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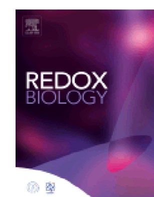

Research Paper

Quercetin affects glutathione levels and redox ratio in human aortic endothelial cells not through oxidation but formation and cellular export of quercetin-glutathione conjugates and upregulation of glutamate-cysteine ligase

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ABSTRACT

Endothelial dysfunction due to vascular inflammation and oxidative stress critically contributes to the etiology of atherosclerosis. The intracellular redox environment plays a key role in regulating endothelial cell function and is intimately linked to cellular thiol status, including and foremost glutathione (GSH). In the present study we investigated whether and how the dietary flavonoid, quercetin, affects GSH status of human aortic endothelial cells (HAEC) and their response to oxidative stress. We found that treating cells with buthionine sulfoximine to deplete cellular GSH levels significantly reduced the capacity of quercetin to inhibit lipopolysaccharide (LPS)-induced oxidant production. Furthermore, incubation of HAEC with quercetin caused a transient decrease and then full recovery of cellular GSH concentrations. The initial decline in GSH was not accompanied by a corresponding increase in glutathione disulfide (GSSG). To the contrary, GSSG levels, which were less than 0.5% of GSH levels at baseline (0.26 ± 0.01 vs. 64.7 ± 1.9 nmol/mg protein, respectively), decreased by about 25% during incubation with quercetin. As a result, the GSH: GSSG ratio increased by about 70%, from 253 ± 7 to 372 ± 23. These quercetin-induced changes in GSH and GSSG levels were not affected by treating HAEC with 500 μM ascorbic acid phosphate for 24 h to increase intracellular ascorbate levels. Incubation of HAEC with quercetin also led to the appearance of extracellular quercetin-glutathione conjugates, which was paralleled by upregulation of the multidrug resistance protein 1 (MRP1). Furthermore, quercetin slightly but significantly increased mRNA and protein levels of glutamate-cysteine ligase (GCL) catalytic and modifier subunits. Taken together, our results suggest that quercetin causes loss of GSH in HAEC, not because of oxidation but due to formation and cellular export of quercetin-glutathione conjugates. Induction by quercetin of GCL subsequently restores GSH levels, thereby suppressing LPS-induced oxidant production.

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1. Introduction

Activation of the vascular endothelium leading to increased cellular adhesion molecule expression and monocyte recruitment to the arterial wall are critical initializing steps in the development of atherosclerosis. Reactive oxygen species (ROS) have been shown to be involved in induction of endothelial dysfunction and vascular inflammation in various animal models of human atherosclerosis [10,24,25]. ROS induce endothelial injury [32], mediate obesity-related endothelial dysfunction [13,26], and are involved in endothelial activation and adhesion molecule expression upon exposure of endothelial cells to lipopolysaccharide (LPS) or angiotensin II [25,27]. ROS also exert pro-atherogenic effects by depleting NO and limiting its protective effects [12].

Glutathione (GSH) is an important intracellular, small-molecule antioxidant and detoxifying agent. Its relatively high concentration in cells [29] makes GSH the main intracellular redox buffer to protect cells from oxidants originating intra- and extracellularly. As an oxidative stress-related disease, atherosclerosis is substantially affected by glutathione status. For example, intraperitoneal injection of GSH to apolipoprotein E-deficient mice

Abbreviations: AAP, ascorbic acid phosphate; BSO, buthionine sulfoximine; BPDS, bathophenanthrolinedisulfonic acid; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; GCL, glutamate-cysteine ligase; GCLC, catalytic subunit of glutamate-cysteine ligase; GCLM, modifier subunit of glutamate-cysteine ligase; GSH, glutathione; GSSG, glutathione disulfide; HAEC, human aortic endothelial cells; IAA, iodoacetic acid; LC-MS, liquid chromatography-mass spectrometry; LPS, lipopolysaccharide; MRM, multiple reaction monitoring; MRP1, multidrug resistance protein 1; NO, nitric oxide; Nrf2, nuclear factor erythroid 2-related factor 2; p38, p38 mitogen-activated protein kinase; ROS, reactive oxygen species

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reversed impairment of endothelial function induced by hypoxia and reduced the size of atherosclerotic plaques [33]. Deficiency in the modifier subunit (GCLM) of glutamate-cysteine ligase (GCL) increased the rate of aortic lesion development in apolipoprotein E-deficient mice, which was reduced by overexpression of the catalytic subunit of GCL (GCLC) [6].

GCL catalyzes the synthesis of γ-glutamylcysteine from glutamate and cysteine, which is the rate-limiting step in the de novo synthesis of GSH. The GCLC subunit binds glutamate and cysteine and catalyzes their ligation, while GCLM regulates the binding affinity of GCLC to its substrates [35]. Inhibition of GCL activity by buthionine sulfoximine (BSO) or nitric oxide (NO) results in cellular GSH depletion [7,19,31]. On the other hand, induction of GCL is associated with an increase in GSH levels [1,6].

We previously described the antioxidant and anti-inflammatory effects of the dietary flavonoid, quercetin (3,3′,4′,5,7-pentahydroxyflavone), in human aortic endothelial cells (HAEC), but whether GSH plays a role in these effects of quercetin is unknown. Reports on intracellular GSH concentrations in HAEC are limited, as are studies on the effects of quercetin on GSH levels in endothelial cells.

Therefore, in the present study, we hypothesized that cellular GSH plays a critical role in mediating the antioxidant effects of quercetin in endothelial cells, and investigated the effects of incubating HAEC with quercetin on GSH oxidation, conjugation, and cellular export, as well as the induction of GCL.

2. Materials and methods

2.1. Materials

Quercetin, GSH and GSSG, tyrosinase (EC 1.14.18.1) (from mushroom), lipopolysaccharide (LPS), and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma-Aldrich (St. Louis, MO). All the other chemicals were of the highest grade available from Sigma-Aldrich.

2.2. Synthesis of quercetin-glutathione conjugates

Quercetin-glutathione conjugates were prepared according to methods reported by Awad *et al.* [2]. GSH was dissolved in phosphate-buffered saline pH 7.6 (final concentration, 1 mM). 100 U/ml tyrosinase and 150 μM quercetin (100 mM stock solution in DMSO) were added and the solution was incubated at 37 °C for 8 min and then freeze-dried and extracted with a mixture of 1:1:1 ethanol, methanol and water. Following centrifugation of the solution at 12,000g for 5 min, the supernatant was analyzed by liquid chromatography-mass spectrometry (LC-MS).

To purify the quercetin-glutathione conjugates, the solution was loaded onto an Alltech high-capacity C18 reversed-phase extract-clean column (Grace, Columbia, Maryland, USA). The column was then washed with 200 ml water. The quercetin-glutathione conjugates were eluted with 80 ml 10% (v/v) acetonitrile in water. Purity of quercetin-glutathione conjugates in 10% (v/v) acetonitrile was confirmed by HPLC with a Dionex AD20 absorbance detector. The acetonitrile solution containing the conjugates was then freeze-dried, weighed, and reconstituted with 1:1:1 ethanol, methanol and water to make a 7 mM standard solution.

2.3. Endothelial cells

Human aortic endothelial cells were obtained from Lonza (Walkersville, MD). Upon receipt, the cells were seeded in 75-cm^2 flasks precoated with 1% (w/v) bovine gelatin (Sigma-Aldrich) at a ratio of 1:2 and were grown in EBM basal medium (Lonza) containing bovine brain extract, ascorbic acid, hydrocortisone, epidermal growth factor, 2% (v/v) fetal bovine serum (FBS; Sigma-Aldrich), and gentamicin/amphotericin-B at 37 °C under 5% CO_2 in a humidified atmosphere. Medium was replaced periodically until cells reached 80–90% confluence; cells were then detached with 0.05% (w/v) trypsin-0.02% (w/v) EDTA (Sigma-Aldrich) and subcultured in gelatin-precoated 75 cm^2 flasks at a 1:3 ratio. Cells at passage 7 were used for experiments.

2.4. Experiments

Human aortic endothelial cells at passage 7 were plated in 1% (w/v) gelatin-precoated 96-well plates or 10-cm dishes with Medium 199 (Sigma-Aldrich) supplemented with 20% (v/v) FBS (Sigma-Aldrich), 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 1 ng/ml human basic fibroblast growth factor (Roche). The cells were then allowed to grow for 3–4 days until they reached confluence.

Confluent HAEC were incubated without or with different concentrations (5, 10, or 20 μM) of quercetin for up to 18 h [18]. Quercetin was added from stock solutions freshly prepared in DMSO and diluted with Medium 199 containing 20% (v/v) FBS. The final concentrations of DMSO in the medium were $\leq$0.1% (v/v). Appropriate controls with the vehicle DMSO were included in all experiments.

In some experiments, HAEC grown in 10-cm dishes were pre-incubated without or with 15 μM of the p38 inhibitor, SB203580 (Sigma-Aldrich), for 1 h before the addition of quercetin and incubation for up to 18 h. In other experiments, HAEC were pre-incubated with 500 μM ascorbic acid phosphate for 24 h before adding quercetin, or cells were co-incubated with 500 μM buthionine sulfoximine (BSO) and 20 μM quercetin for 18 h.

2.5. Real-time quantitative polymerase chain reaction

Total RNA was isolated from HAEC with TRIzol reagent (Life Technologies, Carlsbad, CA). cDNA was synthesized using a high-capacity cDNA archive kit (Life technologies). mRNA levels of GCLC and GCLM were determined by real-time qPCR with an ABI Prism 7500 Sequence Detection System (Life Technologies). Primers and probes used were purchased from Life Technologies as Assays on Demand, which contained a 20x mixture of PCR primers and TaqMan 6-FAM dye-labeled probes. The PCR reactions were set up with TaqMan Universal PCR Master Mix (Life Technologies). β-Actin was used as internal control gene. A standard curve of β-actin and a standard curve of each target gene were constructed to quantify the level of the target genes relative to the control gene.

2.6. Immunoblotting

Whole cell extracts of HAEC were prepared with a cell extraction kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. Equal amounts of protein (20–25 μg) were separated on 8% or 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and then transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked in 5% (w/v) nonfat dry milk in phosphate-buffered saline containing 0.1% (v/v) Tween 20 for 1 h at room temperature and then incubated overnight at 4 °C with specific primary antibodies to GCLC, GCLM, multidrug resistance protein 1 (MRP1), or β-actin (Abcam, Eugene, OR) followed by incubation for 1 h at room temperature with a horseradish peroxidase–conjugated secondary antibody (Abcam). The membrane was then incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Eugene, OR) and exposed to films for visualization.

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