



ELSEVIER

Contents lists available at [ScienceDirect](http://ScienceDirect)

## Redox Biology

journal homepage: [www.elsevier.com/locate/redox](http://www.elsevier.com/locate/redox)

## 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



Chuan Li, Wei-Jian Zhang, Jaewoo Choi, Balz Frei\*

Linus Pauling Institute and Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331, USA

## ARTICLE INFO

## Article history:

Received 24 June 2016

Received in revised form

28 July 2016

Accepted 18 August 2016

Available online 21 August 2016

## Keywords:

Quercetin

Glutathione

Endothelial cells

Oxidants

## 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 ( $0.26 \pm 0.01$  vs.  $64.7 \pm 1.9$  nmol/mg protein, respectively), decreased by about 25% during incubation with quercetin. As a result, the GSH: GSSG ratio increased by about 70%, from  $253 \pm 7$  to  $372 \pm 23$ . These quercetin-induced changes in GSH and GSSG levels were not affected by treating HAEC with  $500 \mu\text{M}$  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.

© 2016 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## 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

**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

\* Corresponding author.

E-mail address: [balz.frei@oregonstate.edu](mailto:balz.frei@oregonstate.edu) (B. Frei).

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

<http://dx.doi.org/10.1016/j.redox.2016.08.012>

2213-2317/© 2016 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

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  $\gamma$ -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'-dichlorofluorescein 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  $\mu$ M 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<sup>2</sup> 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<sub>2</sub> 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<sup>2</sup> 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  $\mu$ g/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  $\mu$ M) 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  $\mu$ M 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  $\mu$ M ascorbic acid phosphate for 24 h before adding quercetin, or cells were co-incubated with 500  $\mu$ M buthionine sulfoximine (BSO) and 20  $\mu$ M 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).  $\beta$ -Actin was used as internal control gene. A standard curve of  $\beta$ -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  $\mu$ g) 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  $\beta$ -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.

## 2.7. HPLC analysis of intracellular glutathione

Following incubations, HAEC were harvested with trypsin-EDTA (Sigma-Aldrich), and 1 mM bathophenanthrolinedisulfonic acid (BPDS)/10% (v/v) perchloric acid was added to the cell suspension. The samples were then centrifuged for 3 min at 15,000 × g. The cell pellet was used for total protein measurement. To 250 μl supernatant, 50 μl 100 mM iodoacetic acid (IAA)/0.2 mM m-cresol purple and 250 μl KOH (2.0 M)-KHCO<sub>3</sub> (2.4 M) were sequentially added. After 1 h incubation in the dark at room temperature, 500 μl 1% (v/v) 1-fluoro-2,4-dinitrobenzene was added and the mixture was incubated in the dark for 1 h at room temperature and then overnight at 4 °C. After incubation, the sample was centrifuged at 15,000 rpm and the supernatant was used for HPLC analysis.

HPLC analysis was performed with a Shimadzu liquid chromatography system. A Thermo Scientific APS-2 Hypersil column (250 × 4.6 mm, 5 μm) was used. The column was eluted at 1.0 ml/min with mobile phase A (80% (v/v) methanol in water) and 20% mobile phase B (0.5 M sodium acetate in 80% (v/v) methanol) for 8 min, followed by a linear gradient from 20% mobile phase B to 99% mobile phase B in 20 min. The percentage of mobile phase B was kept for 5 min and then a linear gradient from 99% to 20% mobile phase B in 2 min was used. The percentage of mobile B was kept at 20% for another 7 min. The injection volume was 80 μl. Derivatized GSH and GSSG were detected with a Shimadzu SPD-10Avp detector at 365 nm. For quantification, standard curves were constructed by running GSH and GSSG standards together with the samples.

## 2.8. LC-MS analysis of quercetin-glutathione conjugates

Following incubation, HAEC were washed and then extracted with a mixture of 1:1:1 ethanol, methanol and water. The medium was freeze-dried and extracted with the same mixture. Samples were then centrifuged at 12,000g for 5 min. The supernatant was stored at –80 °C until analysis. To quantify quercetin-glutathione conjugates, standard was synthesized and purified as described above. Sample separation was carried out using a Shimadzu HPLC system (Columbia, MD) with an Agilent Porshell 120 EC-C8 column (2.1 × 50 mm, 2.7 μm) coupled with a Sciex API 3000 triple quadrupole mass spectrometer with a Turbolon Spray source operated with mass analyzer set at unit resolution in negative ion mode (LC-MS/MS, Sciex API 3000, Foster City, CA). The sample was separated using a gradient with mobile phase A containing water with 0.1% (v/v) formic acid and mobile phase B containing acetonitrile with 0.1% (v/v) formic acid. The sample was eluted with a linear gradient from 5% mobile phase B to 10% mobile phase B in 5 min, then a linear gradient from 10% to 12% mobile phase B in 3 min and then a gradient from 12% to 15% mobile phase B in 2 min. The percentage of mobile phase B was kept for 5 min. This was followed by another linear gradient from 15% to 55% mobile phase B in 3 min and a gradient from 55% to 90% mobile phase B in 2 min. The percentage of mobile phase B was kept for 1 min and then a linear gradient from 90% to 5% mobile phase B in 1 min was used and maintained for 3 min to re-equilibrate the column. The total elution time was 24.5 min. The flow rate was 0.3 ml/min and the injection volume was 3 μl. Mass spectrometer source parameters were as follows: The Nebulizer, curtain, and collision (CAD) gas parameters were set at 11, 9, and 5 psi, respectively. Source temperature was at 400 °C. All gases were high purity nitrogen supplied by a custom liquid nitrogen system (Polar Cryogenics, Portland, OR). The ionizing voltage was –4000 V, and the declustering, focusing, entrance, and exit potentials were –50, –220, –10, and –12 V, respectively. A standard curve was constructed using purified quercetin-glutathione conjugate and

multiple reaction monitoring (MRM) to quantify the conjugates in samples.

## 2.9. Intracellular oxidant production

Intracellular oxidant production was measured using 2',7'-dichlorofluorescein diacetate (DCFH-DA). Following incubations, HAEC in 96-well plates were washed with HBSS buffer, and then incubated with 10 μM DCFH-DA for 20 min. After washing the cells again, they were incubated with 10 μg/ml LPS in medium containing 0.1% (v/v) FBS. Fluorescence was measured immediately after adding LPS and then every hour up to 4 h using a Spectromax Gemini XS multiplate fluorometer (Molecular Devices, Sunnyvale, CA) with excitation and emission settings of 485 nm and 530 nm, respectively.

## 2.10. Cell viability

Cell viability was determined with Cell Proliferation Reagent WST-1 (Sigma-Aldrich) according to the manufacturer's instructions.

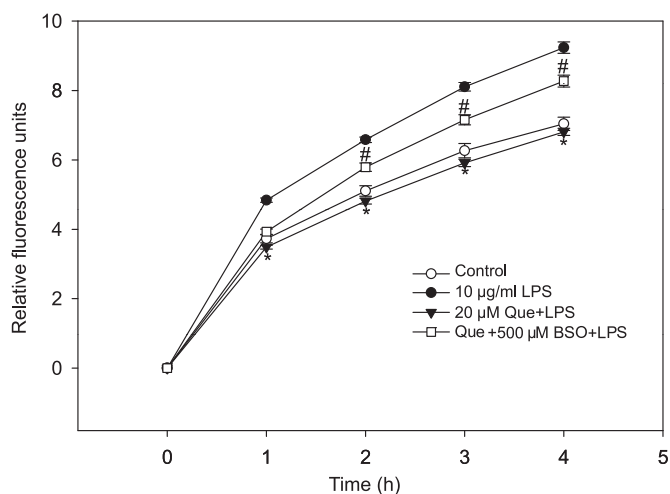
## 2.11. Statistical analysis

Data are expressed as means ± SEM of at least three independent experiments run in triplicate. Data were analyzed by factorial analysis of variance (ANOVA) or unpaired student's *t*-test. Significance was accepted at *P* < 0.05.

## 3. Results and discussion

### 3.1. GSH is involved in the antioxidant effect of quercetin against LPS-induced oxidant production

To investigate whether GSH is involved in the antioxidant effect of quercetin, BSO was used to inhibit GCL and deplete intracellular GSH levels [5,11,34]. HAEC were incubated with 20 μM quercetin alone or 20 μM quercetin and 500 μM BSO for 18 h, washed to



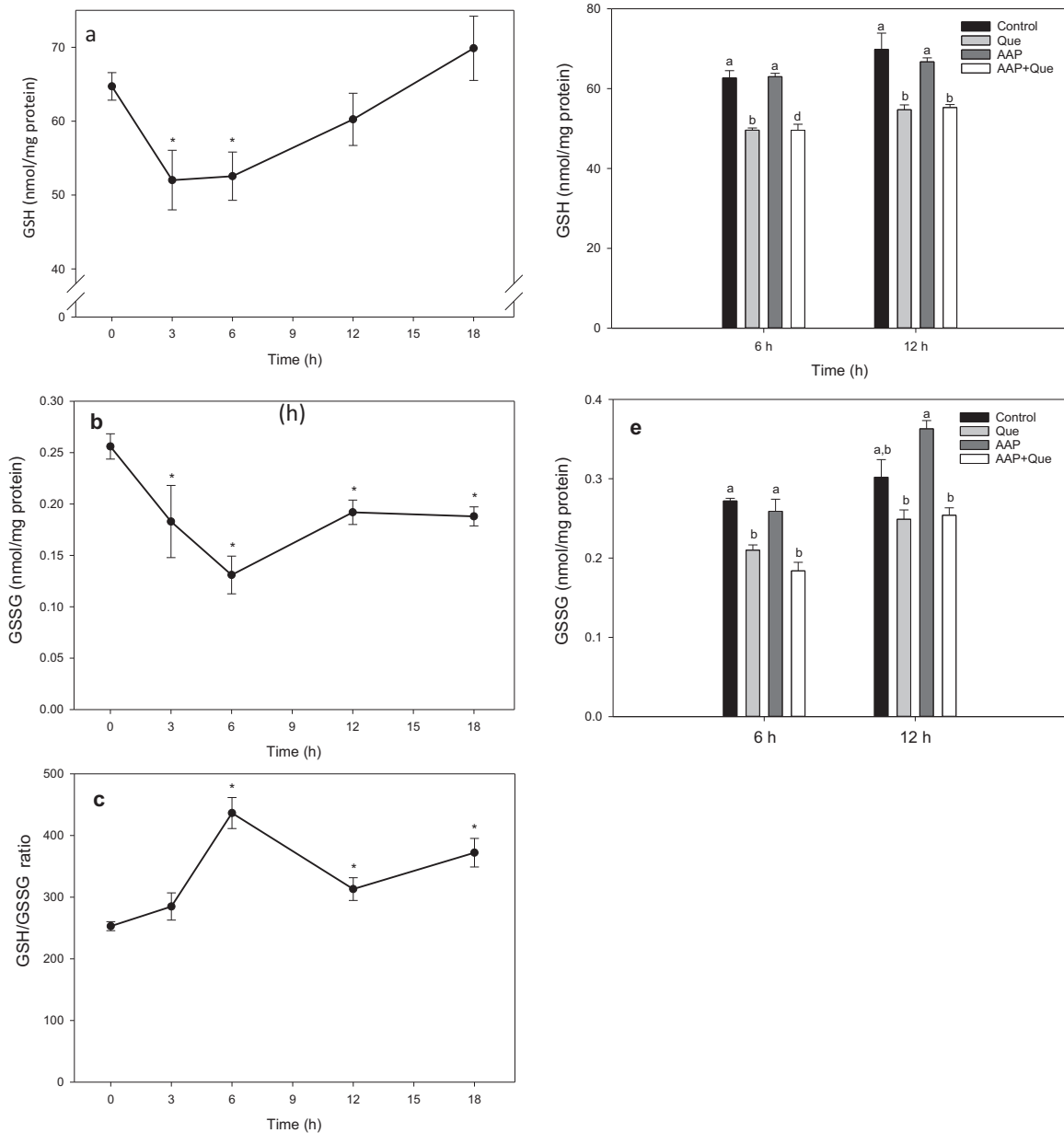
**Fig. 1. GSH is involved in the antioxidant effect of quercetin against LPS-induced oxidant production.** HAEC were incubated without (Control, LPS) or with 20 μM quercetin (Que+LPS) and 500 μM BSO (Que+BSO+LPS) for 18 h, and then were washed with HBSS and incubated with 10 μM DCFH-DA for 20 min. After incubation, DCFH-DA was washed off and medium containing 0.1% (v/v) FBS without or with 10 μg/ml LPS was added. Fluorescence was measured immediately after adding LPS and every hour up to 4 h. \*Denotes significant difference from LPS-stimulated cells ("10 μg/ml LPS"), *P* < 0.05; #denotes significant difference from cells incubated with quercetin and stimulated with LPS ("20 μM Que + LPS"), *P* < 0.05.

remove any remaining quercetin, and then stimulated with LPS. As shown in Fig. 1, intracellular oxidant production, as measured by DCFH-DA fluorescence, was significantly increased by LPS compared to unstimulated control cells. The increase in DCFH fluorescence by LPS was completely suppressed by quercetin. However, in the presence of BSO, the inhibitory effect of quercetin on oxidant production was significantly reduced, suggesting that GSH, in part, mediates the antioxidant effect of quercetin in HAEC.

### 3.2. Quercetin affects cellular glutathione levels and redox ratio

To investigate the effect of quercetin treatment on intracellular GSH status, HAEC were incubated with 20  $\mu$ M quercetin for different time periods, and the concentrations of GSH and GSSG were determined by HPLC with spectrophotometric detection. We found that GSH levels decreased by 20%, from  $64.7 \pm 1.9$  to

$52.0 \pm 4.0$  nmol/mg protein, during the first 3 h of incubation with quercetin; remained constant for the next 3 h; and then gradually increased to reach initial levels after 18 h of incubation (Fig. 2a). GSSG levels, which were less than 0.5% of GSH levels at baseline ( $0.26 \pm 0.01$  nmol/mg protein), decreased by about 50% during the first 6 h of incubation with quercetin; and then slightly increased after 12 and 18 h, but remained significantly lower than baseline GSSG levels (Fig. 2b). As a result of the very low cellular GSSG levels, the GSH: GSSG ratio was very high at baseline ( $253 \pm 27$ ), indicative of a highly reducing intracellular redox environment in HAEC. The GSH: GSSG ratio further increased during incubation with quercetin (Fig. 2c); this increase was mainly due to the decrease in GSSG, which was most pronounced at 6 h of incubation (Fig. 2b), coinciding with the highest GSH: GSSG ratio (Fig. 2c). A higher GSH: GSSG ratio indicates a more negative redox potential of the GSSG:2GSH couple, which might make the cells more



**Fig. 2. Quercetin affects cellular glutathione levels and redox ratio.** HAEC were incubated with 20  $\mu$ M quercetin for the indicated time periods (a, b, c), or were pre-incubated without (Control, Que) or with 500  $\mu$ M ascorbic acid phosphate (AAP) for 24 h and then incubated without or with quercetin (AAP+Que) for 6 h or 12 h (d, e). GSH (a, d) and GSSG (b, e) levels were determined by HPLC, and the GSH/GSSG ratio for each incubation period was calculated (c). \*Denotes significant difference from 0 h,  $P < 0.05$  (a, b, c). Results marked with a different letter differ significantly,  $P < 0.05$  (d, e).

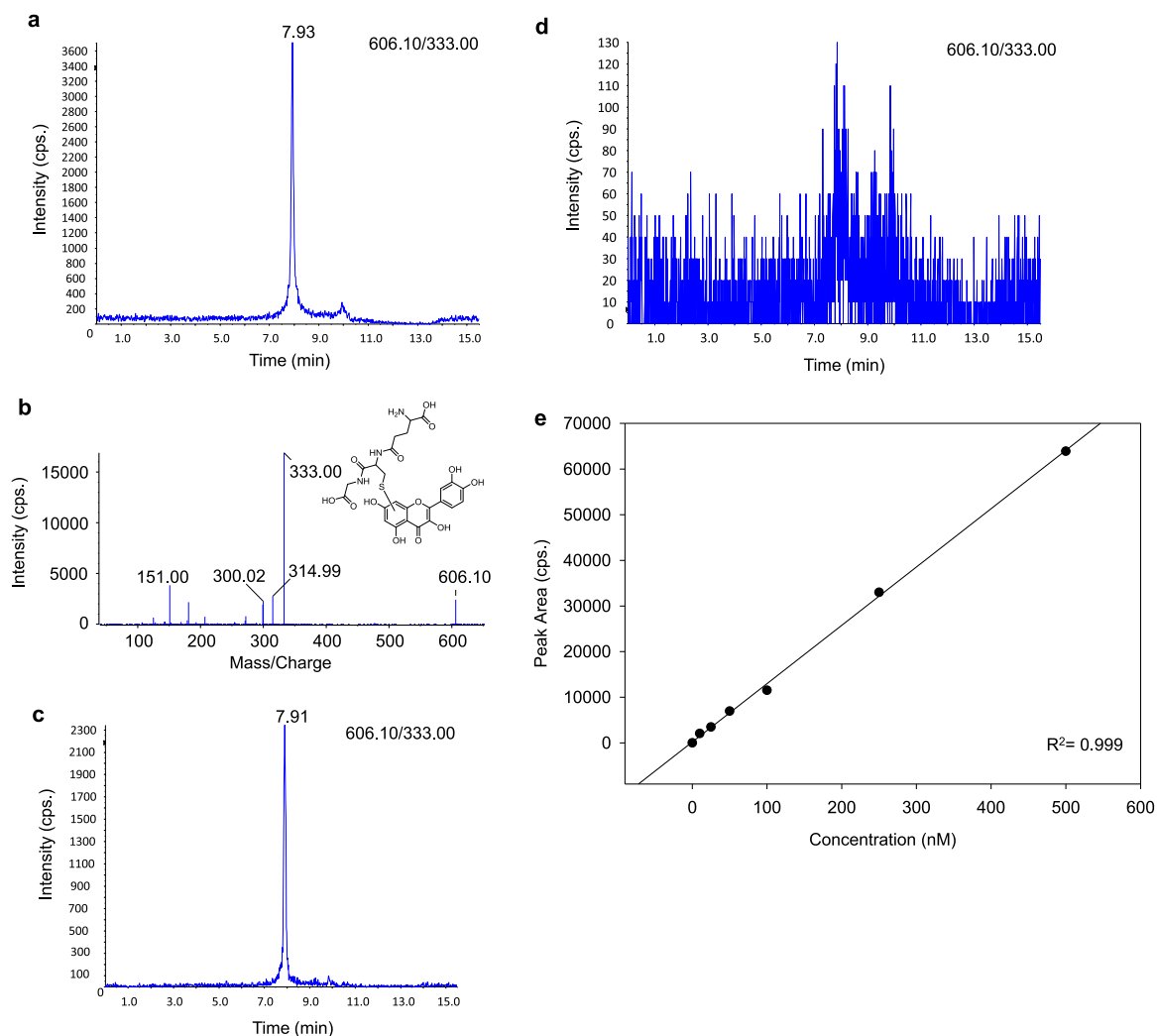
resistant to oxidative stress [28] and compensate for the decrease in GSH levels.

The finding that GSSG levels did not increase, but rather decreased, upon incubation of HAEC with quercetin suggests that the observed decrease in GSH levels was not due to oxidation. To further investigate this notion, HAEC were treated with ascorbic acid phosphate (AAP) before incubation with quercetin. We and others have shown previously that HAEC are depleted of ascorbic acid under standard cell culture conditions but can be enriched with ascorbate upon incubation with AAP; this, in turn, decreases background levels of ROS [29] and protects cells against ROS-induced glutathione depletion [14]. Incubation of HAEC with AAP successfully increased intracellular ascorbate concentrations from non-detectable levels ( $< 2$  pmol/mg protein) to  $1.91 \pm 0.08$  nmol/mg protein, as assessed by HPLC analysis with electrochemical detection. However, pre-incubation with AAP did not prevent the loss of GSH and GSSG caused by quercetin treatment, both after 6 and 12 h of incubation (Fig. 2d and e). These results are consistent with the notion that the quercetin-induced loss of GSH (Fig. 2a) is not due to oxidation, suggesting alternative mechanisms.

### 3.3. Formation of quercetin-glutathione conjugates

Quercetin, like all flavonoids, are metabolized by humans as xenobiotics, and conjugation to GSH is an important reaction for cellular detoxification and disposal of xenobiotics [20]. Quercetin-glutathione conjugates have been found in several cell types upon incubation with quercetin [23,30], but have never been identified in endothelial cells. To investigate whether quercetin-glutathione conjugates are formed in HAEC during quercetin treatment, cells were incubated with  $20 \mu\text{M}$  quercetin for 12 h. Glutathionyl conjugates of quercetin in cell extracts and the culture medium were identified by LC-MS, using synthetic quercetin-glutathione conjugates as standard.

The  $[\text{M}-\text{H}]^-$  ion of the quercetin-glutathione conjugates was detected at  $m/z$  606.10. For the purified quercetin-glutathione conjugate standard, a peak appeared with a retention time of 7.93 min in the MRM of  $m/z$  606.10/333.00 (Fig. 3a). According to the fragmentation patterns of its MS/MS spectrum (Fig. 3b), the fragment ion ( $m/z$  333.00) of  $m/z$  606.10 was chosen as the MRM of glutathionyl quercetin. A corresponding peak in the MRM ( $m/z$  606.10/333.00) of the culture medium of quercetin-treated HAEC was observed at 7.91 min (Fig. 3c), which presented the same MS/MS spectrum as the synthesized glutathionyl quercetin standard



**Fig. 3.** Mass spectra of quercetin-glutathione conjugates. Synthetic quercetin-glutathione standard (a) and reaction product from the culture medium of HAEC incubated with  $20 \mu\text{M}$  quercetin for 12 h (c) were analyzed by multiple reaction monitoring (MRM) HPLC. The quercetin-glutathione conjugates ( $m/z$  606.10) were identified by MS/MS in negative ion mode (b). The MRM of specific  $m/z=606.10$  value representing quercetin-glutathione conjugates was not detected in cell culture medium of HAEC incubated without quercetin for 12 h (d). A standard curve of quercetin-glutathione conjugates was built using purified synthetic quercetin-glutathione conjugates (e). cps.: counts per second.

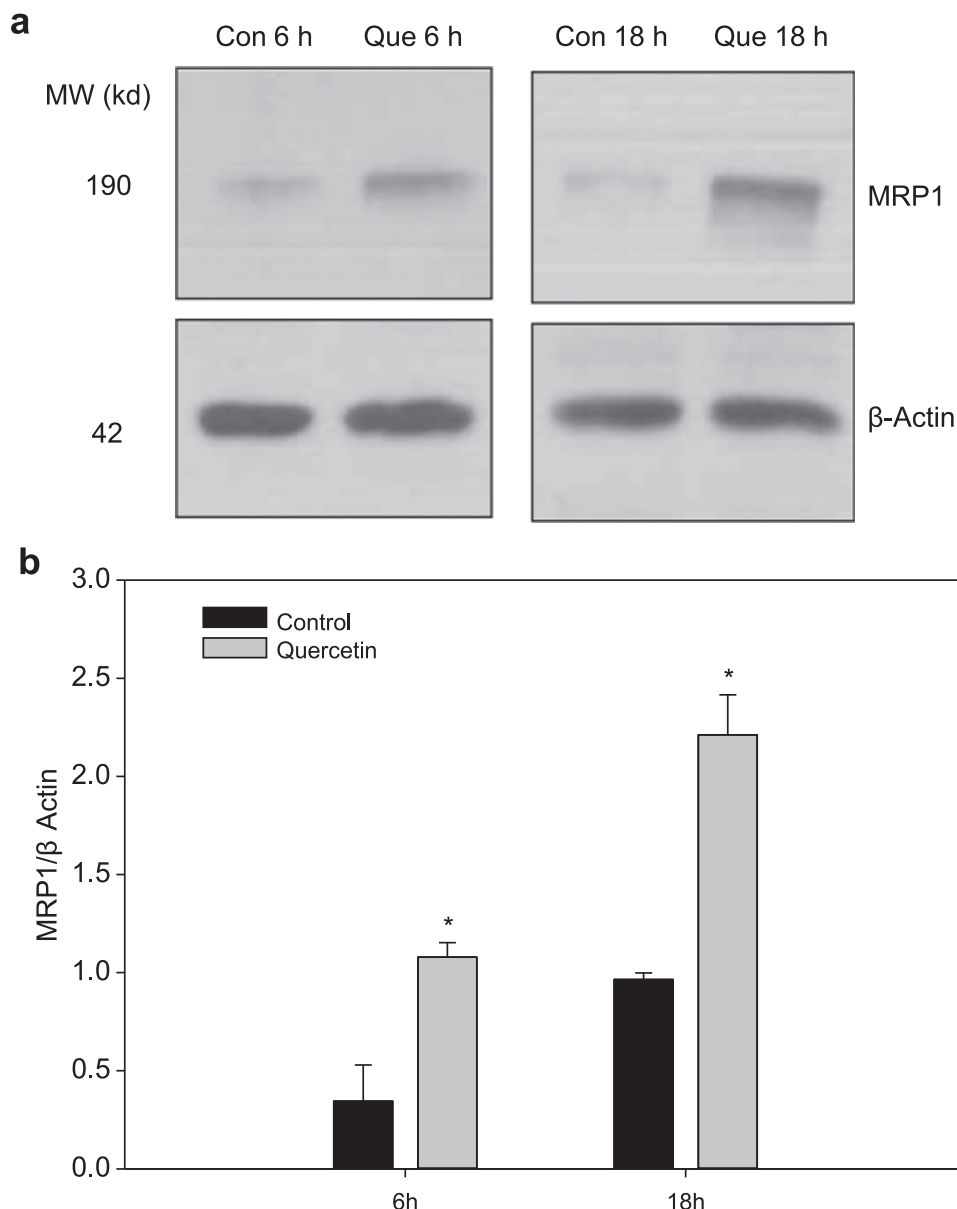


(Fig. 3b). Therefore, the existence of the quercetin-glutathione conjugate in the culture medium of quercetin-treated cells was confirmed. The specific position of the glutathionyl group on quercetin was not determined, but previous reports suggest that GSH reacts with quercetin at the 6- or 8-position [2,3] (Fig. 3b).

In contrast, the chromatogram representing the quercetin-glutathione conjugate was not detected in the medium of HAEC incubated without quercetin (Fig. 3d). Furthermore, no quercetin-glutathione conjugates were detected in the cell extracts of HAEC incubated with quercetin; in fresh cell culture medium; or in culture medium to which quercetin had been added (data not shown). These data strongly suggest that quercetin-glutathione conjugates are formed inside HAEC upon incubation with quercetin and rapidly excreted into the extracellular milieu; and hence are only detectable in the medium, but not in cellular extracts.

To quantify the quercetin-glutathione conjugates released by HAEC, a standard curve was constructed with purified standards (Fig. 3e) and the concentration of conjugates in the medium was

calculated. The quercetin-glutathione conjugates were detected in the medium of quercetin-treated HAEC at an average concentration of  $9.4 \pm 1.1$  nM, which corresponds to  $0.21 \pm 0.03$  nmol/mg protein of HAEC. As discussed above, quercetin treatment caused GSH levels in HAEC to initially drop by 12.7 nmol/mg protein (Fig. 2a). Therefore, the amount of quercetin-glutathione conjugates detected in the medium only accounts for about 1.7% of the decrease in intracellular GSH levels. However, quercetin-conjugates are not stable, with a reported half-life of only minutes in aqueous solution [4]. It is, therefore, likely that a substantial portion of the quercetin-conjugates was degraded and lost during incubation and subsequent sample work up. In addition, measurements by us (data not shown) and others [36] have shown that quercetin itself is not stable in aqueous solution and almost completely lost after 18 h of incubation in cell culture medium. This may lead to decreased formation of quercetin-glutathione conjugates in HAEC upon prolonged incubation, further contributing to the low yield in the extracellular medium.



**Fig. 4. Quercetin induces MRP1 expression.** HAEC were incubated for 6 or 18 h without (Con) or with 20  $\mu$ M quercetin (Que). MRP1 protein levels in cell extracts were measured by immunoblotting (a) and quantitatively analyzed (b). \*Denotes significant difference from the corresponding Control,  $P < 0.05$ .

### 3.4. Quercetin induces MRP1 expression

The multidrug resistance protein 1 (MRP1) is an ATP-binding cassette transporter on membranes known to be involved in cross-membrane cellular export of glutathione and glutathione conjugates [9,16,21,22]. To investigate whether MRP1 is involved in the effect of quercetin on cellular glutathione status, HAEC were incubated with 20  $\mu$ M quercetin for up to 18 h, and MRP1 protein expression was determined by immunoblotting. Quercetin induced significant increases in MRP1 protein levels at both 6 and 18 h of incubation (Fig. 4). These results suggest that MRP1-mediated export of quercetin-glutathione conjugates, and possibly GSH and GSSG, plays a significant role in the quercetin-induced changes in cellular glutathione status of HAEC.

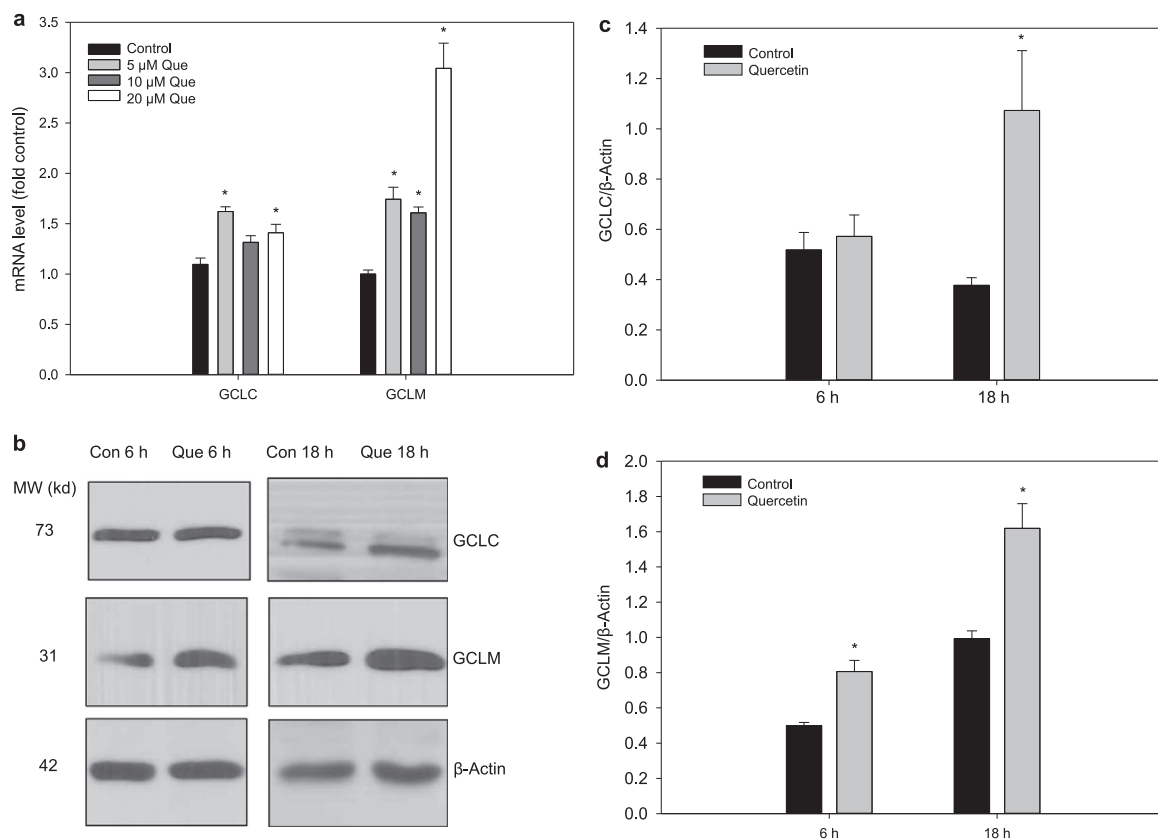
### 3.5. Quercetin induces GCLC and GCLM expression

GCL catalyzes the formation of  $\gamma$ -glutamylcysteine from cysteine and glutamate, which is the rate-limiting step of glutathione synthesis. To investigate whether GCL is involved in quercetin's effects on cellular glutathione levels, mRNA and protein levels of GCLC and GCLM in quercetin-treated HAEC were determined. Incubation for 18 h with quercetin dose-dependently increased mRNA levels of GCLM. Quercetin at 5  $\mu$ M and 20  $\mu$ M significantly increased mRNA levels of GCLC, but no obvious dose-response was observed (Fig. 5a). Furthermore, incubation of HAEC with 20  $\mu$ M quercetin for 6 or 18 h caused a significant increase in GCLM protein levels, while GCLC protein levels were only significantly elevated after 18 h of incubation (Fig. 5b–d).

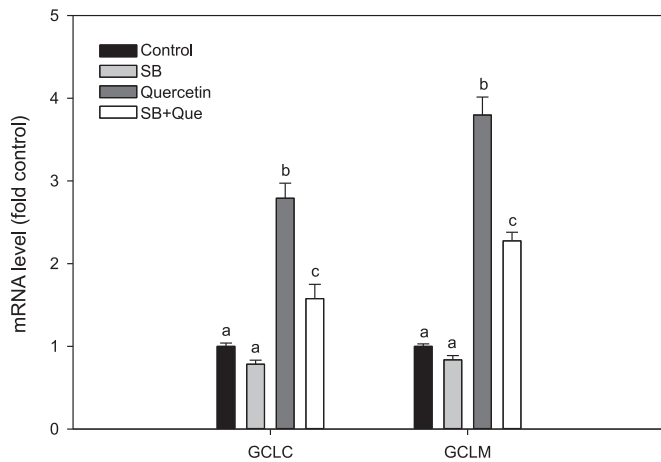
### 3.6. The protein kinase p38 is involved in quercetin-induced GCLC and GCLM expression

We have previously shown that quercetin induces the antioxidant enzymes, heme oxygenase 1 and NAD(P)H: quinone oxidoreductase 1, in HAEC through a mechanism involving activation of the p38 mitogen-activated protein kinase (p38) and the transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2) [17]. To investigate the role of p38 in mediating the upregulation of GCL by quercetin, HAEC were pre-incubated with 15  $\mu$ M SB203580, a specific p38 inhibitor, for 1 h, and then were incubated for another 12 h with 20  $\mu$ M quercetin. SB203580 significantly inhibited quercetin-induced GCLC and GCLM mRNA expression (Fig. 6), suggesting that quercetin induces GCLC/GCLM through p38. These results are consistent with previous reports of the involvement of p38 in regulating antioxidant enzyme expression [8,15].

As mentioned above, quercetin almost completely degrades in cell culture medium within 18 h of incubation. According to our findings in the present and previous [17] studies, it takes a certain time for p38 and Nrf2 to be activated by quercetin and for gene transcription and protein expression of Nrf2 target proteins (MRP-1, GCLC and GCLM) to be induced, with maximal protein expression observed after 18 h of incubation. Quercetin might also accumulate and be present in cells beyond 18 h of incubation, which would allow it to continue to exert biological effects despite the fact that extracellular quercetin has been completely degraded. However, the concentration and time course of quercetin's intracellular accumulation in HAEC are not known and require further investigation.



**Fig. 5. Quercetin induces GCLC and GCLM expression.** HAEC were incubated for 18 h without (Control) or with the indicated concentrations of quercetin (Que) and mRNA levels of GCLC and GCLM were measured by real-time qPCR (a). Furthermore, HAEC were incubated for 6 h or 18 h without (Con) or with 20  $\mu$ M quercetin (Que), and protein levels of GCLC and GCLM were determined by immunoblotting (b) and quantitatively analyzed (c, d). \*Denotes significant difference from the corresponding Control,  $P < 0.05$ .



**Fig. 6. The protein kinase p38 is involved in quercetin induced GCLC and GCLM expression.** HAEC were incubated for 1 h without (Control) or with 15  $\mu$ M of the p38 inhibitor, SB203580 (SB), and then were incubated without or with 20  $\mu$ M quercetin (Que) for 12 h. mRNA levels of GCLC and GCLM were measured by real-time qPCR. Results marked with different letters differ significantly,  $P < 0.05$ .

In summary, the results presented here show that quercetin significantly affects glutathione levels and redox ratio of HAEC. Quercetin caused a temporary decrease and then full recovery of intracellular GSH concentrations. The initial drop of GSH levels was not due oxidation to GSSG, but instead due to formation of quercetin-glutathione conjugates—demonstrated here for the first time in an endothelial cell type using LC-MS—and the rapid cellular export of these conjugates via MRP-1. At the same time, quercetin significantly lowered intracellular GSSG levels, likely due to MRP1 mediated export, thus increasing the GSH: GSSG ratio and making the intracellular redox environment even more reducing. The recovery of GSH levels upon prolonged incubation of HAEC with quercetin was preceded by upregulation of GCL, the rate-limiting enzyme in the *de novo* synthesis of GSH, due to quercetin-induced Nrf2 activation by a pathway involving p38. The induction of GCL might give HAEC an increased ability to rapidly synthesize GSH during an oxidant challenge. These quercetin-induced changes in glutathione levels and redox ratio may explain the antioxidant effects of quercetin against LPS-induced oxidant production in endothelial cells.

## Acknowledgment

This research was supported by the donor-funded Research Innovation Fund of the Linus Pauling Institute at Oregon State University; it did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

## References

- T. Akaboshi, R. Yamanishi, Certain carotenoids enhance the intracellular glutathione level in a murine cultured macrophage cell line by inducing glutamate-cysteine-ligase, *Mol. Nutr. Food Res.* 58 (6) (2014) 1291–1300.
- H.M. Awad, M.G. Boersma, S. Boeren, H. van der Woude, J. van Zanden, P.J. van Bladeren, J. Vervoort, I.M. Rietjens, Identification of o-quinone/quinone methide metabolites of quercetin in a cellular in vitro system, *FEBS Lett.* 520 (1–3) (2002) 30–34.
- M.G. Boersma, J. Vervoort, H. Szymusiak, K. Lemanska, B. Tyrakowska, N. Cenaz, J. Segura-Aguilar, I.M. Rietjens, Regioselectivity and reversibility of the glutathione conjugation of quercetin quinone methide, *Chem. Res. Toxicol.* 13 (3) (2000) 185–191.
- A.W. Boots, J.M. Balk, A. Bast, G.R. Haenen, The reversibility of the glutathionyl-quercetin adduct spreads oxidized quercetin-induced toxicity, *Biochem. Biophys. Res. Commun.* 338 (2) (2005) 923–929.
- C. Busu, V. Atanasiu, G. Caldito, T.Y. Aw, Influence of GSH synthesis inhibition on temporal distribution of NAD<sup>+</sup>/NADH during vascular endothelial cells proliferation, *J. Med. Life* 7 (4) (2014) 611–618.
- A. Callegari, Y. Liu, C.C. White, A. Chait, P. Gough, E.W. Raines, D. Cox, T. J. Kavanagh, M.E. Rosenfeld, Gain and loss of function for glutathione synthesis: impact on advanced atherosclerosis in apolipoprotein E-deficient mice, *Arterioscler. Thromb. Vasc. Biol.* 31 (11) (2011) 2473–2482.
- S. Canals, M.J. Casarejos, S. de Bernardo, E. Rodríguez-Martín, M.A. Mena, Nitric oxide triggers the toxicity due to glutathione depletion in midbrain cultures through 12-lipoxygenase, *J. Biol. Chem.* 278 (24) (2003) 21542–21549.
- J.S. Chen, P.H. Huang, C.H. Wang, F.Y. Lin, H.Y. Tsai, T.C. Wu, S.J. Lin, J.W. Chen, Nrf-2 mediated heme oxygenase-1 expression, an antioxidant-independent mechanism, contributes to anti-atherogenesis and vascular protective effects of Ginkgo biloba extract, *Atherosclerosis* 214 (2) (2011) 301–309.
- S.P. Cole, Targeting multidrug resistance protein 1 (MRP1, ABCC1): past, present, and future, *Annu. Rev. Pharmacol. Toxicol.* 54 (2014) 95–117.
- A. Costanzo, F. Moretti, V.L. Burgio, C. Bravi, F. Guido, M. Levrero, P.L. Puri, Endothelial activation by angiotensin II through NfκB and p38 pathways: Involvement of NfκB-inducible kinase (NIK), free oxygen radicals, and selective inhibition by aspirin, *J. Cell Physiol.* 195 (3) (2003) 402–410.
- M.L. Diaz-Hung, L. Blanco, N. Pavón, R. León, B. Estupiñán, E. Orta, K. Martínez, I. Fernández, Sensory-motor performance after acute glutathione depletion by L-buthionine sulfoximine injection into substantia nigra pars compacta, *Behav. Brain Res.* 271 (2014) 286–293.
- U. Förstermann, T. Münzel, Endothelial nitric oxide synthase in vascular disease: from marvel to menace, *Circulation* 113 (13) (2006) 1708–1714.
- C.J. Huang, M.J. McAllister, A.L. Slusher, H.E. Webb, J.T. Mock, E.O. Acevedo, Obesity-Related Oxidative Stress: the Impact of Physical Activity and Diet Manipulation, *Sports Med. Open* 1 (1) (2015) 32.
- A.M. Jenner, J.E. Ruiz, C. Dunster, B. Halliwell, G.E. Mann, R.C. Siow, Vitamin C protects against hypochlorous Acid-induced glutathione depletion and DNA base and protein damage in human vascular smooth muscle cells, *Arterioscler. Thromb. Vasc. Biol.* 22 (4) (2002) 574–580.
- K.W. Kang, S.J. Lee, S.G. Kim, Molecular mechanism of nrf2 activation by oxidative stress, *Antioxid. Redox Signal* 7 (11–12) (2005) 1664–1673.
- E.M. Leslie, R.G. Deeley, S.P. Cole, Bioflavonoid stimulation of glutathione transport by the 190-kDa multidrug resistance protein 1 (MRP1), *Drug Metab. Dispos.* 31 (1) (2003) 11–15.
- C. Li, W.J. Zhang, B. Frei, Quercetin inhibits LPS-induced adhesion molecule expression and oxidant production in human aortic endothelial cells by p38-mediated Nrf2 activation and antioxidant enzyme induction, *Redox Biol.* (2016), <http://dx.doi.org/10.1016/j.redox.2016.06.006>.
- S.B. Lotito, W.J. Zhang, C.S. Yang, A. Crozier, B. Frei, Metabolic conversion of dietary flavonoids alters their anti-inflammatory and antioxidant properties, *Free Radic. Biol. Med.* 51 (2) (2011) 454–463.
- S.C. Lu, Regulation of glutathione synthesis, *Curr. Top. Cell Regul.* 36 (2000) 95–116.
- B. Lundgren, J.W. DePierre, The metabolism of xenobiotics and its relationship to toxicity/genotoxicity: studies with human lymphocytes, *Acta Physiol. Scand. Suppl.* 592 (1990) 49–59.
- T. Minich, J. Riemer, J.B. Schulz, P. Wieling, J. Wijnholds, R. Dringen, The multidrug resistance protein 1 (Mrp1), but not Mrp5, mediates export of glutathione and glutathione disulfide from brain astrocytes, *J. Neurochem.* 97 (2) (2006) 373–384.
- C.F. Mueller, J.D. Widder, J.S. McNally, L. McCann, D.P. Jones, D.G. Harrison, The role of the multidrug resistance protein-1 in modulation of endothelial cell oxidative stress, *Circ. Res.* 97 (7) (2005) 637–644.
- K. Omar, M.H. Grant, C. Henderson, D.G. Watson, The complex degradation and metabolism of quercetin in rat hepatocyte incubations, *Xenobiotica* 44 (12) (2014) 1074–1082.
- B. Pamukcu, G.Y. Lip, E. Shantsila, The nuclear factor- $\kappa$ B pathway in atherosclerosis: a potential therapeutic target for atherothrombotic vascular disease, *Thromb. Res.* 128 (2) (2011) 117–123.
- H.S. Park, J.N. Chun, H.Y. Jung, C. Choi, Y.S. Bae, Role of NADPH oxidase 4 in lipopolysaccharide-induced proinflammatory responses by human aortic endothelial cells, *Cardiovasc. Res.* 72 (3) (2006) 447–455.
- F. Perticone, R. Ceravolo, M. Candigliota, G. Ventura, S. Iacopino, F. Sinopoli, P. L. Mattioli, Obesity and body fat distribution induce endothelial dysfunction by oxidative stress: protective effect of vitamin C, *Diabetes* 50 (1) (2001) 159–165.
- M.E. Pueyo, W. Gonzalez, A. Nicoletti, F. Savoie, J.F. Arnal, J.B. Michel, Angiotensin II stimulates endothelial vascular cell adhesion molecule-1 via nuclear factor- $\kappa$ B activation induced by intracellular oxidative stress, *Arterioscler. Thromb. Vasc. Biol.* 20 (3) (2000) 645–651.
- F.Q. Schafer, G.R. Buettner, Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple, *Free Radic. Biol. Med.* 30 (11) (2001) 1191–1212.
- A.R. Smith, F. Visioli, T.M. Hagen, Vitamin C matters: increased oxidative stress in cultured human aortic endothelial cells without supplemental ascorbic acid, *FASEB J.* 16 (9) (2002) 1102–1104.
- J.P. Spencer, G.G. Kuhnle, R.J. Williams, C. Rice-Evans, Intracellular metabolism and bioactivity of quercetin and its in vivo metabolites, *Biochem. J.* 372 (Pt 1) (2003) 173–181.
- D.M. Townsend, K.D. Tew, H. Tapiero, The importance of glutathione in human disease, *Biomed. Pharmacol.* 57 (3–4) (2003) 145–155.



- [32] K. Tsukimori, K. Fukushima, A. Tsushima, H. Nakano, Generation of reactive oxygen species by neutrophils and endothelial cell injury in normal and preeclamptic pregnancies, *Hypertension* 46 (4) (2005) 696–700.
- [33] I. Tuleta, C.N. França, D. Wenzel, B. Fleischmann, G. Nickenig, N. Werner, D. Skowasch, Hypoxia-induced endothelial dysfunction in apolipoprotein E-deficient mice; effects of infliximab and L-glutathione, *Atherosclerosis* 236 (2) (2014) 400–410.
- [34] F. Vargas, I. Rodríguez-Gómez, R. Pérez-Abud, P. Vargas Tendero, Y. Baca, R. Wangenstein, Cardiovascular and renal manifestations of glutathione depletion induced by buthionine sulfoximine, *Am. J. Hypertens.* 25 (6) (2012) 629–635.
- [35] G. Wu, Y.Z. Fang, S. Yang, J.R. Lupton, N.D. Turner, Glutathione metabolism and its implications for health, *J. Nutr.* 134 (3) (2004) 489–492.
- [36] L. Zhang, E. Angst, J.L. Park, A. Moro, D.W. Dawson, H.A. Reber, G. Eibl, O. J. Hines, V.L. Go, Q.Y. Lu, Quercetin aglycone is bioavailable in murine pancreas and pancreatic xenografts, *J. Agric. Food Chem.* 58 (12) (2010) 7252–7257.