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Quercetin Metabolites Up-Regulate the Antioxidant Response in Osteoblasts Isolated From Fetal Rat Calvaria

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ABSTRACT

Oxidative stress contributes to osteoporosis by suppressing differentiation of osteoblasts, suggesting the osteoblast antioxidant response may be a viable strategy for osteoporosis prevention. Quercetin, an antioxidant flavonol, up-regulates the antioxidant response in many cell types, but studies are needed to understand the effects of quercetin plasma metabolites on the osteoblast antioxidant response. The first specific aim was to examine antioxidant response genes and proteins in osteoblasts exposed to plasma quercetin metabolites. The second specific aim was to identify potential signaling pathways in the osteoblast antioxidant response that mediate the effect of quercetin, specifically Nrf2, ERK1/2, and NFκB p65. Osteoblasts isolated from fetal rat calvaria were treated with doses up to 20 μM of three different quercetin metabolites found in blood plasma after consumption of quercetin-rich foods or supplements: quercetin aglycone (QRC), isorhamnetin (ISO), or quercetin 3-O-glucuronide (Q3G). Alternatively, some cells received a 2:1:1 mixture of all three metabolites (10 μM Q3G: 5 μM ISO: 5 μM QRC) to evaluate synergistic effects. Antioxidant response genes and proteins known to be up-regulated by quercetin were analyzed along with Nrf2, ERK1/2, and NFκB proteins. Both QRC and ISO, but not Q3G, up-regulated heme oxygenase-1 (HO-1) and γ-glutamate cysteine ligase catalytic subunit (GCLC) at the mRNA and protein level. Synergistic effects of metabolites were not observed. Up-regulation of HO-1 and GCLC was associated with suppression of phosphorylated ERK1/2 and NFκB, but no alterations in Nrf2 protein levels were observed. This study shows that the antioxidant response of osteoblasts is differentially stimulated by quercetin metabolites. J. Cell. Biochem. 116: 1857–1866, 2015. © 2015 Wiley Periodicals, Inc.

KEY WORDS: OSTEOBLAST; ANTIOXIDANT RESPONSE; HEME OXYGENASE-1; QUERCETIN AGLYCONE; ISORHAMNETIN; QUERCETIN-3-O-GLUCURONIDE

Oxidative stress has been implicated as a major contributing factor in a number of chronic diseases, including osteoporosis [Almeida et al., 2007]. Oxidative stress directly suppresses the maturation and function of bone-forming osteoblasts [Hinoi et al., 2006] which along with increased osteoclastic bone resorption, leads to low bone mass and bone fragility observed in osteoporosis. These findings suggest that preventive strategies for osteoporosis may lie in targeting the antioxidant stress response of osteoblasts. Quercetin, a common antioxidant flavonoid found abundantly in many plant-based foods, has been investigated as an antioxidant that may prevent chronic diseases associated with oxidative stress [Boots et al., 2008]. Furthermore, quercetin improves bone density [Tsuji et al., 2009], bone strength [Huang et al., 2008], and microarchitecture [Siddiqui et al., 2010] in ovariectomized rodent models of estrogen deficiency bone loss, and quercetin-rich diets have been shown to be associated with higher bone density in human populations [Welch and Hardcastle, 2014]. Despite these findings, little is known about the effect of quercetin, or its plasma metabolites, on the osteoblast antioxidant response. Therefore, the overall goal of this study was to address these gaps in knowledge.

Quercetin aglycone has been shown to stimulate the expression of antioxidant response genes and proteins in various cell types, and these proteins may prevent damage from subsequent oxidative insult. For example, quercetin aglycone up-regulates heme oxy-

Abbreviations: GCLC, γ-glutamate cysteine ligase catalytic subunit; HO-1, heme oxygenase-1; ISO, isorhamnetin; NQO-1, NADPH:quinone oxidoreductase-1; Prdx, peroxiredoxins 3 and 5; QRC, quercetin aglycone; Q3G, quercetin 3-O-glucuronide; tBHQ, tert-butylhydroquinone.

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genase-1 (HO-1) in RAW264.7 macrophages [Boesch-Saadatmandi et al., 2011] and glioma C6 cells [Chen et al., 2005], γ-glutamate-cysteine ligase catalytic synthetase (GCLC) in primary neurons [Arredondo et al., 2010], catalase, peroxiredoxins (Prdx) 3 and 5 in trabecular meshwork cells of the eye [Miyamoto et al., 2011] and NADPH:quinone oxidoreductase-1 (NQO-1) in HepG2 cells [Tanigawa et al., 2007]. The most commonly reported mediator of quercetin glycone's effects is the redox sensitive transcription factor, nuclear factor (erythroid-derived 2)-like 2 (Nrf2), which binds to antioxidant response elements and initiates transcription of genes involved in protecting cells from oxidative stressors [Ma, 2013]. While the effect of quercetin aglycone on Nrf2 accumulation and subsequent up-regulation of antioxidant response genes has been reported in a number of cell types, this has not been described in cultures of primary osteoblast-like cells. Furthermore, other signaling pathways with overlapping roles in osteoblast development and stress signaling have been identified, including mitogen activated protein kinase extracellular-signal related kinase (ERK) 1/2 [Xiao et al., 2002; Bai et al., 2004] and the p65 subunit of nuclear factor-κB (NFκB), [Bai et al., 2004; Chen et al., 2013] but the effect of quercetin agylcone on these pathways has not been investigated fully in osteoblasts.

Most in vitro studies focus on quercetin aglycone even though quercetin and other flavonoids consumed in foods or supplements are extensively modified by enzymes in the gut and liver to produce a mixture of methylated, sulfated, and glucuronidated conjugates in the blood plasma [Kemperman et al., 2010]. In order to obtain a more complete picture of the effect of quercetin plasma metabolites on the osteoblast antioxidant response, *in vitro* studies are needed that describe the effects of multiple metabolites. Therefore, the first specific aim of this study was to examine antioxidant response genes and proteins (HO-1, GCLC, catalase, Prdx3, and 5, NQO-1) in osteoblasts exposed to quercetin aglycone and enzymatically conjugated quercetin metabolites (isorhamnetin and quercetin-3-O-glucuronide) in osteoblast-like cells isolated from fetal rat calvaria. The second specific aim of this study was to identify signaling pathways in the osteoblast antioxidant response that mediate the effect of quercetin metabolites, specifically targeting Nrf2, ERK1/2, and NFκB p65. We hypothesized that one or more quercetin metabolites would up-regulate antioxidant response genes and proteins, and that this would involve accumulation of Nrf2 and alterations in ERK1/2 and NFκB signaling.

MATERIALS AND METHODS

STUDY DESIGN

These studies were designed to investigate the extent that quercetin metabolites up-regulated the antioxidant response in healthy osteoblast-like cells during early differentiation. To assess the acute effects of quercetin metabolites on the osteoblast antioxidant response, cells were treated starting at D5 (confluence) with three different quercetin metabolites, quercetin aglycone (QRC), isorhamnetin (ISO), a methylated metabolite, or quercetin-3-O-glucuronide (Q3G), at doses up to 20 μM for a total of 48 h. Alternatively, some cells received a mixture of all three metabolites simultaneously (2:1:1 mixture, 10 μM Q3G: 5 μM ISO: 5 μM QRC). Samples were collected along a time course (0 to 48 h) and antioxidant response genes and proteins, and cell signaling proteins, were analyzed as described below. As a positive control, some cells were treated with tert-butylhydroquinone (tBHQ), a known inducer of the antioxidant response, at doses of 0, 20, or 40 μM for 3, 6, or 12 h starting on D10. To ensure that the quercetin doses and time frame of treatment did not disrupt the development of the osteoblast phenotype, alkaline phosphatase expression, an early marker of osteoblast phenotypic development, was assessed by alkaline phosphatase staining.

REAGENTS

Alpha-modified minimal essential media, fetal bovine serum, gentamicin, fungizone, and dimethylsulfoxide (DMSO) were purchased from Thermo Fisher Scientific (Waltham, MA). Penicillin, collagenase, ascorbic acid, β-glycerophosphate, dexamethasone, Red Violet LB salt, Naphthol AS MX-PO_4, hydrogen peroxide, tBHQ, QRC, and ISO were purchased from Sigma (St. Louis, MO, USA). Quercetin-3-O-glucuronide was purchased from Extrasynthese (Genay, France)

ANIMALS AND DIET

Timed-pregnant Sprague-Dawley rats were obtained on D 4 of pregnancy (Harlan Laboratories, Indianapolis, IN, USA) and housed with a 12 h light/dark cycle, and ad libitum access to water and Teklad soy protein-free rodent diet (Harlan, 2920X). A soy-free diet was used to avoid potential confounding effects of isoflavones found in soy on osteoblast development in utero. Total isoflavone concentrations were measured by the manufacturer for the lots used in these studies, and were near the lower limit of detection (3 and 8 mg isoflavones /kg of diet). Dams were euthanized on D21 of gestation by overexposure to CO_2. Fetuses were removed and placed in a petri dish on ice to induce hypothermia before decapitation and removal of calvariae (frontal and parietal bones) for cell isolation. All procedures were reviewed and approved by the University of North Carolina at Greensboro Institutional Animal Care and Use Committee.

CELL CULTURE

Fetal rat calvarial cultures proliferate until confluence at approximately D5, which is followed by a 2-week differentiation phase (approximately D5-21), culminating in formation of discrete nodules that stain positive for cell-surface protein alkaline phosphatase, and secrete a collagen-based protein matrix that becomes mineralized. This is accompanied by temporal up-regulation of osteoblast phenotypic genes, including collagen type 1a, alkaline phosphatase, bone sialoprotein , and osteocalcin, as well as the master regulator of osteoblast differentiation, transcription factor Runx2 [Aubin, 1998].

Isolation of osteoblast-like cultures from fetal rat calvaria have been previously described [Messer et al., 2010]. Cells were plated at 3,000 cell/cm^2 in 6-well culture dishes and incubated at 37 °C under 5% CO_2 for the duration of the experiment. Cell culture media was changed every 2–3 days and contained alpha-modified minimal essential media supplemented with 10% fetal bovine serum, 10% antibiotic solution, ascorbic acid (50 μg/μL), β-glycerophosphate (10 mM), and dexamethasone (10^{-8} M) to induce osteogenesis.

ALKALINE PHOSPHATASE STAINING

These staining methods have been previously described [Bonnelye et al., 2008]. Briefly, cells were washed in PBS, fixed in 10% neutral formalin buffer for 15 min, and rinsed with deionized water. Alkaline phosphatase-positive cells were stained using Naphthol AS MX-PO_4 as substrate and Red Violet LB salt as coupler. Alkaline phosphatase is expressed in osteoblasts and cells that have the potential to become osteoblastic.

PREPARATION OF STOCK SOLUTIONS

QRC (Fig. 1A), ISO (Fig. 1B), a methylated metabolite, or Q3G (Fig. 1C), a glucuronidated metabolite were dissolved in degassed DMSO and stored at −20 °C. For experiments using a mixture of metabolites, stocks were diluted at a 2:1:1 ratio of 10 μM Q3G: 5 μM QRC: 5 μM ISO. This ratio contained mostly conjugated metabolites, since this appears to be most prevalent in blood plasma after conjugation by enzymes in the gut and liver [Mullen et al., 2008]. Hydrogen peroxide (30% w/v) was freshly diluted in sterile, deionized water just before use. Quercetin metabolites at final concentrations up to 20 μM, and hydrogen peroxide at final concentration of 75, 150, or 300 μM were administered with fresh media. Some cells were treated with tBHQ at final concentrations of 0, 20, or 40 μM as a positive control. The vehicle control (0 μM) for hydrogen peroxide was sterile, deionized water, and the vehicle control for quercetin metabolites and tBHQ was 0.1% (v/v) DMSO.

QUANTITATIVE REAL-TIME PCR

Cells were lysed in 900 μL of Qiazol and RNA was isolated using RNeasy Universal Minikit (Qiagen, Valencia, CA, USA) and quantified using a Nanodrop spectrophotometer. Two micrograms of RNA was reverse-transcribed using a High Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using Taqman Gene Expression Assays and Taqman Fast Universal Mastermix (Applied Biosystems). Gene expression was quantified using the standard curve method with ribosomal 18 S as the endogenous control. RNA was collected from three separate wells for each treatment group and expression of HO-1, GCLC, NQO1, catalase, Prdx3, and Prdx5 were assessed.

WESTERN BLOTTING

These methods were previously described [Messer et al., 2010]. Briefly, protein lysates were resolved on NuPage 4–12% bis-tris gels (Invitrogen), transferred to a polyvinylidene membrane, and blocked for 1 h in 5% (w/v) fat-free milk in Tris-buffered saline solution with 0.05% (v/v) Tween-20 (TBS-T). Membranes were then probed overnight with primary antibody diluted in 5% (w/v) bovine serum albumin in TBS-T. Primary antibodies and dilutions for antioxidant response proteins included HO-1 (1:2000, Millipore, Temecula, CA, USA), GCLC (1:2000, Abcam, Cambridge, MA, USA), and catalase (1:1000, Abcam). Nrf2 (H300) antibody was purchased from Santa Cruz (1:800, Santa Cruz, CA, USA). Phosphorylated (1:2000) and total ERK1/2 (1:1000), and NFκB p65 (1:600) antibodies were purchased from Cell Signaling (Danvers, MD, USA). β-actin (1:25000, Sigma) or GAPDH (1:500, Santa Cruz) antibodies were used as the loading control. For fractionated proteins (see below), nucleoporin (1:5000, BD Biosciences) was used for nuclear fraction control and GAPDH was used as the cytosolic control. HRP-conjugated secondary antibodies were diluted to 1:5000 and included donkey anti-mouse (Santa Cruz), rabbit anti-goat (Santa Cruz) and goat anti-rabbit (Cell Signaling). Recombinant Nrf2 (Abnova, Taipei, Taiwan) was used as a positive control for the anti-Nrf2 antibody. Signal was detected with Western Lightning Chemiluminescence Reagent Plus kit (PerkinElmer, Waltham, MA, USA).

PROTEIN FRACTIONATION

To analyze nuclear accumulation of Nrf2, cell protein lysates were fractionated into cytosolic and nuclear compartments using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) per manufacturer's instructions. Cells from six wells were trypsinized with 0.2% trypsin, pooled, centrifuged at $500 \times g$ for 5 min and the pellet was washed with PBS. Cell pellets were lysed and fractionated according to instructions for a 20 μL packed cell volume. Samples were stored in −80 °C freezer.

STATISTICS

Data are expressed as mean +/− SEM. Statistical differences were determined using one-way analysis of variance (ANOVA) with Tukey post hoc analysis (SPSS version 17.0 for Windows, Chicago, IL, USA). Univariate analysis was performed to check for interactions when there were two main effects of dose and time. Student's *t*-tests were performed between control and treatment groups within each day when main effects were significant. The α level was set at 0.05.

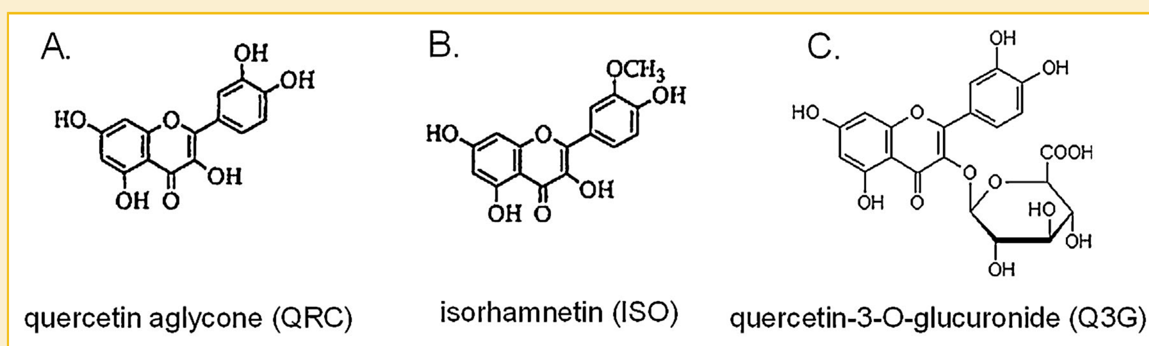

Fig. 1. Quercetin metabolites. (A) Quercetin aglycone, (B) methylated metabolite, isorhamnetin, (C) and glucuronidated metabolite, quercetin-3-O-glucuronide.

RESULTS

QUERCETIN UP TO 20 μM DID NOT DOWN-REGULATE OSTEOBLAST PHENOTYPE

Since alkaline phosphatase expression is an early marker for cells that have the potential to form mature osteoblasts, staining was used to assess potential QRC-induced suppression of osteoblast phenotypic development. Alkaline phosphatase staining was not down-regulated after a 48 h treatment with quercetin at doses up to 20 μM. However, the 40 μM dose suppressed alkaline phosphatase staining (Fig. 2A). Microscopic evaluation of stained wells under bright field illumination (Fig. 2B) indicated that the 40 μM dose resulted in cells with more diffuse alkaline phosphatase staining, and there were fewer cells arranged in discrete multi-layered, foci. Phase contrast images (Fig. 2C) show presence of cells in wells at all doses. Therefore, the doses used to stimulate the antioxidant response did not exceed 20 μM.

QUERCETIN METABOLITES UP-REGULATE ANTIOXIDANT GENES AND PROTEINS

Treating cells up to 48 h with 20 μM QRC resulted in marked up-regulation in expression of several genes involved in the antioxidant response, including HO-1, GCLC, and catalase (Fig. 3A). HO-1 and GCLC were significantly ($P < 0.05$) up-regulated as early as 3 h after beginning 20 μM QRC treatment compared to 0 μM control, and this was sustained for all time points for the entire 48 h period. In contrast, catalase gene expression was not markedly altered until 12 h after treatment and up-regulation was sustained at 24 and 48 h. Alterations in gene expression levels of NQO-1 and Prdx 3 were not detected, and levels of Prdx 5 were only slightly up-regulated by quercetin and only at 24 and 48 h. Protein levels of HO-1 and GCLC were up-regulated similarly to gene expression (Fig. 3B), but catalase protein expression appeared to be down-regulated after 24 and 48 h after 20 μM QRC treatment compared to control (0 μM), and was not similar to gene expression patterns.

Gene and protein expression of HO-1, GCLC, and catalase were also analyzed after treatment with either ISO, a methylated metabolite, Q3G, a glucuronidated metabolite (Fig. 4), or a 2:1:1 mixture of all three metabolites (10 μM Q3G, 5 μM QRC, and 5 μM ISO) (Fig. 5). Prdx 3 and 5, and NQO-1 were not analyzed since mRNA and protein levels were not altered by QRC at any time point. Treating cells with a 20 μM dose of ISO up-regulated levels of HO-1 and GCLC at both the mRNA (Fig. 4A) and protein levels (Fig. 4B), but these effects were less robust compared to the effects of QRC. There were no major alterations in catalase expression after treating with ISO. There was no marked up-regulation of HO-1, GCLC, or catalase after treatment with Q3G at the mRNA (Fig. 4C) or protein level (Fig. 4D). The metabolite mixture resulted in up-regulation of HO-1 and GCLC at the mRNA and protein level (Fig. 5), but there did not appear to be an additive, synergistic effect, such that the combination of metabolites resulted in more robust up-regulation compared to QRC alone.

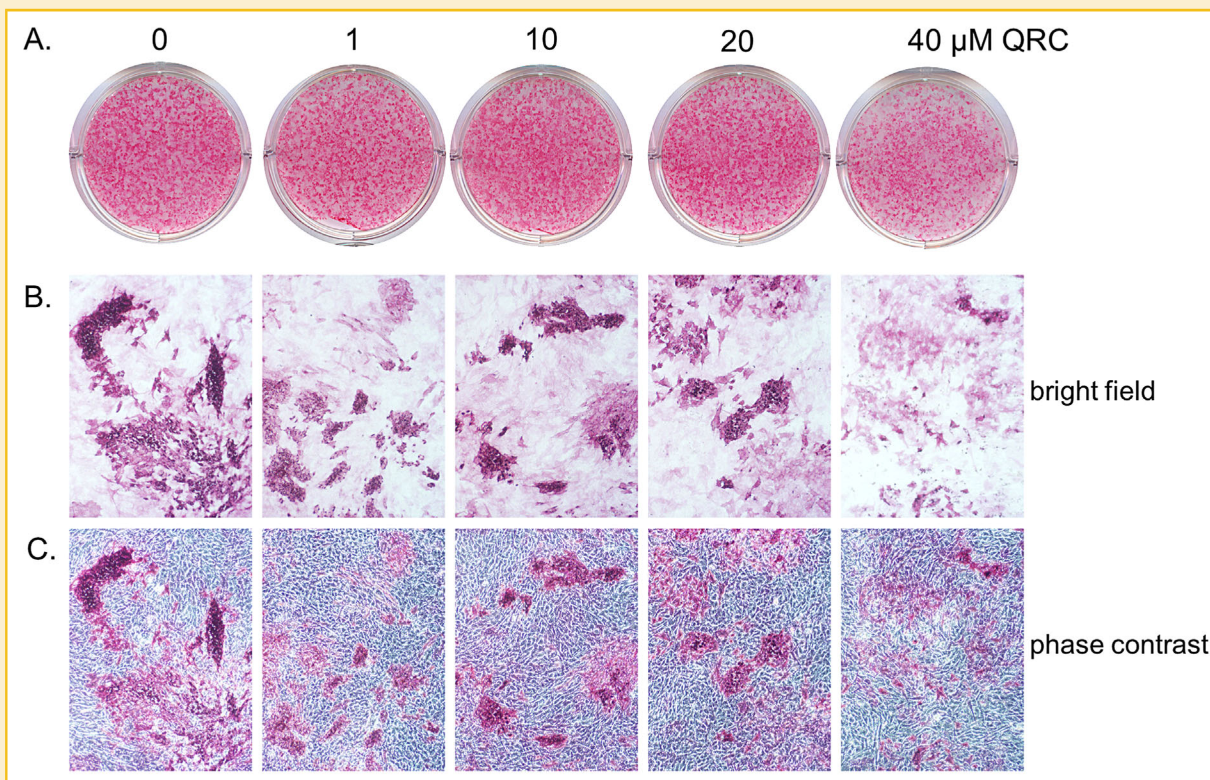

Fig. 2. Acute treatment with 20 μM quercetin aglycone during early differentiation does not reduce alkaline phosphatase staining. (A) Representative wells of alkaline phosphatase stained (pink) osteoblast-like cultures after treatment with 0–40 μM quercetin starting at D5 and treated for 48 h. (B) Bright field and (C) phase contrast micrographs of stained wells show that 40 μM quercetin aglycone results in more diffuse staining that is not localized to discrete foci.

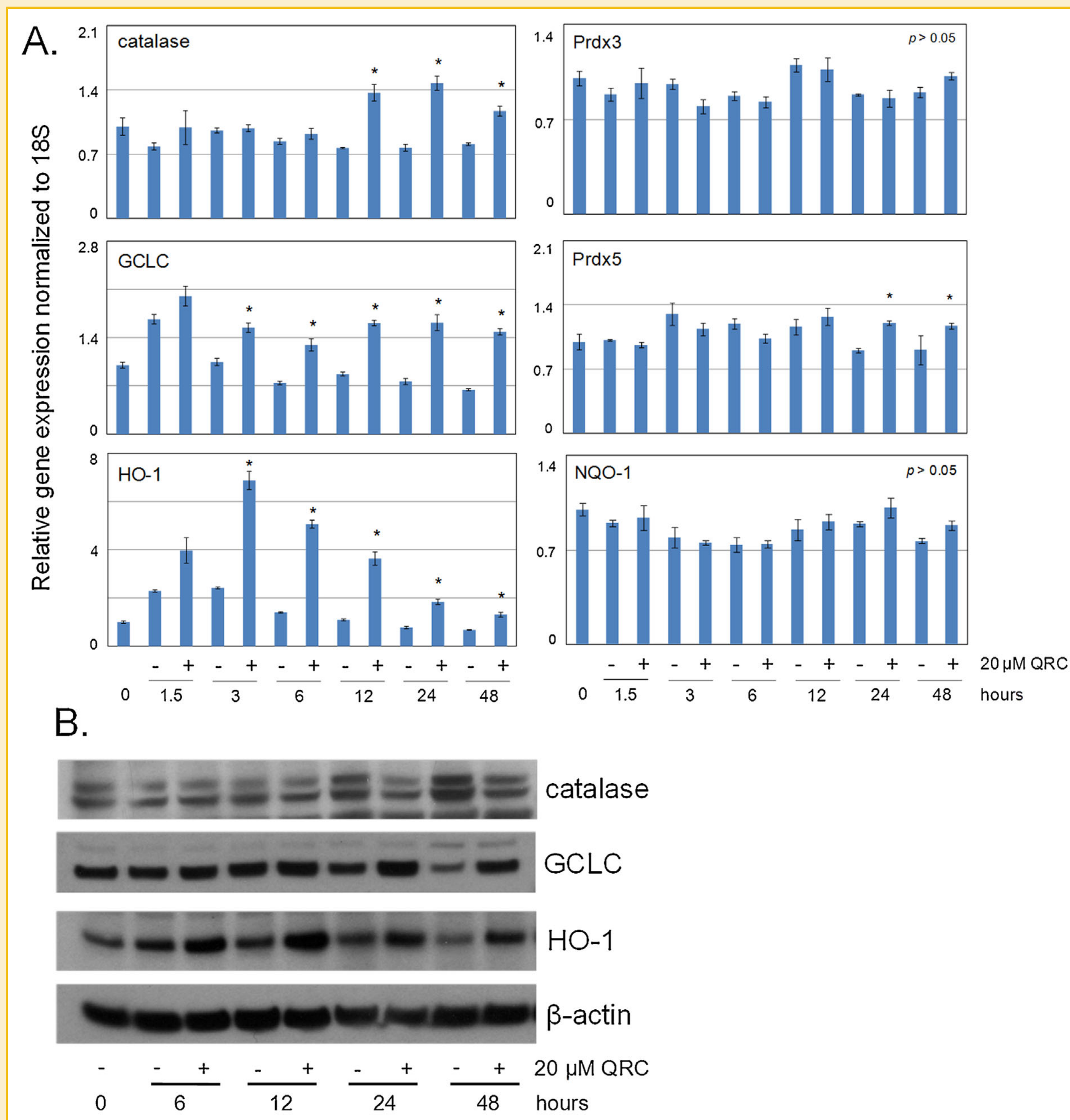

Fig. 3. Expression of antioxidant response genes and proteins in osteoblast-like colonies after treatment with 20 μM quercetin aglycone up to 48 h starting at confluence (day 5). (A) Real-time PCR amplification of heme oxygenase-1 (HO-1), γ-glutamyl-cysteine ligase catalytic subunit (GCLC) and catalase after up to 48 h of treatment with quercetin agylcone. Means +/− SEM, n = 3 separate wells of RNA from the same experiment. Univariate analysis of PCR revealed a significant interaction between time and quercetin treatment ($P < 0.05$). Significance between quercetin and vehicle control was assessed by student's *t*-tests within each time point. Asterisks indicate significant difference ($P < 0.05$) compared to control within each time point. (B) Protein levels of HO-1, GCLC, and catalase as assessed by Western blotting after up to 48 h treatment with quercetin. Similar results were observed in three independent studies.

QUERCETIN AGLYCONE DID NOT ALTER LEVELS OF NRF2

Since 20 μM QRC robustly up-regulated the expression of three Nrf2 target genes (HO-1, GCLC, and catalase), protein levels of transcription factor Nrf2 were assessed. The Nrf2 antibody detected two doublets that migrated to about 100 kDa and 70 kDa. It has been shown that endogenous Nrf2 does not migrate to its predicted molecular weight during electrophoresis, and the 95-100 kDa band is thought to represent biologically active Nrf2 [Lau et al., 2013; Pi et al., 2013]. Cells treated with 20 μM quercetin aglycone did not show any alterations in Nrf2 protein expression at any time point up to 48 h after treatment in whole cell lysates (Fig. 6). To address whether Nrf2 accumulated in the nucleus, Western blots were performed on nuclear protein fractions at doses up to 60 μM for up to 12 h, but QRC treatment did not result in detectable Nrf2 accumulation in the nucleus or the cytoplasm (data not shown). Similarly, treatment of cells with tBHQ did not result in

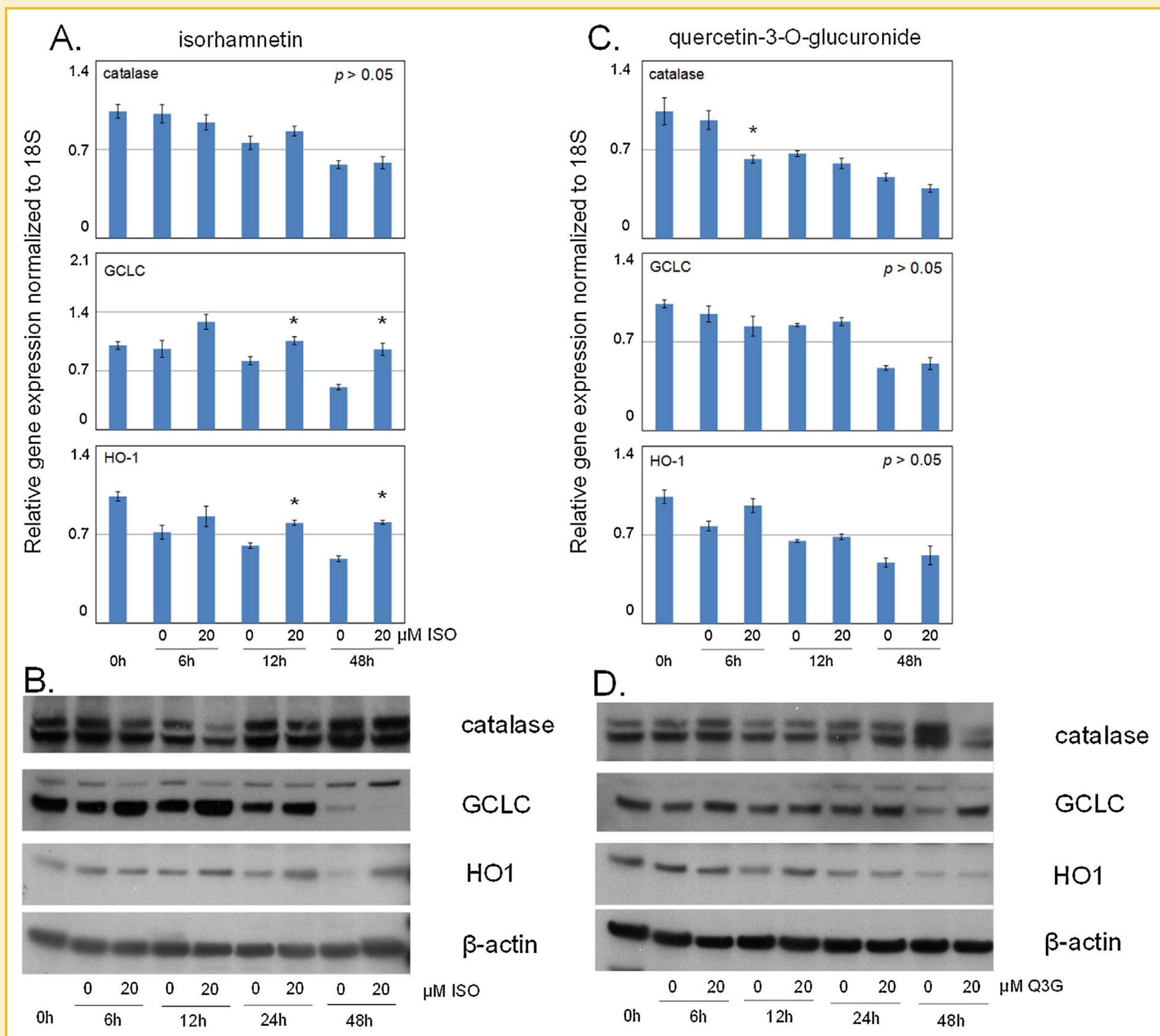

Fig. 4. Expression of antioxidant response genes and proteins in osteoblast-like colonies after treatment with 20 µM isorhamnetin, or 20 µM quercetin-3-O-glucuronide for up to 48 h starting at confluence (day 5). Real-time PCR amplification of heme oxygenase-1 (HO-1), γ-glutamyl-cysteine ligase catalytic subunit (GCLC) and catalase after up to 48 h of treatment with (A) isorhamnetin or C) quercetin-3-O-glucuronide. Means +/− SEM, n = 3 independent wells of RNA from the same experiment. Western blotting of heme oxygenase-1 (HO-1), γ-glutamyl-cysteine ligase catalytic subunit (GCLC) and catalase after up to 48 h of treatment with (B) isorhamnetin and (C) quercetin-3-O-glucuronide. Univariate analysis of PCR revealed no interaction ($P > 0.05$) between time and quercetin metabolite treatment. Significance between quercetin and vehicle control was assessed by Student's *t*-tests within each time point. Similar results were observed in at least two independent studies.

Nrf2 accumulation at any time point, in spite of dose-dependent up-regulation of HO-1 after 6 and 12 h of treatment (data not shown).

QUERCETIN ALTERED LEVELS OF PHOSPHORYLATED ERK1/2 AND NFκB

Since ERK1/2 has been shown to be involved in stress signaling and quercetin-induced responses in other cell types, phosphorylated and total ERK1/2 protein levels were assessed by Western blotting. A 20 µM dose of quercetin aglycone down-regulated levels of phosphorylated ERK1/2 within 1.5 h of treatment below basal levels, and this was sustained 6 h after treatment (Fig. 7A). Similarly, total levels of NFκB p65 were down-regulated at each of the time points analyzed (Fig. 7b). We did not detect alterations in the accumulation of NFκB p65 in Western blotting of nuclear fractions (data not shown).

DISCUSSION

These studies provide novel observations of the osteoblast antioxidant response after stimulation with three quercetin metabolites. A 20 µM dose of two quercetin metabolites, QRC and ISO, markedly up-regulated expression of three antioxidant genes (HO-1, GCLC, and catalase) in primary osteoblasts, with QRC producing the most robust effect. Up-regulation of HO-1 and GCLC mRNA resulted in congruent up-regulation in protein levels, but this

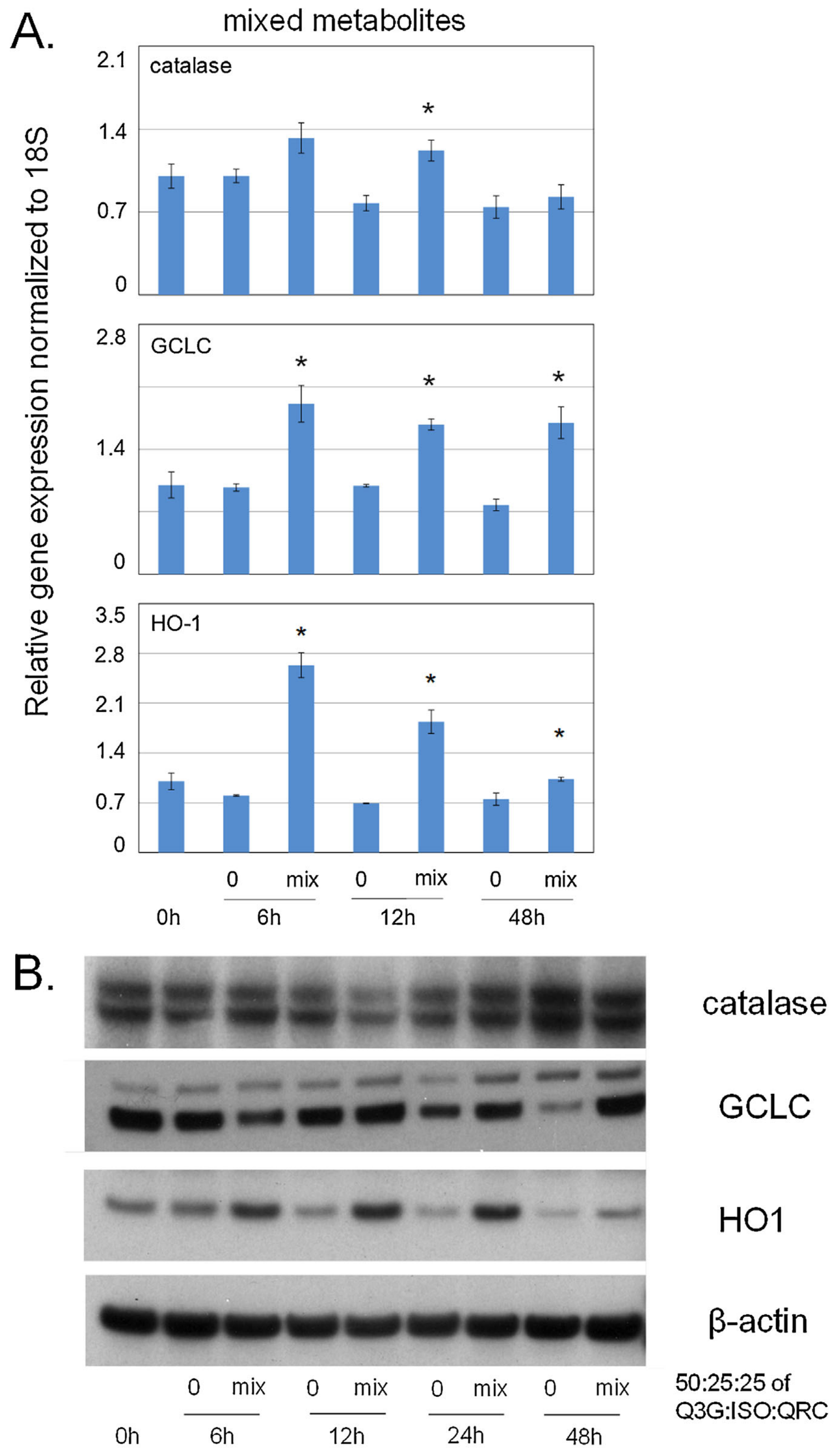

Fig. 5. Expression of antioxidant response genes and proteins in osteoblast-like colonies after treatment with a 2:1:1 mixture of 10 μM quercetin-3-O-glucuronide, 5 μM isorhamnetin, and 5 μM quercetin aglycone up to 48 h starting at confluence (day 5). (A) Real-time PCR amplification and (B) Western blotting analysis of heme oxygenase-1 (HO-1), γ-glutamyl-cysteine ligase catalytic subunit (GCLC) and catalase after up to 48 h of treatment with a 2:1:1 mixture quercetin metabolites. For PCR, means +/− SEM, n = 3 independent wells of RNA from the same experiment. Univariate analysis revealed no interaction ($P > 0.05$) between time and quercetin treatment for catalase and GCLC. Univariate analysis of HO-1 revealed a significant interaction. Significance between quercetin and vehicle control was assessed by Student's *t*-tests within each time point. Asterisks indicate significant difference ($P < 0.05$) compared to control within each time point. Similar results were observed in at least two independent studies.

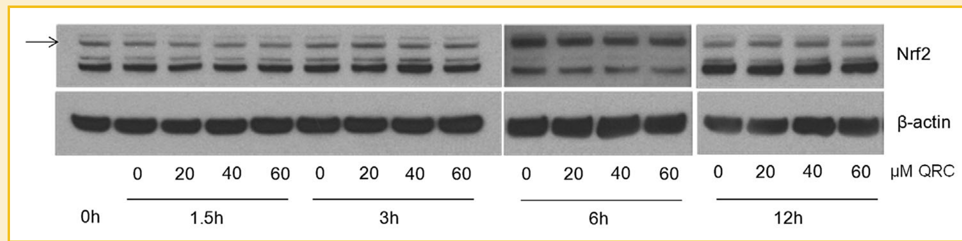

Fig. 6. Western blot of Nrf2 protein expression in whole lysates of osteoblast-like cells after treatment with 0 to 60 μM quercetin aglycone for up to 48 h starting at confluence (day 5).

was not observed for catalase, suggesting that other mechanisms are involved in regulating the final level of catalase protein in osteoblasts. The similar patterns of up-regulation of antioxidant gene expression after treatment with both QRC and ISO suggest that these two metabolites may stimulate similar pathways to induce the antioxidant response. In contrast to ISO and QRC, Q3G did not up-regulate expression of any antioxidant genes or proteins. Furthermore, results from cells treated with the mixture of metabolites, show up-regulation of gene expression that is consistent with the presence of the 5 μM dose of QRC in the mixture, and perhaps to a lesser extent ISO, but not Q3G. Taken together these data suggest that conjugation of the aglycone molecule diminishes the stimulatory effect on the osteoblast antioxidant response. Poor up-take of glucuronidated metabolites has been reported in neurons due to the relatively large size of the glucuronic acid conjugate [Spencer et al., 2003a]. It is not clear from the current studies if the conjugation of the aglycone molecule with methyl groups also partially prevents up-take, which may explain the less robust response of ISO.

Some antioxidant response genes assessed in this study were not up-regulated by any of the quercetin metabolites, including Prdx3, Prdx5, and NQO1. This suggests that quercetin metabolites do not produce a global effect on the expression of all antioxidant genes, rather there appear to be mechanisms in place to regulate the specificity of the genes that are transcribed. Based on this study, osteoblasts exposed to quercetin metabolites during early differentiation activate pathways that up-regulate expression of HO-1 and GCLC genes and proteins, and the functional outcome of the quercetin-induced up-regulation of the osteoblast antioxidant response may therefore rely on the activities of these two proteins.

These data are generally consistent with studies in other cell types that show up-regulation of antioxidant gene expression in response to QRC treatment, however, unlike other studies, this did not appear to coincide with detectable accumulation of Nrf2 protein, even at doses 2–3 times higher than that used to stimulate transcription of Nrf2 target genes (HO-1 and GCLC). The absence of Nrf2 accumulation was also observed in this study after treatment with tBHQ, a well-known inducer of Nrf2 accumulation and activation

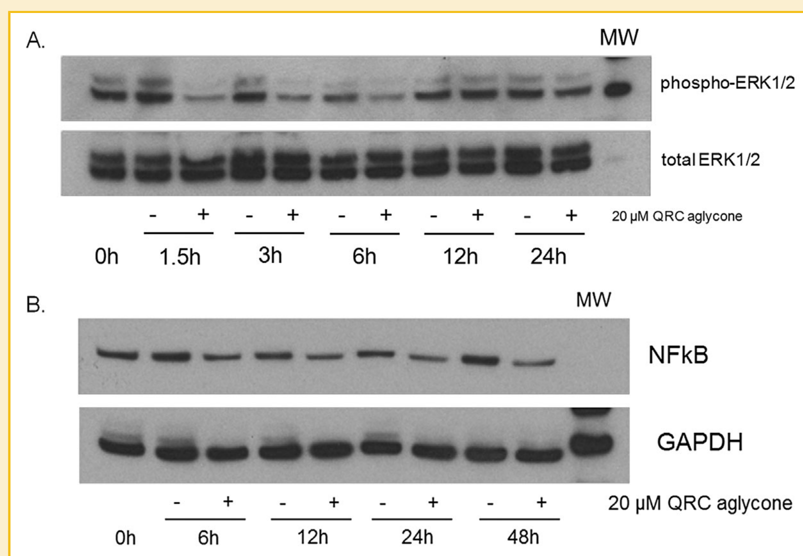

Fig. 7. (A) Western blot of ERK1/2 and phosphorylated ERK1/2 after treatment with 20 μM quercetin aglycone up to 24 h starting at confluence (D5); (B) Western blot of NFκB p65 after treatment with 20 μM quercetin aglycone up to 48 h starting at confluence (D5).

[Lau et al., 2013]. It is not clear why Nrf2 accumulation was not detected alongside up-regulation of antioxidant genes. The control of Nrf2 activation and subsequent transcription of antioxidant genes is complex and involves multiple post-translational modifications of both Nrf2 and its cytoplasmic sequestering protein, Keap 1, including phosphorylation, alterations in redox sensitive cysteine residues, and dimerization of Nrf2 with nuclear proteins [Ma, 2013]. These post-translational mechanisms appear to be differentially altered depending on the stimulus and the cell type, and result in variability in the level of Nrf2 accumulation and kinetics of the Nrf2-mediated transcription [Kobayashi et al., 2009; Lau et al., 2013; Pi et al., 2013]. Further experiments to examine Nrf2 post-translational modifications and Nrf2 activity levels in osteoblasts are needed before the role of Nrf2 in the quercetin-induced up-regulation of the antioxidant response can be ruled out.

These data showed suppression of phosphorylated ERK1/2 and NFκB p65, which suggests a role for these cell signaling proteins in initiating or sustaining quercetin-induced up-regulation of the osteoblast antioxidant response. There are currently only two studies examining the effect of QRC on ERK signaling pathways in osteoblast cell lines. In MC3T3-L1 osteoblasts QRC induced phosphorylation of ERK1/2 up to 6 h after treatment with a 20 μM dose [Nam et al., 2008], and in MG-63 osteosarcoma cells, QRC induced a transient phosphorylation of ERK1/2 after only 5 minutes with a 50 μM dose [Prouillet et al., 2004]. Both of these studies are in direct contrast to the effects that were observed in the current study. In other cell types, alterations in ERK1/2 phosphorylation by QRC have been described, but results are inconsistent despite similar doses (20–30 μM) and time points (up to 24 h) among studies. For example, QRC suppressed phosphorylated ERK1/2 in primary cortical neurons [Spencer et al., 2003b], but up-regulated phosphorylated ERK1/2 in BEAS-2B bronchial epithelial cells [Lee et al., 2011]. Like ERK1/2, QRC appears to suppress NFκB p65 protein levels. Several lines of evidence suggest that Nrf2 activators down-regulate NFκB signaling [Li et al., 2008], and this has also been shown in MC3T3-E1 osteoblasts [Yamaguchi et al., 2011], which suggests that activation of the antioxidant response may also be anti-inflammatory. This observation is of particular importance to bone, since inflammation has been shown to down-regulate development of the osteoblast phenotype and activate osteoclastic resorption. The effect of QRC on cell signaling pathways is markedly different depending on cell type and culture conditions. Future studies are needed to establish the exact role of ERK1/2 and NFκB p65 in the osteoblast stress response, and to what extent these signaling pathways are required for up-regulation of antioxidant genes.

The quercetin-induced antioxidant response in fetal rat calvaria cells occurred in early stages of differentiation (D5–D7). This suggests that osteoprogenitors and pre-osteoblasts in oxidative stress environments may benefit from stimulation with antioxidants that offer protective effects on osteoblast phenotypic development. Although this study demonstrates that plasma metabolites of quercetin stimulate the osteoblast antioxidant response, inherent difficulties in translating the in vitro setting to physiological conditions limits the interpretation of data in an in vivo context. The doses required to detect effects in this study are generally considered higher than that found in the blood plasma after consumption [Mullen et al., 2008, 2006] of QRC or quercetin-rich foods, and high inter-individual variability has been described among humans that consume quercetin-rich foods [Egert et al., 2008]. Additionally, roughly 20 plasma quercetin metabolites have been reported after consuming quercetin or quercetin-rich foods [Mullen et al., 2006] but we are only aware of three metabolites that are commercially available. These studies have provided evidence of differential effects of quercetin metabolites on the osteoblast antioxidant response and have identified potential molecular targets in the osteoblast antioxidant response that may be altered by flavonols.

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