulating conjugated forms show weak acute activity in vitro (as confirmed here for Q3GA in human arteries), an inconsistency that has been termed “the flavonoid paradox” [22,24]. However, glucurono-conjugates can be hydrolized at the vascular level by β-glucuronidase, yielding the parent aglycone which due to its higher lipid solubility accumulates in tissues [21]. In fact, inhibition of β-glucuronidase prevents the in vivo effects of both intravenous Q3GA and oral quercetin in rats [23]. Therefore, we proposed that quercetinol derivatives transport quercetin and its methylated form in plasma, and deliver to the tissues the free aglycones, which are the final effectors [22,24].

β-Glucuronidase is a lysosomal enzyme involved in the cleavage of glycosaminoglycans whose total deficiency leads to severe mucopolysaccharidosis type VII. A wide inter-individual variability in the activity of this enzyme has also been reported which may be accounted for by changes on its expression or by variations in its gene sequence [38]. Thus, we hypothesized that β-glucuronidase activity may influence the effectiveness of quercetin. Because inhibition of β-glucuronidase is not feasible in humans, precluding the proper analysis of a cause–effect relationship, we measured its activity in an attempt to correlate it with the functional effects of quercetin. We measured glucuronidase activity in plasma which is considerably low, but it may be used as a surrogate for activity in tissues [38].

After oral administration of pure quercetin, total flavonoids in plasma peak at 60–360 min [25,27,11]. We measured plasma metabolites at 2 h which can be used as an indicator of quercetin bioavailability. Based on animal studies we would not expect that the biological effects and the plasma concentrations of Q3GA follow a similar time-course, the former being much more prolonged than the latter [23]. We found that the effect on arterial diameter did not correlate with either the early changes in plasma flavonoids or the plasma glucuronidase activity. However, these effects were correlated with a composite factor of both, the product of early plasma Q3GA concentrations by β-glucuronidase activity. These data are consistent with the view that the effects are proportional to the bioavailability of quercetin and the rate of deconjugation of the glucuronide in tissues.

In conclusion, quercetin exerts acute vasodilator effects in vivo in normotensive, normocholesterolemic human subjects. These results are consistent with the effects being due to the deconjugation of the metabolite Q3GA.