Original Contribution

# Metabolic conversion of dietary flavonoids alters their anti-inflammatory and antioxidant properties

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## A R T I C L E   I N F O

## A B S T R A C T

The notion that dietary flavonoids exert beneficial health effects in humans is often based on in vitro studies using the glycoside or aglycone forms of these flavonoids. However, flavonoids are extensively metabolized in humans, resulting in the formation of glucuronide, methyl, and sulfate derivatives, which may have different properties than their parent compounds. The goal of this study was to investigate whether different chemical modifications of the same flavonoid molecule affect its biological and antioxidant activities. Hence, we studied the anti-inflammatory effects of several major human metabolites of quercetin and (−)-epigallocatechin-3-O-gallate (EGCG) by assessing their inhibitory effects on tumor necrosis factor $\alpha$ (TNF$\alpha$)-induced protein expression of cellular adhesion molecules in human aortic endothelial cells (HAEC). HAEC were incubated with 1–30 µM quercetin, 3′- or 4′-O-methyl-quercetin, quercetin-3-O-glucuronide, and quercetin-3′-O-sulfate or 20–100 µM EGCG, 4″-O-methyl-EGCG, and 4′,4″-di-O-methyl-EGCG, prior to coincubation with 100 U/ml of TNF$\alpha$. 3′-O-Methyl-quercetin, 4′-O-methyl-quercetin, and their parent aglycone compound, quercetin, all effectively inhibited expression of intercellular adhesion molecule-1 (ICAM-1) with IC$_{50}$ values (concentration required for 50% inhibition) of 8.0, 5.0, and 4.4 µM, respectively; E-selectin expression was suppressed to a somewhat lesser but still significant degree by all three compounds, whereas vascular cell adhesion molecule-1 (VCAM-1) was not affected. In contrast, quercetin-3-O-glucuronide (20–100 µM), quercetin-3′-O-sulfate (10–30 µM), and phenolic acid metabolites of quercetin (20–100 µM) did not inhibit adhesion molecule expression. 4′,4″-Di-O-methyl-EGCG selectively inhibited ICAM-1 expression with an IC$_{50}$ value of 94 µM, whereas EGCG (20–60 µM) and 4″-O-methyl-EGCG (20–100 µM) had no effect. The inhibitory effects of 3′-O-methyl-quercetin and 4′,4″-di-O-methyl-EGCG on adhesion molecule expression were not related either to inhibition of NF-$\kappa$B activation or to their antioxidant reducing capacity. Our data indicate that flavonoid metabolites have different biological and antioxidant properties than their parent compounds, and suggest that data from in vitro studies using nonmetabolites of flavonoids are of limited relevance in vivo.

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## Introduction

The health benefits of consuming fruits and vegetables are often attributed, in part, to their high content of polyphenolic compounds [1,2]. The consumption of polyphenols in a plant-derived diet can be several times higher than the consumption of other phytochemicals and vitamins, including ascorbic acid (vitamin C), $\alpha$-tocopherol (vitamin E), or carotenoids [3,4]. Among the polyphenols, flavonoids have attracted considerable attention. Epidemiological studies have found that an increased intake of dietary flavonoids is associated with a decreased risk of chronic diseases, including certain cancers and cardiovascular diseases [5–8]. Dietary flavonoids have many interesting in vitro properties, including antioxidant and anti-inflammatory effects, which have been postulated to underlie their beneficial health effects [9–12].

Flavonoids undergo extensive first-pass metabolism, and the chemical forms of flavonoids present in fruits and vegetables, usually glycosides or aglycones, are quite different from their in vivo metabolites. In the intestinal mucosa and the liver, flavonoids are subjected to extensive glucuronidation, methylation, and sulfation [4,13]. Thus, after intake of flavonoid-rich foods, these flavonoid metabolites are the main forms found in the circulatory system, where they are

present for up to 4–6 h (e.g., catechins) [14,15] or may remain longer, probably as a result of enterohepatic circulation (e.g., quercetin) [16–18]. As with pharmaceutical drugs and other xenobiotics [19], biotransformation greatly affects the physical and chemical properties of flavonoids, making them more water-soluble and readily excreted in bile and urine.

Therefore, in this study we investigated whether different chemical modifications of the same flavonoid molecule alter its biological activity and antioxidant capacity. To this end, we compared the effects of different derivatives of two representative flavonoids, the flavonol, quercetin, and the flavan-3-ol, (−)-epigallocatechin-3-O-gallate (EGCG), to their respective parent aglycones. Specifically, we studied whether O-methylation of the flavonoid B ring, glucuronidation or sulfation of quercetin, and O-methylation of EGCG affect the antioxidant properties of quercetin and EGCG and their anti-inflammatory effects on tumor necrosis factor α (TNFα)-induced activation of human aortic endothelial cells (HAEC). Endothelial activation was characterized by surface protein expression of cellular adhesion molecules and release of monocyte chemotactic protein-1 (MCP-1), which is known to critically contribute to monocyte recruitment to the vascular wall and, hence, initiation of vascular inflammation and atherosclerotic lesion formation [20,21]. Our results show that biotransformation of dietary flavonoids can result in loss or gain of biological or antioxidant activity, which cannot be predicted from the chemical nature of the flavonoid.

## Materials and methods

### Materials

3′-O-Methyl-quercetin (isorhamnetin) and 4′-O-methyl-quercetin (tamarixetin) were obtained from Indofine (Hillsborough, NJ). Quercetin and EGCG were purchased from Sigma-Aldrich (St. Louis, MO). TNFα was purchased from Roche (Mannheim, Germany). All other chemicals were of the highest grade available.

### Metabolites of quercetin

Quercetin-3′-O-sulfate was synthesized according to the method described by Day et al. [22], while quercetin-3-O-glucuronide extracted from developing seeds of French bean (Phaseolus vulgaris) with methanol was purified by liquid–liquid partitioning and preparative HPLC. The phenolic acids 3′,4′-dihydroxyphenylacetic acid, 3,4-dihydroxybenzoic acid, 3′-hydroxyphenylacetic acid, and 3′-methoxy-4′-hydroxyphenylacetic acid were obtained from Sigma-Aldrich. The 3-(3′-hydroxyphenyl)propionic acid was obtained from Fluorochem (Derby, UK). The structures of quercetin and related compounds are shown in Scheme I.

### Metabolites of (−)-epigallocatechin-3-O-gallate

The metabolites 4″-O-methyl-EGCG and 4′,4″-di-O-methyl-EGCG were prepared by methylating EGCG with methyl iodide, as described previously [23]. The structures of EGCG and related compounds studied in this work are shown in Scheme II.

### Endothelial cells

Human aortic endothelial cells were obtained from Lonza (Walkersville, MD) at third passage. Upon receipt, the cells were seeded at a ratio of 1:3 in 75-cm² flasks (precoated with 1% bovine gelatin; Sigma-Aldrich) and grown at 37 °C, under 95% air–5% CO₂ and in a humidified atmosphere in endothelial cell growth medium (Lonza) containing bovine brain extract, human epithelial growth factor, hydrocortisone, amphotericin B, gentamicin sulfate, and 2% fetal bovine serum (FBS). The medium was periodically renewed until the cells reached 70–90% confluence, at which point they were treated with 0.05% trypsin–0.02% EDTA (Sigma-Aldrich). Subsequently, the cells were expanded in 75-cm² precoated flasks at a ratio of 1:5 until passages 5–6, when they were plated and the experiments were carried out.

### Experiments

Human aortic endothelial cells were plated in 96-well plates (precoated with 1% gelatin) at an average density of $5 \times 10^4$ cells/ml medium. The medium consisted of Medium 199 (Sigma-Aldrich) supplemented with 20% FBS (GIBCO Invitrogen, Grand Island, NY), 1 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 0.1 μg/ml amphotericin B (Sigma-Aldrich), and 1 ng/ml human basic fibroblast growth factor (Roche). The cells were allowed to attach to the plates overnight (18 h), after which they were washed with Hanks' balanced salt solution (Sigma-Aldrich) and the medium was renewed. The cells were incubated at 37 °C, under 95% air–5% CO₂ and in a humidified atmosphere until they reached confluence, typically 24–48 h after seeding.

For experiments, HAEC were incubated for 18 h with medium (100 μl) containing different concentrations of quercetin or its derivatives, 3′-O-methyl-quercetin, 4′-O-methyl-quercetin, quercetin-3-O-glucuronide, and quercetin-3′-O-sulfate, or phenolic acid metabolites of quercetin; or for 1 h with EGCG or its O-methyl derivatives, 4″-O-methyl-EGCG, or 4′,4″-di-O-methyl-EGCG, prior to the addition of 100 U/ml of TNFα. We chose longer incubation times for quercetin and its derivatives to mimic enterohepatic circulation. In addition, preliminary experiments indicated that a 18-h preincubation was required for maximal inhibition of adhesion molecule expression. The solutions were freshly prepared by dissolving the flavonoids in either DMSO or deionized water and subsequent dilution in culture medium containing 20% serum. The final concentration of DMSO in the medium did not exceed 0.1%. Proper controls with the vehicle DMSO or water were carried out.

The cells were examined regularly using an inverted optical microscope. No changes in cell morphology were observed with any of the treatments. Cell viability was evaluated by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, using the Cell Proliferation kit I, according to the manufacturer's instructions (Roche). In addition, to test for potential effects of artificially generated reactive oxygen species, catalase (0.1 μM) and/or superoxide dismutase (3 μM) was added and/or phenol-red free medium was used.

### Cellular adhesion molecule expression

Surface protein levels of the cellular adhesion molecules, E-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1), were determined by ELISA performed on HAEC monolayers in flat-bottom 96-well plates. Following treatment, the cells were fixed in PBS containing 0.1% glutaraldehyde. For cell ELISA, plates were blocked at 37 °C for 1 h with 5% skim milk powder in PBS and then incubated overnight at 4 °C with a primary antibody to either E-selectin, ICAM-1 (R&D Systems, Minneapolis, MN) or VCAM-1 (Dako, Carpinteria, CA). The plates were then incubated for 1 h with a horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (Amersham, Piscataway, NJ) at 37 °C. The expression of E-selectin, ICAM-1, and VCAM-1 was assessed by the addition of o-phenylendiaminehydrochloride (Sigma-Aldrich). The absorbance at 492 nm was recorded in a plate-reader spectrophotometer (Spectromax 190, Molecular Devices, Sunnyvale, CA).

### Monocyte chemotactic protein-1 expression

Monocyte chemotactic protein-1 (MCP-1) was measured in the conditioned medium of cell cultures by the quantitative sandwich