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Oxidative stress plays a major role in development of osteoporosis, in part, by suppressing the differentiation and function of bone forming osteoblasts. This finding suggests that strategies to prevent or reverse osteoporosis may lie in targeting the osteoblast antioxidant response. Quercetin is an antioxidant flavonoid found abundantly in the diet and in supplements, and is known to induce expression of antioxidant response genes and proteins in a variety of cell types. The purpose of these studies was to examine the extent that quercetin metabolites up-regulate the antioxidant response, to identify cell signaling pathways that might be involved, and to examine the extent that quercetin preserves development of the osteoblast phenotype when cells are cultured in an oxidative stress environment. We hypothesized that quercetin metabolites would up-regulate the antioxidant response, and that this up-regulation would protect cells from oxidative stress-induced suppression. Studies were performed in osteoblast-like cultures isolated from fetal rat calvaria that were treated with 0 to 20 μM quercetin aglycone (QRC), isorhamnetin (ISO), quercetin-3-O-glucuronide (Q3G) or a 2:1:1 mixture of all three metabolites (10 μM Q3G: 5 μM QRC: 5 μM ISO). The antioxidant response was assessed by measuring expression of antioxidant genes and proteins. Results indicated that QRC and ISO robustly up-regulated expression of two antioxidant response genes and proteins, heme oxygenase-1 (HO-1) and the catalytic subunit of γ-glutamate cysteine ligase, but Q3G had no effect. Cell signaling protein, ERK1/2, and transcription factor NFκB proteins were also down-regulated by quercetin. To examine the effect of

quercetin on oxidative stress-induced suppression of osteoblast phenotype, cells were pretreated 12h with 20 μM QRC followed by incubation with 0 or 300 μM hydrogen peroxide, a known inducer of oxidative stress. Differentiation was assessed by alkaline phosphatase staining and expression of osteoblast phenotypic gene markers. Pretreating cells with 20 μM QRC partially blocked hydrogen peroxide-induced suppression of osteoblast phenotype, as indicated by higher levels of alkaline phosphatase staining and gene expression of osteoblast phenotype markers compared to cells pretreated with 0 μM QRC. QRC also partially blocked hydrogen peroxide-induced up-regulation of HO-1. These results suggest that quercetin produces a low grade antioxidant response that "primes" cells to withstand a subsequent oxidative stress event, which protects development of osteoblast phenotype. These findings offer important insight into the osteoblast antioxidant stress response, and support a link between osteoblast stress signaling and phenotypic development.

THE EFFECT OF QUERCETIN ON THE ANTIOXIDANT RESPONSE AND PHENOTYPIC DEVELOPMENT OF OSTEOBLASTS

by

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

Overview

More than 40 million Americans are at high risk to develop, or have been diagnosed with osteoporosis (1). Osteoporosis results in debilitating fragility fractures, and direct medical costs associated with these fractures are expected to exceed $25 billion by 2025 (2). Oxidative stress contributes to bone fragility, in part, by directly suppressing the phenotypic development and function of bone-forming osteoblasts (3-5). Concurrently, a diet high in antioxidant-rich, plant-based foods appears to be associated with higher bone density (6,7). Taken together this evidence supports the investigation of the effects of antioxidant flavonoids on the antioxidant response and phenotypic development of osteoblasts.

Quercetin is one of the most abundant flavonoids found in plant-based foods, and has been shown to protect bone health in animal studies. In ovariectomized rodent models of estrogen deficiency-induced bone loss, quercetin or quercetin-rich food extracts administered in the diet resulted in higher bone density (8), higher bone strength (9), and improved microarchitecture (10) compared to estrogen deficient ovariectomized controls that did not receive quercetin. Therefore, quercetin appears to be a viable target molecule to investigate the extent that flavonoids prevent or reverse osteoporosis via osteoblast-specific mechanisms.

Previous studies investigating the effects of quercetin on osteoblasts are limited and contradictory, where some studies show stimulation of alkaline phosphatase activity (11), up-regulated osteoblast phenotypic gene markers, and calcium deposition (12), but other studies show that quercetin stimulates apoptotic cell death (13,14). Additionally, many *in vitro* studies have primarily included only one quercetin metabolite, despite the fact that quercetin is enzymatically modified in the gut and liver such that there are multiple metabolites found in blood plasma after ingesting quercetin. There have also been no studies, to our knowledge, specifically examining the effect of quercetin on the osteoblast antioxidant response and its signaling pathways during oxidative stress.

In other cell types, quercetin appears to protect cells from oxidative stress by activating the transcription of antioxidant response genes, which code for proteins involved in redox homeostasis (15-17). This response is classically thought to be mediated by transcription factor Nrf2, which binds to antioxidant response elements in the promoter region of these genes. However, other proteins, such as the mitogen-activated protein kinase ERK1/2, and transcription factor NFκB have been shown to have overlapping roles in the antioxidant response and osteoblast phenotype development (18-20), but the relationship of these proteins to the osteoblast antioxidant stress response and quercetin treatment is largely unknown.

Research Hypothesis and Specific Aims

The overall objective of this project is to examine the potential benefits of dietary antioxidant flavonoids, such as quercetin, on phenotypic development and function of osteoblasts, and to examine the mechanism by which quercetin interacts with the

osteoblast antioxidant response. The central *hypothesis* is that quercetin metabolites will up-regulate the antioxidant response and preserve osteoblast development in fetal rat calvarial cells cultured in a high oxidative stress environment.

The *first specific aim* is to **c**haracterize the extent to which plasma quercetin metabolites up-regulate the antioxidant response of osteoblast-like fetal rat calvarial cells, with the hypothesis that one or more quercetin metabolites will up-regulate the antioxidant response. The *second specific aim* is to identify potential signaling pathways involved in quercetin-induced alterations in the antioxidant response. We hypothesize that the antioxidant response will involve alterations in at least one of the following pathways: Nrf2, ERK1/2, and NFκB, which are all involved in stress signaling and have overlapping roles in osteoblast phenotypic development. The *third specific aim* is to examine the extent that quercetin preserves phenotypic development and alters the antioxidant response of osteoblasts cultured in an oxidative stress environment, with the hypothesis that quercetin will preserve the osteoblast phenotype and block activation of the antioxidant response when cells are in a suppressive, oxidative stress environment.

This study is the first to use a primary osteoblast cell model treated with several quercetin metabolites to investigate the effects of quercetin on the antioxidant response and phenotypic development in osteoblasts cultured in an oxidative stress environment. This study provides a greater understanding of how flavonoids interact with bone cells under oxidative stress, which will provide evidence that supports development of antioxidant interventions that prevent or reverse osteoporosis. Additionally, this work

contributes to a broader understanding of how specific food components interact with cells to prevent or reverse human disease.

CHAPTER II

REVIEW OF THE LITERATURE

Oxidative Stress Suppresses Phenotypic Development of Osteoblasts

Oxidative stress plays a major role in the development of osteoporosis. The aging process (21,22) and menopausal estrogen deficiency (4,5), two risk factors associated with the development of osteoporosis, both result in oxidative stress in the osteoblast. Oxidative stress in osteoblasts is characterized by increased production of free radicals that results in damage to various cellular organelles, and can lead to cell death (19,23). In this condition, cells generally respond by up-regulating genes and proteins involved in maintaining redox homeostasis and protecting the cell from the damaging free radicals (24-26). Evidence demonstrates that transcriptional pathways that confer redox homeostasis also interfere with transcriptional pathways that regulate the osteoblast differentiation program (24), thereby suppressing osteoblast development and function. For example, the Forkhead box O (FoxO) family of transcription factors diverts an important scaffolding protein, β-catenin, away from the Wnt-signaling pathway, thus reducing Wnt/β-catenin signaling that is required for osteoblast differentiation (4). Similarly, the antioxidant response transcription factor, Nrf2, down-regulates osteoblast phenotype gene marker osteocalcin by physically binding to transcription factor Runx2, the master regulator of osteoblast differentiation and preventing its activity (3). Evidence also suggests that oxidative stress suppresses Maf transcription factors, shifting the

differentiation of osteoblast precursor cells away from osteogenesis, and towards adipogenesis, further compromising the bone integrity (27). Oxidative stress, therefore, appears to contribute to bone fragility by suppressing multiple differentiation pathways in osteoblasts and their precursors, which would inhibit formation of new bone. Therefore, interventions that target alleviation of oxidative stress in osteoblasts may be an important strategy for preventing or reducing bone fragility seen in osteoporosis.

Plant-based Diets Rich in Antioxidant Flavonoids Benefit Bone Health

Plant-based foods that are high in antioxidants may impart protective effects on the skeleton. Supporting this hypothesis, cross-sectional and epidemiological evidence indicate a positive association between high intake of antioxidant rich plant foods and bone health. Prynne et al (6) showed that a 2-fold increase in intake of fruits and vegetables was associated with an approximately 6% increase in bone mineral density in the spine in girls, a 4% increase in the spine in boys, and an approximately 5% increase in the bone mineral content of the spine in older women. Similarly, a cross-sectional study in post-menopausal Chinese women found a positive association between fruit and vegetable intake and bone density in the whole body and lumbar spine (28). Studies investigating the relationship between bone health parameters and intake of specific antioxidant-rich foods show similar results. Intake of onions, abundant in the antioxidant flavonol quercetin, was associated with approximately 5% higher BMD at the lumbar spine, total body, and total leg in peri- and post-menopausal, non-Hispanic, white women living in the United States (29). Black tea, the primary source of flavonol intake in the United States (30), is also protective of bone mineral density in elderly women (31).

Only one study has directly evaluated the specific intake of flavonoids and bone health, and found a positive association between total flavonoid intake and bone mineral density in the femoral neck and lumbar spine in peri-menopausal women (32). These studies show that plant-based diets appear to be protective of bone health across the lifespan and in various ethnic populations. Taken together, these data provide evidence that plant-based flavonoids might be an effective way to prevent or reverse osteoporosis.

Antioxidant Flavonoid Quercetin may Protect Bone Health

Despite the positive association between plant-based foods and bone health, the cause of these observed effects remains unknown. It has been hypothesized that antioxidant flavonoids in plant-based foods may ameliorate oxidative stress in osteoblasts. Quercetin has received attention as a flavonoid that may impart multiple disease-preventive health benefits, whether administered as a supplement or in the diet (33). Quercetin is an abundant flavonoid in the Western diet (34), and is found in many types of commonly consumed foods, such as onions (35), apples (36), and tea (30). Studies specific to osteoporosis typically have focused on the extent that quercetin or quercetin-rich foods prevent bone loss in ovariectomized rat models of post-menopausal osteoporosis. These studies largely support the hypothesis that quercetin benefits bone health. Quercetin-rich onion powder extract supplemented in the diet of ovariectomized rats resulted in lower trabecular separation and increased bone strength compared to ovariectomized controls (9). Direct supplementation of quercetin aglycone in the diet resulted in higher bone density in the femur and L4 vertebra in ovariectomized mice (8), and improved microarchitecture (bone volume, trabecular separation, and trabecular

number) in femur epiphyses of ovariectomized rats (10). Additionally, biomechanical quality was improved by quercetin in osteopenic rat models of diabetes (37). Studies such as these lend support to the idea that quercetin may be, at least in part, responsible for the positive association between plant-based food consumption and bone health. Furthermore, these studies make quercetin a viable target molecule for investigating the extent to which quercetin protects phenotypic development of osteoblasts during oxidative stress.

Although animal studies suggest that quercetin protects bone health, the cellular target of quercetin in bone has not been fully elucidated. In studies conducted with cultures of bone-resorbing osteoclasts, quercetin aglycone appears to induce apoptosis and reduce osteoclastic-activity (38,39), suggesting that quercetin may, in part, protect bone integrity by limiting bone resorption. However, studies conducted in osteoblast cell models are often not congruent with evidence from animal studies, and report increased apoptotic cell death. For example, in MC3T3-E1 cells, 20 μM quercetin aglycone stimulated Bax up-regulation and apoptosis (13), and 10 μM quercetin aglycone enhanced apoptosis in the MC3T3-E1 osteoblast cell line, but not in primary calvarial osteoblasts, when co-incubated alongside pro-apoptotic cytokine TNF-α (14). In contrast, other studies show that quercetin directly stimulates osteogenic markers. A 50 μM dose of quercetin aglycone stimulated alkaline phosphatase activity in MG-63 cells, a human osteoblast line (11), and in human primary mesenchymal stem cells, pretreating with 5 μM quercetin aglycone before incubation with osteogenic media resulted in higher Alizarin red-stained calcium deposits and higher expression of osteoblast gene markers

(e.g. osteopontin, alkaline phosphatase, and Runx2) compared to cells without quercetin pretreatment (12). The paradoxical evidence that quercetin aglycone causes both cell death and enhances osteogenesis, highlights the importance of further experiments to examine the relationship between quercetin, oxidative stress, and development of the osteoblast phenotype.

The primary limitations of the previous studies in bone cells are the reliance on only one quercetin metabolite (the aglycone form), and doses that are outside of the range that is physiologically achievable in blood plasma from the diet. Dietary quercetin is extensively metabolized by enzymes in the small intestine, liver, and kidneys to produce plasma metabolites that include not only the parent molecule, quercetin aglycone, but several sulfated, methylated, and glucuronidated conjugates (40). Plasma concentrations of quercetin metabolites are difficult to absolutely determine, due to wide variability in experimental methodology between studies (e.g. administration method, animal species, detection method, and the molecular form of quercetin administered during treatment). For example, administering 50 mg quercetin aglycone per kg body weight by gavage resulted in peak total plasma metabolite concentration of 2.5 μM (41) in one study, but 13.2 μM in a different study (42) after 1h in rats. This same dose, when administered as 0.1% (w/w) quercetin aglycone in the rats' diet, resulted in 7μM total plasma metabolites (consisting of about 1.5 μM non-methylated metabolites and about 5 μM methylated metabolites) after 4 weeks of treatment (41) or up to 23.4 μM total plasma metabolites (consisting of 7.7 μM quercetin aglycone and 15.7 μM isorhamnetin) after 11 weeks of treatment (43). Conversely, only 0.81μM total plasma quercetin metabolites were

measured in pigs fed 50 mg quercetin aglycone per kg body weight in a meals for 4 weeks (44). Median intake of quercetin in humans has been reported to range between 7-33 mg/day (45), but has also been difficult to absolutely establish given variability in study designs, as well as individual and cultural food preferences (46). Therefore, it is not clear as to whether the doses administered in pharmacokinetic animal studies are congruent with amounts of quercetin that would be regularly achievable in the human diet. In clinical trials, high inter-individual variability in peak total quercetin plasma concentrations has been noted, and peak concentration for each plasma metabolite is also variable, but in the range of 45 to 600nM (35,47), and perhaps as high as 5 μM (48).

Although this wide variability makes the physiological concentration of quercetin metabolites in the blood plasma difficult to determine with absolute certainty, these studies demonstrate that dietary or supplemented quercetin consumed orally will result in a maximum concentration in the blood plasma in the high nanomolar to low micromolar range. Therefore, these experiments focused on doses of multiple quercetin metabolites, which are more reasonably achievable plasma concentrations attained from ingestion of quercetin or quercetin-containing foods.

Quercetin Protects Cells from Oxidative Stress

There are currently no studies that have explored the relationship between quercetin and the oxidative stress response in osteoblast-like cells. Studies in other cell types, however, provide evidence of the mechanism by which quercetin or its metabolites impact the cellular stress response of cells. One of the primary targets of quercetin appears to be nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Nrf2 is a transcription

factor which, when activated by oxidative stressors, initiates transcription of stress response genes by binding to the antioxidant response elements (AREs) in the promoter region of these genes (49). Relatively short-term treatment (0.5h to 48 h) of cells with quercetin aglycone (ranging from 1 to 100 μM) has been shown to up-regulate Nrf2 at both the transcriptional and post-translational level (15,17,26). The quercetin-mediated up-regulation of Nrf2 protein results in increased binding to AREs, and subsequent transcription of genes involved in the stress response, such as NADPH:quinone oxidoreductase (15), heme oxygenase-1 (16,25), peroxiredoxins 3 and 5 (17), and the catalytic subunit of ɣ-glutamate-cysteine ligase (26). In addition to up-regulating genes and proteins involved in the stress response, evidence also indicates that quercetin may detoxify free radicals by direct scavenging and subsequent interaction with the intracellular tri-peptide thiol, glutathione (50,51). Functionally, quercetin is able to decrease free radical damage (50) and increase cell survival and function (25,25,51) in the face of oxidative stress by activating the cellular antioxidant response.

In addition to Nrf2, quercetin has been shown to alter phosphorylation of MAP kinase ERK1/2 and the p65 subunit of NFκB. In previous studies in osteoblast cell lines quercetin appears to induce phosphorylation of ERK1/2. For example quercetin induced phosphorylation of ERK1/2 up to 6h after treatment with 20 μM quercetin in MC3T3-E1 osteoblasts (13), while in MG-63 osteosarcoma cells (11), 50 μM quercetin induced a transient phosphorylation of ERK1/2 after only 5 minutes. In other cell types results are inconsistent despite similar doses (20-30uM) and time points (up to 24h) among studies. For example, quercetin suppressed phosphorylated ERK1/2 in primary cortical neurons

(52), but up-regulated phosphorylated ERK1/2 in BEAS-2B bronchial epithelial cells (53). Further investigation of ERK1/2 is necessary since this pathway appears to have overlapping roles in both the antioxidant response of osteoblasts (19), and osteoblast differentiation (54). Like ERK1/2, the p65 subunit of NFκB has been shown to be altered by the antioxidant response. Several lines of evidence suggest that Nrf2 activators down-regulate NFκB signaling (55), and this has also been shown in MC3T3-E1 osteoblasts (56), which suggests that activation of the antioxidant response may also be anti-inflammatory. This finding is of particular importance to bone, since inflammation has been shown to down-regulate development of the osteoblast phenotype and activate osteoclastic resorption.

In bone, quercetin may alleviate oxidative stress in osteoblasts and play a role in preventing osteoporosis. Protection of the osteoblast phenotype by quercetin may seem paradoxical since the stress response pathways that quercetin activates have also been implicated in the suppression of osteoblast differentiation (e.g. Nrf2, 3). Indeed, this paradoxical effect has been observed in studies in bone cells that show quercetin is both pro-apoptotic and stimulatory of osteoblast phenotype markers. This hormetic effect on cells, such that both protective anti-oxidant and damaging pro-oxidant effects are observed, depends on the dose and duration of treatment (57). In this regard, it is hypothesized that consuming quercetin or quercetin-rich foods would result in low concentrations of plasma quercetin metabolites that may stimulate a low-grade antioxidant response. Exposure to the relatively low quercetin doses would effectively prime the cells for a subsequent oxidative insult (e.g. hydrogen peroxide) without

producing accompanying suppression of osteoblast phenotype development or cell survival.

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CHAPTER III

QUERCETIN METABOLITES UP-REGULATE THE ANTIOXIDANT RESPONSE IN OSTEOBLASTS ISOLATED FROM FETAL RAT CALVARIA

Abstract

Oxidative stress contributes to osteoporosis by directly suppressing differentiation of bone-forming osteoblasts, suggesting that antioxidants may prevent osteoporosis by targeting the osteoblast antioxidant response. Quercetin, a flavonol found in many types of plant-based foods, is under investigation as an antioxidant with potential as a therapeutic strategy to prevent chronic diseases. Little is known about its effects on the osteoblast antioxidant response. This study aimed to examine the extent that quercetin metabolites alter antioxidant response genes and proteins in osteoblasts, and to examine the extent that quercetin alters cell signaling pathways, including nuclear factor (erythroid-derived 2)-like 2 (Nrf2), p65 subunit of nuclear factor-kB (NFkB), and extracellular-signal related kinase (ERK) 1/2, all of which are involved in stress signaling and the antioxidant response. Osteoblasts were isolated from fetal rat calvaria and treated with three different commercially available quercetin metabolites found in blood plasma after consumption of quercetin: quercetin aglycone (QRC), isorhamnetin (ISO), a methylated metabolite, or quercetin 3-O-glucuronide (Q3G), a glucuronidated metabolite, using doses up to 20 μM. Alternatively, some cells received a 2:1:1 mixture of all 3 metabolites (10 μM Q3G: 5 μM ISO: 5 μM QRC) to evaluate synergistic effects. The

osteoblast antioxidant response was assessed by analyzing several antioxidant response genes and proteins. Cell signaling proteins (Nrf2, ERK1/2, NFκB) were also analyzed. Results indicated that QRC and ISO, but not Q3G, up-regulated two antioxidant response genes, 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 were associated with suppression of phosphorylated ERK1/2 and down-regulation of NFκB, but no alterations were seen in the levels of Nrf2 protein levels. Additionally, no major alterations were seen in total antioxidant capacity or glutathione levels. This study offers important observations on the osteoblast antioxidant response in the presence of antioxidant quercetin.

Introduction

Oxidative stress has been implicated as a major contributing factor in a number of chronic diseases, including osteoporosis [1]. Oxidative stress directly suppresses the maturation and function of bone-forming osteoblasts [2], 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 [3]. Furthermore, quercetin improves bone density [4], bone strength [5], and microarchitecture [6] in ovariectomized rodent models of estrogen deficiency bone loss. Additionally, quercetin-rich diets have been shown to be associated with higher bone

density in human populations [7]. Despite these findings, little is known about the effect of quercetin, or other antioxidants, 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 antioxidant response in many cell types by activating the redox sensitive transcription factor, nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Nrf2 imparts oxidative stress protection by binding to antioxidant response elements and initiating transcription of genes involved in protecting cells from environmental stressors [8]. Up-regulation of these endogenous antioxidant genes is thought to prime cells so that they may prevent damage from a subsequent oxidative insult. Quercetin has been shown to up-regulate several antioxidant response genes in various cell types. For example, quercetin up-regulates heme oxygenase-1 (HO-1) in RAW264.7 macrophages [9] and glioma C6 cells [10], γ-glutamate-cysteine ligase catalytic synthetase (GCLC) in primary neurons [11], catalase, peroxiredoxins (Prdx) 3 and 5 in trabecular meshwork cells of the eye [12], and NADPH:quinone oxidoreductase-1 (NQO-1) in HepG2 cells [13]. While the effect of quercetin aglycone on Nrf2 activation and up-regulation of the antioxidant response genes has been frequently reported, this has not been described in osteoblasts. 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 [14,15] and the p65 subunit of nuclear factor-κB (NFκB), [15,16], but the effect of quercetin on these pathways has not been investigated fully in osteoblasts .

Most *in vitro* studies have primarily focused 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 [17]. In order to assess a more complete picture of the effect of quercetin metabolites found in blood plasma, *in vitro* studies are needed that describe the effects of multiple metabolites. Therefore, the first specific aim of this study was to examine the antioxidant response and subsequent alterations in antioxidant capacity in osteoblasts exposed to both quercetin aglycone and enzymatically conjugated quercetin metabolites in osteoblast-like cells isolated from fetal rat calvaria. We hypothesized that one or more of these quercetin metabolites would up-regulate antioxidant response genes and proteins in these cells and that antioxidant capacity would therefore be altered. The second specific aim of this study was to identify potential signaling pathways in the osteoblast antioxidant response, specifically alterations in Nrf2, ERK1/2, and NFκB. We hypothesized that one or more of these signaling pathways would be altered by treatment with quercetin. Since quercetin may have preventive effects on oxidative stress, these studies were designed to investigate stimulation of the antioxidant response from basal conditions in "normal," developing osteoblasts.

Materials and Methods

Reagents

Alpha-modified minimal essential media, fetal bovine serum, gentamicin, fungizone, dimethylsulfoxide (DMSO), and silver nitrate were purchased from Thermo

Fisher Scientific (Waltham, MA, USA). Penicillin, collagenase, ascorbic acid, β-glycerophosphate, dexamethasone, red violet LB salt, naphthol AS MX-PO_4, hydrogen peroxide, tert-butylhydroquinone (tBHQ), quercetin aglycone (QRC), and isorhamnetin (ISO) were purchased from Sigma (St. Louis, MO, USA). Quercetin-3-O-glucuronide (Q3G) was purchased from Extrasynthese (Genay, France)

Animals and Diet

Timed-pregnant Sprague-Dawley rats were purchased from Harlan Laboratories (Indianapolis, IN, USA). Dams were obtained on day (D) 4 of pregnancy and housed with a 12-hour 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*. The total isoflavone concentrations, as measured by the manufacturer, were 3 and 8 mg/kg for the batches used in this study, and were considered near the lower limit of detection. 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

To obtain osteoblast-like cells, calvariae were minced and subjected to five, serial incubations with collagenase to release cells from surrounding tissue. Cells from the first incubation were discarded, while cells from the second through fifth digestions were

plated each in separate T75 flasks. The following day cells were trypsinized, pooled, and plated at 3000 cell/cm^2 in 6-well culture dishes. Cells were incubated at 37°C under 5% CO_2 for the duration of the experiment. Cell culture media was changed every 2-3 days and consisted of alpha-modified minimal essential media supplemented with 10% fetal bovine serum, 10% antibiotic solution (penicillin, gentamicin, and fungizone), as well as ascorbic acid (50 μg/μL), β-glycerophosphate (10 mM), and dexamethasone (10^{-8} M) to induce osteogenesis.

Fetal rat calvarial cultures undergo distinct proliferation and differentiation phases. After plating, cells proliferate until confluence (approximately D5), which is followed by a 2-week differentiation phase (approximately D5-21), culminating in formation of discrete bone-like nodules that stain positive for cell-surface protein alkaline phosphatase, and secrete a collagen-based protein matrix that becomes mineralized. These cells also express genes that are characteristic of the osteoblast phenotype, including collagen type 1a, alkaline phosphatase, bone sialoprotein, and osteocalcin, as well as the master regulator of osteoblast differentiation, transcription factor Runx2 [18].

Alkaline Phosphatase and von Kossa Staining

These staining methods have been previously described [19]. Briefly, cells were washed in PBS, fixed in 10% neutral formalin buffer for 15 minutes, 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.

Mineralized nodules were visualized using the von Kossa method. Alkaline phosphatase stained cultures were incubated with 2.5% (w/v) silver nitrate solution for 30

minutes, which results in brown/black staining of mineralized nodules. Von Kossa staining was only performed at the end of culture (D21). Culture dishes were then rinsed in tap water and air dried overnight.

Bone Nodule Assays

Wells were examined microscopically under bright field illumination and mineralized nodules were counted. Mineralized nodules were areas of intense alkaline phosphatase stained, cuboidal cells that were also costained with von Kossa.

Preparation of Stock Solutions

QRC (Figure 1A), ISO (Figure 1B), a methylated metabolite, or Q3G (Figure 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 was based on studies that show conjugated metabolites to be most prevalent in blood plasma after conjugation by enzymes in the gut and liver [20]. 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 using a cell scraper and lysate was transferred to microcentrifuge tubes. RNA was isolated using RNeasy Universal Minikit (Qiagen, Valencia, CA, USA) per manufacturer's instruction 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 and ribosomal 18S was the endogenous control. RNA was collected from 3 separate wells for each treatment group and expression of HO-1, GCLC, NQO1, catalase, Prdx3, and Prdx5 were assessed.

Western Blotting

Cells were collected in RIPA lysis buffer, sonicated and centrifuged, and supernatant was analyzed for protein concentration using the BCA assay. Proteins were resolved on NuPage 4-12% bis-tris gels (Invitrogen) under denatured and reduced conditions, and transferred to a polyvinylidene membrane. Membranes were blocked for 1 hour using 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 dissolved in 5% (w/v) bovine serum albumin in TBS-T. Primary antibodies for antioxidant response proteins included HO-1 (Millipore, Temecula, CA, USA), GCLC (Abcam, Cambridge, MA, USA), and catalase (Abcam). Nrf2 antibody was purchased from R&D systems (Minneapolis, MN, USA); and Nrf2 (H300) was purchased from

Santa Cruz (Santa Cruz, CA, USA). β-actin (Sigma) or GAPDH (Santa Cruz) were used as the loading control. For fractionated proteins (see below), nucleoporin (BD Biosciences) was used for nuclear fraction control and GAPDH was used as the cytosolic control. The following day membranes were washed in TBS-T and probed with secondary antibody conjugated to horseradish peroxidase dissolved in 5% milk/TBS-T. HRP-conjugated secondary antibodies were donkey anti-mouse (Santa Cruz), rabbit anti-goat (Santa Cruz) and goat anti-rabbit (Cell Signaling, Danvers, MD, USA). 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. Briefly, cells from 6 wells were trypsinized with 0.2% trypsin, pooled, and centrifuged at 500 × *g* for 5 minutes 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.

Antioxidant Assay

To measure antioxidant capacity, cells lysates were analyzed using a commercially available, colorimetric antioxidant assay (Sigma). Three wells of cells per treatment group were collected in 1 mL of cold 1X assay buffer and pooled in

microcentrifuge tubes. Lysates were sonicated and centrifuged at 12000 x *g* for 15 minutes at 4 °C. Assay was performed on supernatants in duplicate as directed. Means were generated from 3 separate samples of each.

Glutathione Assay Kit

Total glutathione was measured using a Glutathione Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). Three wells of cells per treatment were collected in cold 50 mM phosphate buffer containing 1 mM EDTA and pooled. Lysates were sonicated and centrifuged at 10,000 x *g* for 15 minutes at 4 °C and deproteinated with 10% (w/v) metaphosphoric acid. Assay was performed in duplicate as directed. Means were generated from 3 separate samples of each. Samples were normalized to milligrams of protein.

Study Design

Preliminary Study. A preliminary study was performed to determine the developmental period in which fetal rat calvarial cells were most responsive to oxidative stress. Cells were treated with 0-300 µM hydrogen peroxide, a common inducer of oxidative stress, starting at confluence (D5). Treatment was continued either throughout differentiation (D5-21), during early differentiation only (D5-13), or during late differentiation only (D13-21). Alkaline phosphatase staining, along with RNA collection for real-time PCR analysis of osteoblast phenotype markers, was performed over a time course throughout differentiation starting at D5. At D21 alkaline phosphatase stained cultures were co-stained with von Kossa technique. These studies determined the time points evaluated in quercetin studies described below.

Quercetin Studies. To assess the effects of quercetin metabolites on the acute antioxidant response, cells were treated starting at D5 with 3 different quercetin metabolites at doses up to 20 μM for a total of 48h. Alternatively, some cells received a mixture of all 3 metabolites simultaneously (2:1:1 mixture, 10 μM Q3G: 5 μM ISO: 5 μM QRC). Samples were collected along a time course (0 to 48h) and antioxidant response genes and proteins and cell signaling proteins were analyzed as described above. As a positive control, some cells were treated with tBHQ, a known inducer of the antioxidant response, at doses of 0, 20, or 40 μM for 3, 6, or 12 hours.

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.

Results

Preliminary Study

Figure 2 shows representative wells co-stained for alkaline phosphatase and von Kossa (2A) and the quantification of mineralized nodules (2B) after treatment with 0-300 μM hydrogen peroxide throughout differentiation (D5-21), or during early (D5-13) or late differentiation only (D13-21). Cells treated with hydrogen peroxide throughout differentiation exhibited dose-dependent suppression of alkaline phosphatase staining,

and the number of mineralized nodules was approximately 40% ($p < 0.05$) and 50% ($p < 0.05$), in wells treated with 150 μM and 300 μM hydrogen peroxide, respectively. There were 50% fewer mineralized nodules ($p < 0.05$) in cells treated with 300 μM hydrogen peroxide during early differentiation only, while in contrast, cells treated only during late differentiation were similar to 0 μM control. Since hydrogen peroxide produced robust, sustained effects on osteoblast phenotypic development after treatment during early differentiation only, the main subsequent quercetin studies focused on treatment effects during this early differentiation period only.

Quercetin Metabolites Up-regulate Antioxidant Genes and Proteins

Treating cells up to 48h 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 (Figure 3A). HO-1 and GCLC were significantly ($p < 0.05$) up-regulated as early as 3h after beginning 20 μM QRC treatment compared to 0 μM control, and up-regulation was sustained for all time points for the entire 48h period. In contrast, catalase gene expression was not markedly altered until 12h after treatment and up-regulation was sustained at 24 and 48h. 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 QRC and only at 24 and 48h. Protein levels of HO-1 and GCLC were up-regulated similarly to gene expression (Figure 3B), but catalase protein expression was not, and appeared to be down-regulated after 24 and 48h after 20 μM QRC treatment compared to control (0uM). 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

(Figure 4), or a 2:1:1 mixture of all three metabolites (10 µM Q3G, 5 µM QRC, and 5 µM ISO) (Figure 5). The 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 (Figure 4A) and protein levels (Figure 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 (Figure 4C) or protein level (Figure 4D). The metabolite mixture resulted in up-regulation of HO-1 and GCLC at the mRNA and protein level (Figure 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.

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 be the biologically active relevant Nrf2 (Lau et at al 2013, Pi et al). Cells treated with 20 µM QRC did not show any alterations in Nrf2 protein expression at any time point up to 48h after treatment (Figure 6). To further address whether Nrf2 accumulated in the nucleus, Western blots were performed on nuclear protein fractions at doses up to 60 µM for up to 12h. Data show that Nrf2 accumulation was not observed in the nucleus or the cytoplasm in any

pattern that corresponded to QRC treatment (Figure 7). Similarly, treatment of cells with tert-butylhydroquinone did not result in the up-regulation of Nrf2 at any time point in spite of dose-dependent up-regulation of HO-1 after 6 and 12h 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 QRC down-regulated levels of phosphorylated ERK1/2 within 1.5h of treatment below basal levels, and was sustained through 6 h after treatment (Figure 8a). Similarly, total levels of NFκB were down-regulated at each of the time points analyzed (Figure 8b).

Quercetin did not Alter Antioxidant Capacity or Glutathione Levels

Since QRC up-regulated expression of antioxidant response genes and proteins, the total antioxidant capacity of the cells was measured to determine if this protein up-regulation resulted in concomitant changes in the antioxidant status of the cell. The overall antioxidant capacity of the cells was not significantly altered by QRC treatment at any of the time points analyzed ($p > 0.05$) (Figure 9A). GCLC is the rate-limiting enzyme in the synthesis of glutathione, a major cellular detoxifying thiol, and since GCLC was up-regulated by 20 μM QRC, total glutathione levels were also assessed. Total glutathione levels were not significantly altered by quercetin treatment at any of the time points analyzed ($p > 0.05$) (Figure 9B).

Discussion and Conclusions

These studies provide novel observations of the osteoblast antioxidant response after stimulation with quercetin metabolites. The 20 μM dose of two quercetin metabolites, QRC and ISO, markedly up-regulated the 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 catalase proteins were not congruently altered with mRNA, 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, but that conjugation of the aglycone molecule diminishes this stimulatory effect, possibly due to alterations in the permeability of the cells due to the conjugation of the metabolites. This theory is supported by results from cells treated with the mixture of metabolites, where the up-regulation of gene expression appeared to be consistent with the presence of the 5 μM dose of QRC in the mixture, and did not result in detectable additive effects due to the presence of the other two metabolites (Figure 5). In contrast to ISO and QRC, Q3G did not up-regulate expression of any antioxidant genes and proteins. This observation is in-line with *in vitro* studies in neurons, and may be due to the relatively large size of the glucuronic acid conjugate, which would prevent it from entering the cell [20].

Not all antioxidant response genes assessed in this study were up-regulated by quercetin metabolites, including Prdx3, Prdx5, and NQO1. These data 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 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, however, unlike other studies, this did not appear to coincide with detectable accumulation of Nrf2 protein, even at doses 2 to 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 [21]. 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 [8]. 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 [21, 22, 23]. 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.

Unlike Nrf2, these data clearly showed suppression of phosphorylated ERK1/2 and NFκB, 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 quercetin aglycone on ERK signaling pathways in osteoblast cell lines. In MC3T3-L1 osteoblasts quercetin induced phosphorylation of ERK1/2 up to 6h after treatment with 20 μM quercetin [24], and in MG-63 osteosarcoma cells [25], 50 μM quercetin induced a transient phosphorylation of ERK1/2 after only 5 minutes. Both of these studies are in direct contrast to the effects that were observed using primary cultures isolated from fetal rat calvaria. In other cell types, alterations in ERK1/2 phosphorylation by quercetin aglycone have been described, but results are inconsistent despite similar doses (20-30uM) and time points (up to 24h) among studies. For example, quercetin suppressed phosphorylated ERK1/2 in primary cortical neurons [26], but up-regulated phosphorylated ERK1/2 in BEAS-2B bronchial epithelial cells [27].

Like ERK1/2, quercetin appears to suppress NFκB protein levels. Several lines of evidence suggest that Nrf2 activators down-regulate NFκB signaling [28], and this has also been shown in MC3T3-E1 osteoblasts [29], which suggests that activation of the antioxidant response may also be anti-inflammatory. This finding 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 quercetin

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 in the osteoblast stress response, and to what extent these signaling pathways are required for up-regulation of antioxidant genes.

Because of the up-regulation of HO-1 and GCLC proteins, the overall antioxidant capacity and total glutathione levels were expected to be higher in QRC-treated cells, however, we did not detect alterations in either of these parameters at any time point. This indicates that QRC did not significantly alter levels of electron-accepting thiols or other endogenous free radical scavengers. Similarly, other studies using comparable doses of QRC have shown that an up-regulation of GCLC [11, 30], is not necessarily followed by increased levels of total glutathione [11]. Quercetin is thought to form glutathione adducts [20] suggesting that the up-regulation of GCLC may be a compensatory mechanism to keep glutathione at steady-state levels, which may explain why we did not see a concomitant rise in both GCLC and levels of total glutathione. The induction of HO-1 is commonly seen as part of the antioxidant response and presumably results in increased catabolism of heme into Fe^{2+}, biliverdin, and CO. Some lines of evidence show that these metabolic products have free radical scavenging ability, but may also have direct effects on cytoprotective cell signaling pathways [31]. Taken alongside the data from the current study, it appears that the functional outcome of HO-1 and GCLC up-regulation is more complex than an overall increase in cellular free radical scavenging capabilities. It may also be possible that osteoblasts in these culture conditions already have a relatively high antioxidant capacity that was not altered to

detectable levels by the relatively low dose of QRC. Since the overall functional outcome of HO-1 and GCLC up-regulation remain unclear, further studies are needed to assess alterations in specific steps involved in glutathione regulation and heme catabolism.

The quercetin-induced antioxidant response in fetal rat calvaria cells occurred in early stages of differentiation (D5-D7), which was the same time that hydrogen peroxide induced marked suppression of phenotype as determined in the preliminary study. Therefore, osteoprogenitors and pre-osteoblasts in oxidative stress environments may benefit from stimulation with antioxidants that offer potentially 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 quercetin consumption [32,33], and high inter-individual variability has been described among human study participants that consume quercetin-rich foods [34]. Additionally, roughly 20 plasma quercetin metabolites have been reported after consuming quercetin or quercetin-rich foods [32], but we are only aware of three metabolites that are commercially available. Nevertheless, these data offers important insights into the overall effect of flavonoids on osteoblast biology and also provides potential pathways to investigate in future studies examining the osteoblast antioxidant response and the role of oxidative stress in osteoporosis.

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Figure Legends

Figure 3.1. Quercetin metabolites. A) Quercetin aglycone, B) methylated metabolite, isorhamnetin, C) and glucuronidated metabolite, quercetin-3-O-glucuronide.

Figure 3.2. Osteoblast-like phenotype development in colonies isolated from fetal rat calvaria after treatment with 0, 75, 150, or 300 μM hydrogen peroxide. A) Representative wells of alkaline phosphatase (pink) and von Kossa (black) co-stained colonies after treatment with 0-300 μM hydrogen peroxide continuously (D5-21), or during early (D5-13) or late (D13-21) differentiation only. B) Number of mineralized nodules present at D21 after treatment with 0-300 μM hydrogen peroxide, expressed as a percentage of 0uM control; Means +/- SEM, n=6 independent wells of RNA from the same experiment. Significance ($p < 0.05$) was assessed by one-way analysis of variance. Means that do not share a common letter are significantly different. Similar results were observed in at least 2 independent studies.

Figure 3.3. Expression of antioxidant response genes and proteins in osteoblast-like colonies after treatment with 20 μM quercetin aglycone up to 48h 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 48h of treatment with quercetin aglycone. 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 48h treatment with quercetin. Similar results were observed in 3 independent studies.

Figure 3.4. 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 48h 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 48h 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 48h of treatment with B) isorhamnetin and D) 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 2 independent studies.

Figure 3.5. 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 48h 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 48h 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 2 independent studies.

Figure 3.6. 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 48h starting at confluence (day 5).

Figure 3.7. Western blot of Nrf2 protein expression in nuclear fractions of osteoblast-like cells after treatment with up to 60 μM quercetin for up to 12h starting at confluence (day 5).

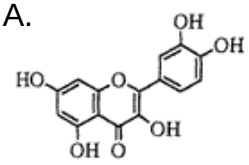
Figure 3.8. Western blots of ERK1/2 and NFκB after acute treatment with 20 μM quercetin aglycone. A) Western blot of ERK1/2 and phosphorylated ERK1/2 after treatment with 20 μM quercetin aglycone up to 24h starting at confluence (D5); B) Western blot of NFκB after treatment with 20 μM quercetin aglycone up to 48h starting at confluence (D5).

Figure 3.9. A) Antioxidant capacity and B) total glutathione levels of cell lysates after treatment with 20 μM quercetin aglycone for up to 12h. Means +/- SEM, n=3 separate samples from the same experiment (3 wells pooled per sample). Univariate analysis revealed no interaction ($p > 0.05$) between time and quercetin treatment for either assay. Significance between quercetin and vehicle control was assessed by student's t-tests within each time point. Similar results were observed in 2 independent studies.

Figures

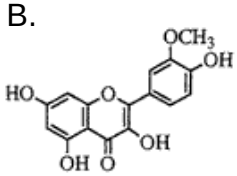
Figure 3.1. Quercetin metabolites. A) Quercetin aglycone, B) methylated metabolite, isorhamnetin, C) and glucuronidated metabolite, quercetin-3-O-glucuronide.

A.



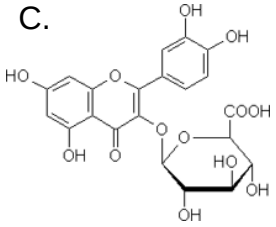
quercetin aglycone (QRC)

B.



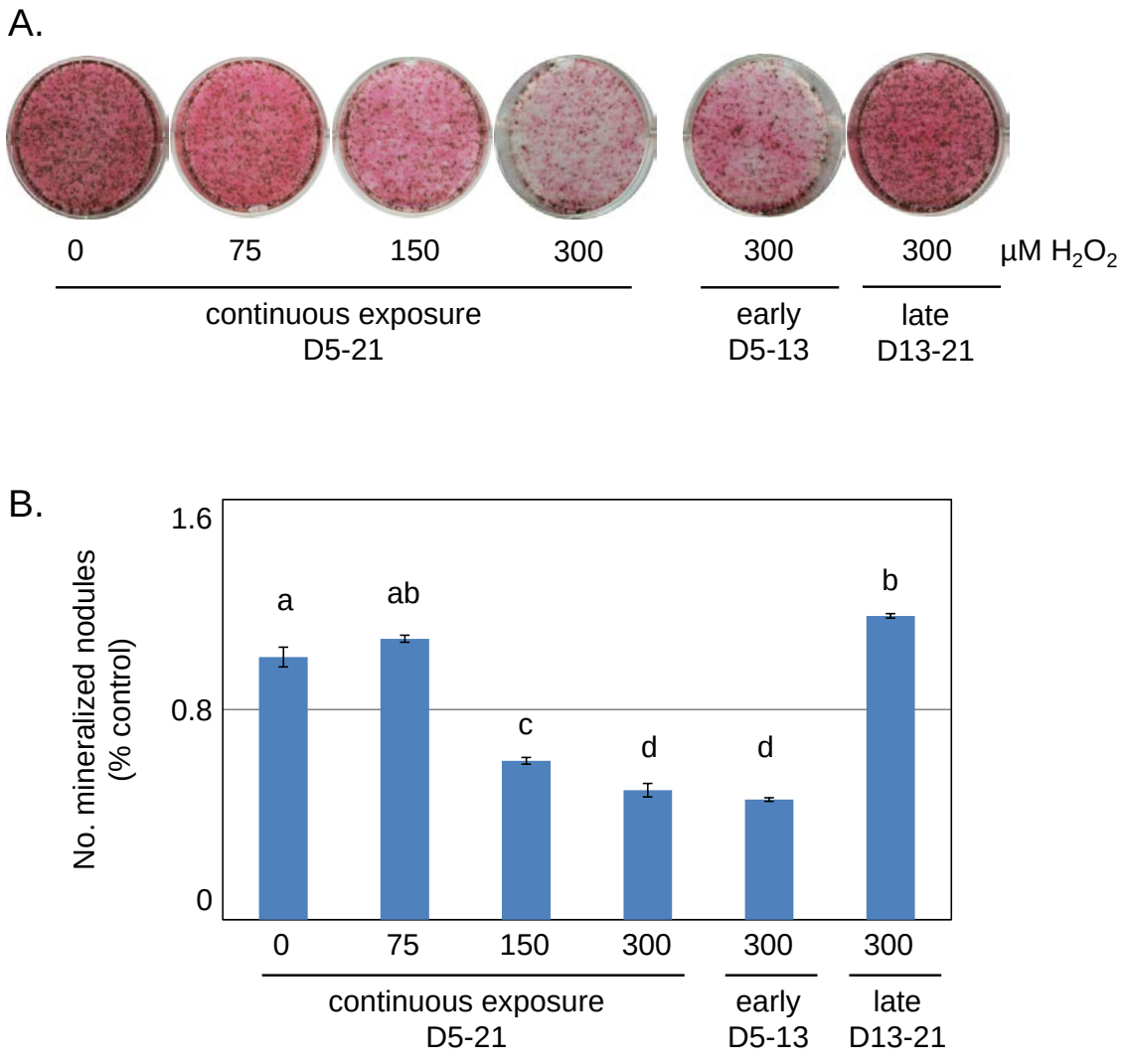
Isorhamnetin (ISO)

C.



quercetin-3-O-glucuronide (Q3G)

Figure 3.2. Osteoblast-like phenotype development in colonies isolated from fetal rat calvaria after treatment with 0, 75, 150, or 300 µM hydrogen peroxide. A) Representative wells of alkaline phosphatase (pink) and von Kossa (black) co-stained colonies after treatment with 0-300 µM hydrogen peroxide continuously (D5-21), or during early (D5-13) or late (D13-21) differentiation only.



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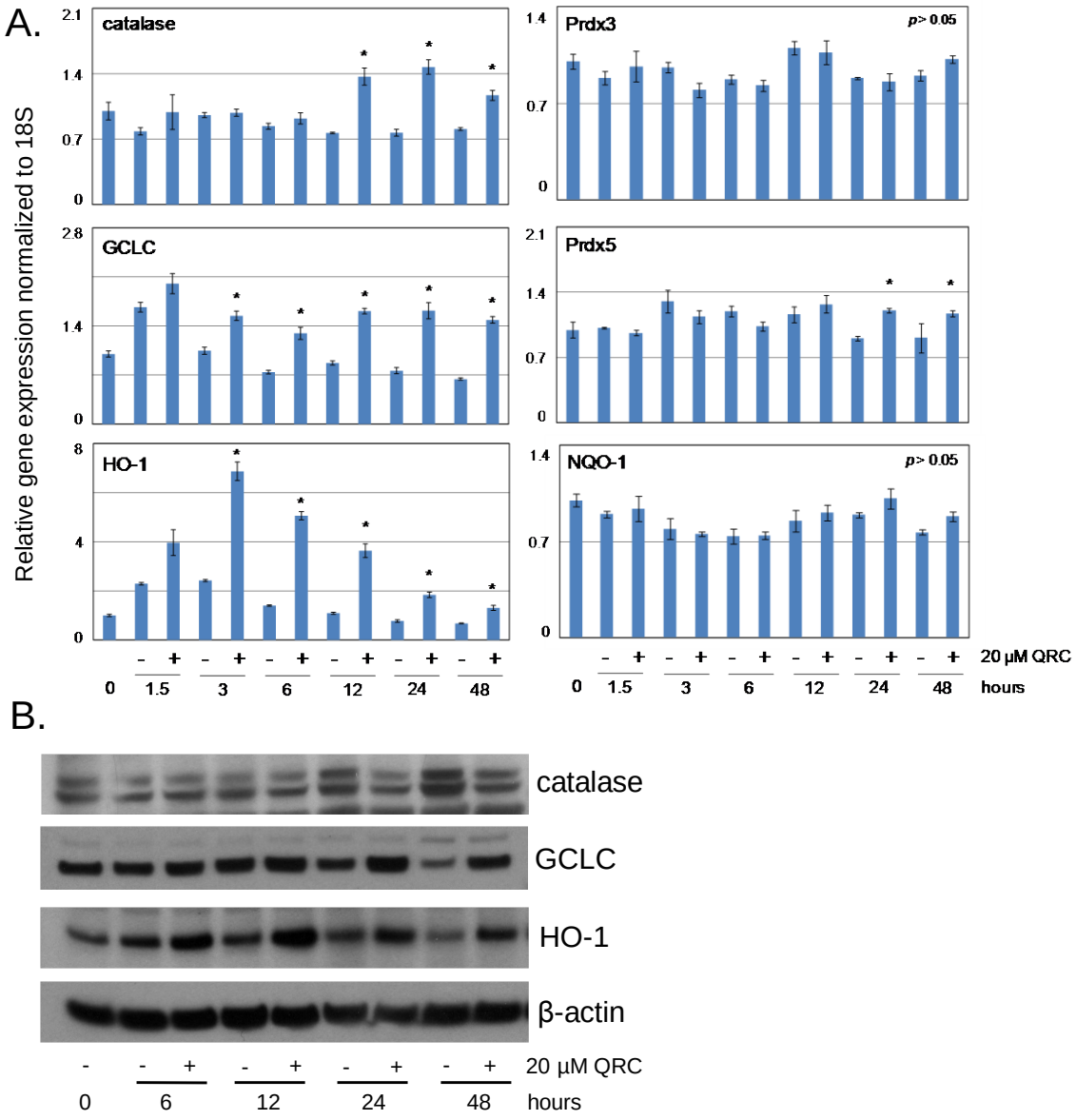
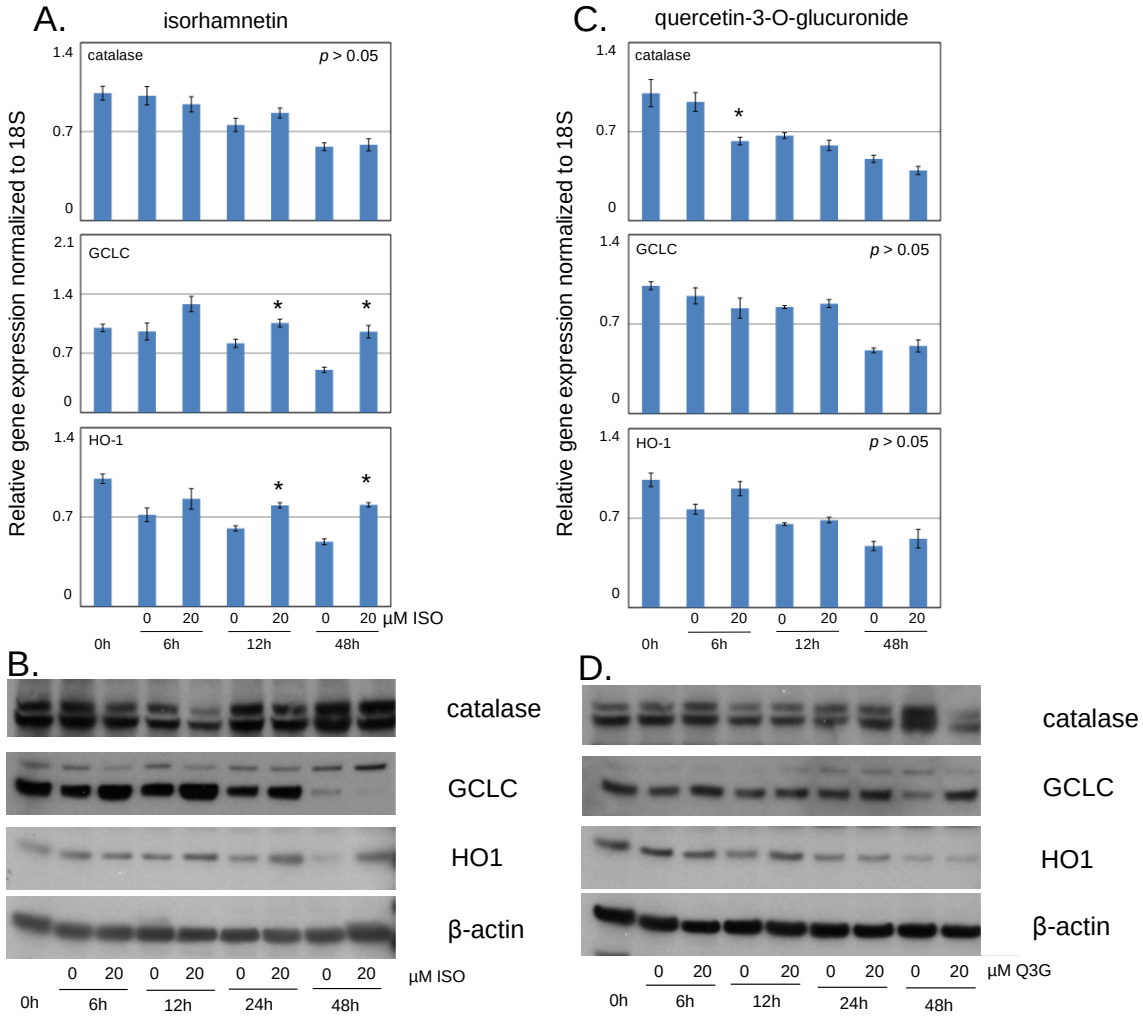


Figure 3.4. Expression of antioxidant response genes and proteins in osteoblast-like colonies after treatment with 20 µM isorhamnetin, or 20 µM quercetin-3-O-glucuronide for up to 48h starting at confluence (day 5)



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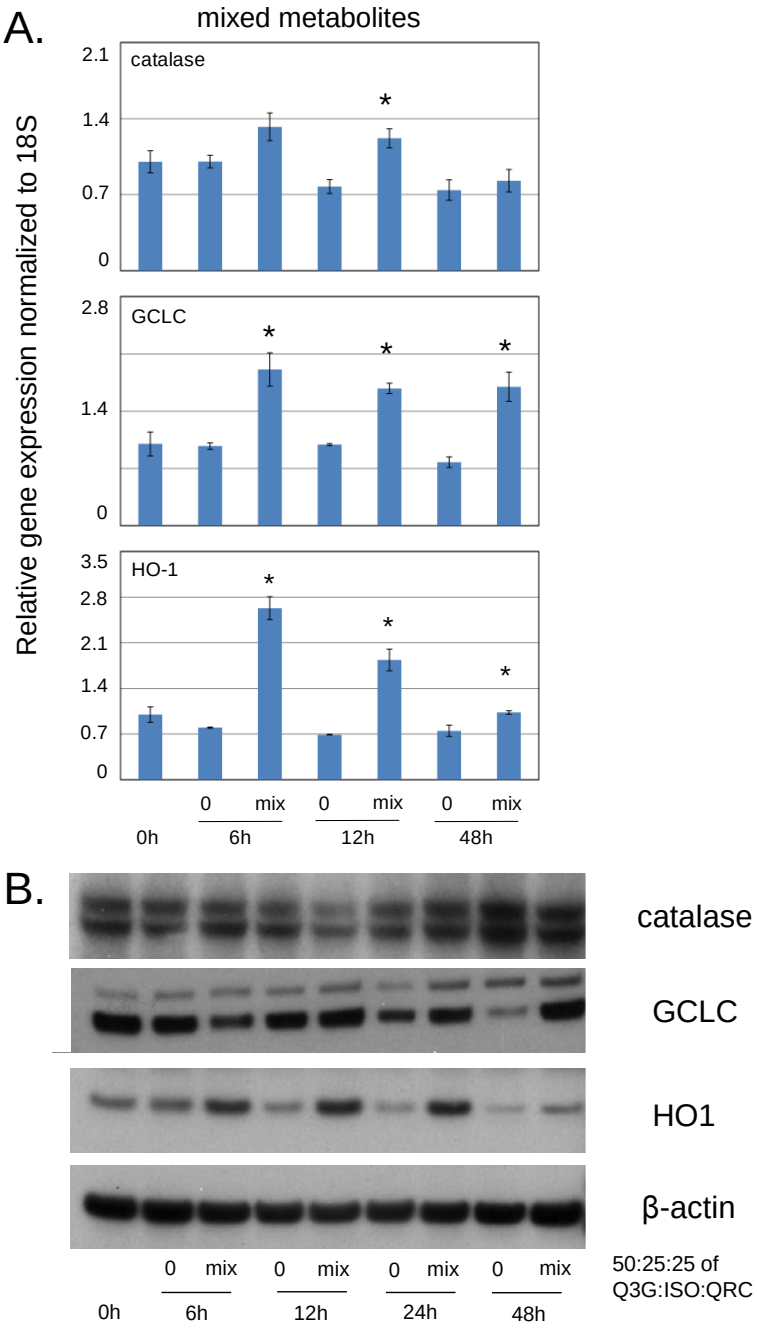


Figure 3.6. Western blot of Nrf2 protein expression in whole lysates of osteoblast-like cells after treatment with 0 to 60 µM quercetin aglycone for up to 48h starting at confluence (day 5).

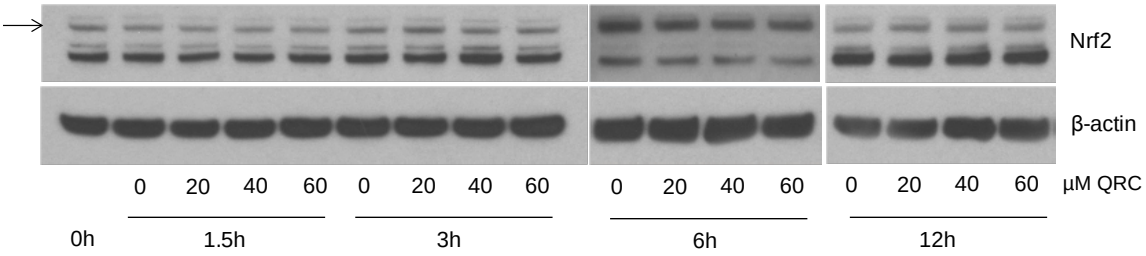
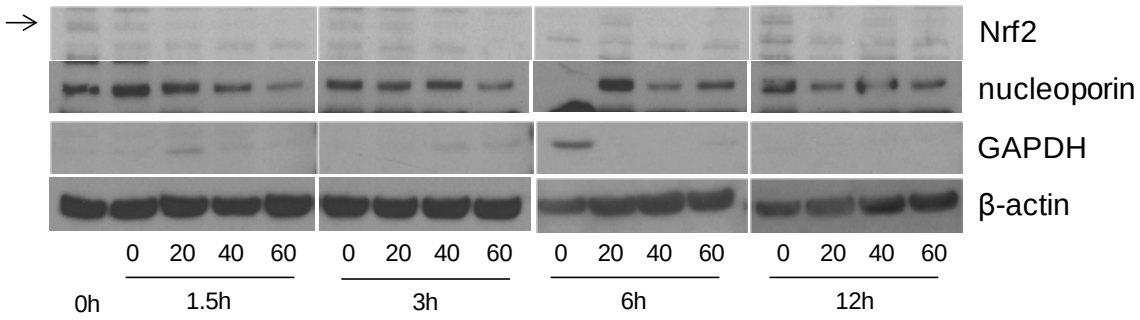


Figure 3.7. Western blot of Nrf2 protein expression in nuclear fractions of osteoblast-like cells after treatment with up to 60 µM quercetin for up to 12h starting at confluence (day 5).



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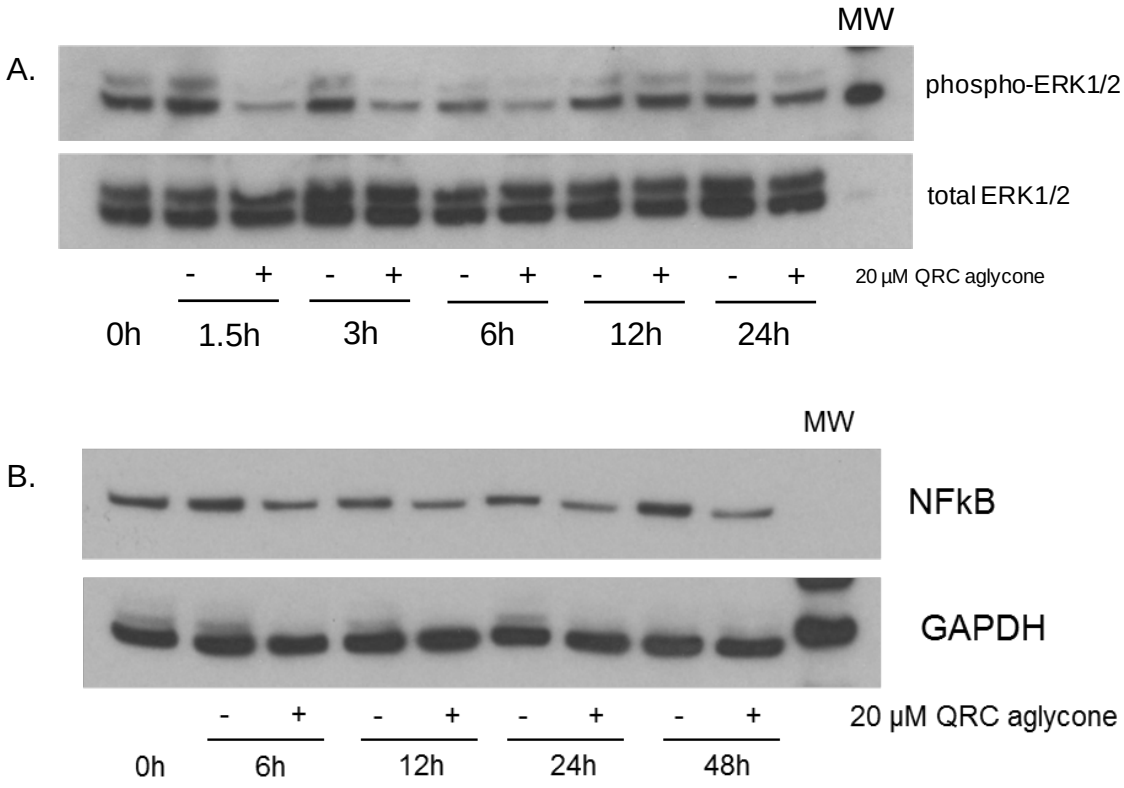
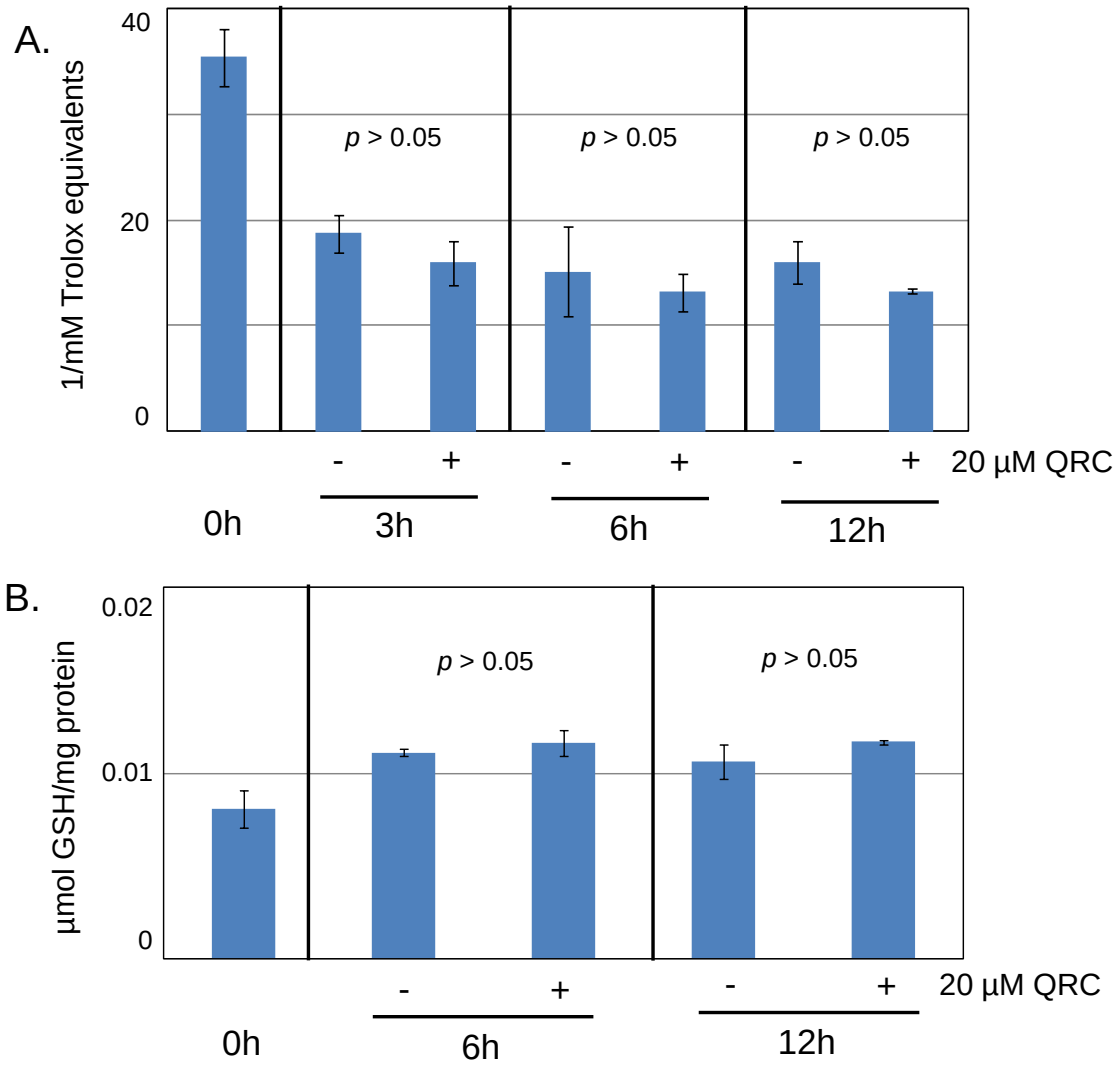


Figure 3.9. A) Antioxidant capacity and B) total glutathione levels of cell lysates after treatment with 20 µM quercetin aglycone for up to 12h.



CHAPTER IV

QUERCETIN PARTIALLY PRESERVES DEVELOPMENT OF THE OSTEOBLAST PHENOTYPE IN FETAL RAT CALVARIAL CELLS IN AN OXIDATIVE STRESS ENVIRONMENT

Abstract

Oxidative stress has been linked to development of osteoporosis, which may be due, in part to suppression of osteoblast differentiation. Because of the link between oxidative stress and osteoblast phenotypic development, targeting the osteoblast antioxidant response may alleviate oxidative stress and support bone health. Quercetin is a common dietary flavonol that up-regulates expression of antioxidant response gene and proteins, heme oxygenase-1 (HO-1) and γ-glutamate cysteine ligase catalytic subunit (GCLC), after acute treatment (up to 12h) during early differentiation, starting on day (D) 5 (confluence). The overall purpose of this study was to characterize the effect of quercetin aglycone (QRC) on the osteoblast antioxidant response and phenotypic development in fetal rat calvarial osteoblasts cultured in an oxidative stress environment. The first aim was to assess the extent that QRC blocked hydrogen peroxide induced suppression of the osteoblast phenotype. Cells treated for 4-days (D5-9) with up to 300 μM hydrogen peroxide had lower levels of alkaline phosphatase staining, altered cell morphology, and significantly lower expression levels of osteoblast phenotypic gene markers ($p < 0.05$), but suppression of these parameters was partially blocked by a 12h pretreatment with 20 μM QRC. The second aim was to characterize alterations in the

osteoblast antioxidant response when cells were cultured with hydrogen peroxide with or without QRC pretreatment. Hydrogen peroxide (300 µM) treatment produced a sustained up-regulation of HO-1 protein expression within 3h after beginning treatment that was also evident at the end of the 4-day incubation period. In cells pretreated with 20 µM QRC, however, expression levels of HO-1 were suppressed. Hydrogen peroxide also blocked QRC-induced up-regulation of GCLC. These results suggest that an acute 12h pretreatment with QRC "primes" cells for a subsequent exposure to hydrogen peroxide, which blocks the hydrogen peroxide-induced activation of the antioxidant response and prevents hydrogen peroxide-induced suppression allowing cells to proceed with differentiation programs.

Introduction

Oxidative stress contributes to the development of osteoporosis, in part, by directly suppressing the number and function of bone-forming osteoblasts. This finding underscores the importance of understanding the osteoblast antioxidant stress response, and suggests that alleviating oxidative stress in osteoblasts may be a promising strategy for preventing osteoporosis. Quercetin (QRC) is an antioxidant flavonoid found abundantly in plant-based foods and supplements, and is a target molecule for research investigating the therapeutic potential of antioxidants in chronic diseases linked to oxidative stress [1]. Several studies in rodent models of estrogen deficient bone loss demonstrate that consumption of QRC or QRC-rich foods results in higher bone density [2], improved microarchitecture [3,4], and higher bone strength [3] compared to estrogen deficient rodents that did not consume QRC. Furthermore, it has been shown that bone

loss from both aging [5] and estrogen deficiency [6] is partly attributed to oxidative stress in osteoblasts. Taken together these studies suggest that antioxidants may preserve the differentiation and function of osteoblasts if cells are in an oxidative stress environment.

Current studies investigating the specific effects of QRC on osteoblasts are limited and contradictory. Studies show stimulation of alkaline phosphatase activity [7] in MC3T3-E1 cells, and up-regulated osteoblast phenotypic gene markers and calcium deposition in multipotent primary cells isolated from adipose tissue [8], but other studies show that QRC stimulates apoptotic cell death of MC3T3-E1 cells [9]. Additionally, 10 μM QRC aglycone enhanced TNFα-induced apoptotic cell death in MC3T3-E1 osteoblast cell lines, but not in primary calvarial cultures [10]. The pro-apoptotic effects of QRC on osteoblasts are not congruent with animal studies that show a protective effect of QRC on bone health. These results are likely due, in part, to variability in cell types and culture conditions, but also suggest that QRC may have hormetic effects, such that low doses or short duration of treatment produce a protective antioxidant response, where higher doses or chronic exposure results in damaging pro-oxidant effects.

In other cell types, the presence of pro-oxidants or electrophiles induces the antioxidant response, which classically involves activation of the transcription factor, Nrf2, which binds to antioxidant response elements in the promoter region of genes coding for proteins that function to maintain redox homeostasis [11]. Previously, we have shown that QRC induces expression of Nrf2-target genes heme oxygenase-1 (HO-1) and γ-glutamate cysteine ligase catalytic subunit (GCLC) in osteoblast cultures isolated from fetal rat calvaria. These alterations were accompanied by down-regulation in

phosphorylated ERK1/2, but not in total protein levels of Nrf2. Activation of these genes suggests that QRC acts as a pro-oxidant in these cell cultures. However, since these genes are considered "protective," we hypothesized that a short duration of QRC at a relatively low dose would prevent damaging effects of a subsequent oxidative event and preserve development of osteoblast phenotype. Because of the link between oxidative stress and phenotypic development of osteoblasts, we also hypothesized that any protective event would coincide with osteoblast antioxidant response pathways. To our knowledge there have been no studies specifically examining the effect of QRC on the antioxidant response and phenotypic development in primary osteoblasts when cells are cultured in a suppressive, oxidative stress environment.

This study had two aims. The first specific aim was to examine the extent to which alterations in the antioxidant response altered development of the osteoblast phenotype. To address the first aim osteoblasts were pretreated for 12h with QRC to stimulate the antioxidant response, followed by treatment with hydrogen peroxide, a commonly used inducer of oxidative stress *in vitro*, to suppress osteoblast phenotype [12,13]. Osteoblast phenotype was assessed by measuring alkaline phosphatase staining and expression of osteoblast phenotypic gene markers. We hypothesized that pretreatment with QRC would preserve osteoblast phenotype when cells were cultured with hydrogen peroxide. The second specific aim was to characterize alterations in the osteoblast antioxidant response when cells were cultured in an oxidative stress environment. To address the second specific aim, cells were pre-treated for 12h with QRC, which was followed by hydrogen peroxide treatment, and the osteoblast

antioxidant response was assessed by measuring expression levels of two antioxidant genes and proteins that are known to be up-regulated by QRC in fetal rat calvaria cultures, and ERK1/2 and Nrf2 proteins that are potentially involved in the quercetin-and hydrogen peroxide-mediated antioxidant response. We hypothesized that QRC would alter the antioxidant response induced by hydrogen peroxide, and this would involve Nrf2 and ERK1/2.

Materials and Methods

Reagents

Alpha-modified minimal essential media, fetal bovine serum, gentamicin, fungizone, dimethylsulfoxide (DMSO), and silver nitrate were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Penicillin, collagenase, ascorbic acid, β-glycerophosphate, dexamethasone, red violet LB salt, naphthol AS MX-PO_4, hydrogen peroxide, tert-butylhydroquinone (tBHQ), QRC aglycone, and were purchased from Sigma (St. Louis, MO, USA).

Animals and Diet

Timed-pregnant Sprague-Dawley rats were obtained from Harlan on D4 of pregnancy and housed with a 12-hour light/dark cycle, and *ad libitum* access to water and 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*. The total isoflavone concentrations, as measured by the manufacturer, were 3 and 8 mg/kg of diet for the batches used in these studies, and were near the level of detection. Dams were euthanized on D21 of gestation by overexposure to CO_2, and fetuses removed.

Fetuses were 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

To obtain osteoblast-like cells, calvariae were minced and subjected to 5, serial incubations with collagenase to release cells from surrounding tissue. Cells from the first incubation were discarded, while cells from the second through fifth digestions were plated each in separate T75 flasks. The following day cells were trypsinized, pooled, and plated at 3000 cell/cm^2 in 6-well culture dishes. Cells were incubated at 37°C under 5% CO_2 for the duration of the experiment. Cell culture media was changed every 2-3 days and consisted of alpha-modified minimal essential media supplemented with 10% fetal bovine serum, 10% antibiotic solution (penicillin, gentamicin, and fungizone), supplemented with ascorbic acid (50 µg/µL), beta-glycerophosphate (10 mM), and dexamethasone (10^{-8} M) to induce osteogenesis. Fetal rat calvarial cultures undergo distinct proliferation and differentiation phases. After plating, cells proliferate until confluence (day 5), which is followed by a 2-week differentiation phase (days 5-21), culminating in formation of discrete bone-like nodules that stain positive for cell-surface protein alkaline phosphatase, and secrete a collagen-based protein matrix that becomes mineralized. Alongside nodule formation, fetal rat calvaria exhibit temporal up-regulation of genes that are characteristic of the osteoblast phenotype, including collagen type 1a (Coll1a), alkaline phosphatase (ALP), bone sialoprotein (BSP), and osteocalcin

(OCN), as well as the master regulator of osteoblast differentiation, transcription factor Runx2 [14].

Alkaline Phosphatase Staining

This method has been previously described [15]. Briefly, cells were washed in PBS, fixed in 10% neutral formalin buffer for 15 minutes, 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. Colonies were examined microscopically under bright field illumination and micrographs were taken with an Olympus DP71 digital camera.

Preparation of Stock Solutions

QRC was dissolved in degassed dimethylsulfoxide (DMSO), aliquoted, and stored at -20°C. Hydrogen peroxide (30% w/v) was freshly diluted in sterile, deionized water just before use. Quercetin at final concentration of 20 µM, and hydrogen peroxide at final concentrations of 150 or 300 µM were administered with fresh media. The vehicle controls (0 µM) for hydrogen peroxide and QRC were sterile, deionized water and 0.1% (v/v) DMSO, respectively.

Quantitative Real-time PCR

Cells were lysed in 900 µL of Qiazol using a cell scraper and lysate was transferred to microcentrifuge tubes. RNA was isolated using RNeasy Universal Minikit (Qiagen) per manufacturer's instruction and quantified using a Nanodrop spectrophotometer. Two micrograms of RNA was reverse-transcribed using a High Capacity Reverse Transcription kit (Applied Biosystems). 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 and ribosomal 18S as the endogenous control. RNA was collected from at least 3 separate wells for each treatment group and used to assess antioxidant response genes HO-1, GCLC, or osteoblast phenotypic genes Runx2, Coll1a, ALP, BSP, and OCN.

Western Blotting

Cells were collected in RIPA lysis buffer supplemented with 10 mM sodium fluoride, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate (Sigma), and protease inhibitor cocktail (Calbiochem). Lysates were sonicated and centrifuged, and supernatant were analyzed for protein concentration using the BCA assay. Proteins were resolved on NuPage 4-12% bis-tris gels (Invitrogen) under denatured and reduced conditions, and transferred to a polyvinylidene membrane. Membranes were blocked for 1 hour using 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 dissolved in 5% (w/v) bovine serum albumin in TBS-T. Primary antibodies for antioxidant response proteins included HO-1 (Millipore), GCLC (company). Nrf2 (H300) was purchased from Santa Cruz. Phosphorylated and total ERK1/2 antibodies were purchased from Cell Signaling. β-actin (Sigma) or GAPDH (Santa Cruz) were used as the loading control. The following day membranes were washed in TBS-T and probed with secondary antibody conjugated to horseradish peroxidase dissolved in 5% milk/TBS-T. HRP-conjugated secondary antibodies include donkey anti-mouse (Santa

Cruz) and goat anti-rabbit (Cell Signaling). Signal was detected with Western Lightning Chemiluminescence Reagent Plus kit (PerkinElmer).

Study Design

Preliminary Study 1. To determine the dose and duration of hydrogen peroxide treatment needed to suppress osteoblast phenotype to detectable levels, cells were treated with 0-300 µM hydrogen peroxide throughout early differentiation only (D5-13). Alkaline phosphatase staining was performed at several time points D5, D9, and D13. Additionally, RNA was collected on D9, and gene expression was analyzed with real-time PCR. Previous data showed that treating cells with 300 µM hydrogen peroxide during the first week of differentiation only (D5-13) produced lasting suppression of osteoblast phenotype that was observed at D21, even if cells were treated with control media (0 µM hydrogen peroxide) during the second week of differentiation (D13-21).

Preliminary Study 2. The assess the extent that an acute dose of QRC suppressed osteoblast phenotype, cells were treated with 0-40 µM QRC for 48h and alkaline phosphatase staining was performed to determine if osteoblast phenotypic development was altered by QRC alone.

Quercetin Studies. To assess the effects of QRC on the osteoblast phenotype and antioxidant response, cells were treated starting at confluence (D5) with 20 µM QRC for a total of 12h. After the 12h pretreatment, cells received fresh media containing either 0 or 300 µM hydrogen peroxide since the 300 µM dose consistently suppress osteoblast phenotype in preliminary studies. Cell cultures received fresh media again on D7, 48h after treatment with hydrogen peroxide began, which contained either 0 or 300 µM

hydrogen peroxide. The expression of antioxidant response genes and proteins, osteoblast phenotypic gene markers, and alkaline phosphatase staining was assessed after 4 days (D5-9) of hydrogen peroxide to assess chronic or lasting effects of QRC. Additionally, antioxidant gene and protein expression was analyzed up to 24h after beginning hydrogen peroxide treatment to assess acute alterations

Statistics

Data are expressed as mean +/- SEM. Statistical differences were determined using one-way analysis of variance (ANOVA) with Tukey post hoc analysis (SPSS Inc., version 17.0 for Windows, Chicago, IL, USA). Univariate analysis was performed to check for interactions when there were two main effects of QRC treatment and hydrogen peroxide treatment. For real-time PCR data, there was a significant interaction between the main effects of QRC pre-treatment and hydrogen peroxide for all oxidative stress genes (Figure 6A) and for ALP (Figure 4). Student's t-tests were performed between 0 μM and 20 μM QRC within each dose of hydrogen peroxide. The α level was set at 0.05.

Results

Preliminary Studies

Hydrogen peroxide dose-dependently suppressed the number of alkaline phosphatase staining (Figure 1A) in fetal rat calvaria cultures when cells were treated starting at confluence (D5), and this effect was evident after only 4 days of treatment (D5-9). Hydrogen peroxide also dose-dependently suppressed osteoblast phenotypic gene markers after 4 days of treatment (Figure 1B). Therefore, a 4-day incubation with

300 μM hydrogen peroxide was used in subsequent studies to suppress osteoblast phenotype.

Treating cells for 48h with up to 20 μM QRC did not markedly alter alkaline phosphatase staining, but staining was suppressed at the 40 μM dose (Figure 2). Therefore, subsequent studies used doses of 20 μM QRC to stimulate the antioxidant response.

Quercetin Pretreatment Partially Preserves Osteoblast Phenotype

Figure 3A shows representative wells of fetal rat calvarial cultures stained for alkaline phosphatase after 12h of QRC pre-treatment (0 or 20 μM) followed by 4 days of incubation with either 0 or 300 μM hydrogen peroxide. Alkaline phosphatase staining was similar to control when cells were pretreated for 12h with QRC only, but cells treated with hydrogen peroxide for 4 days that were not first pretreated with QRC had fewer alkaline phosphatase positive colonies compared to controls. In contrast, cells treated with hydrogen peroxide for 4 days after a 12h QRC pretreatment had more alkaline phosphatase positive colonies compared to hydrogen peroxide treated cells that were not pre-treated.

Microscopic evaluation of stained wells revealed discrete foci of cuboidal cells that were intensely stained for alkaline phosphatase in both control wells, and in wells that were pre-treated with QRC without subsequent hydrogen peroxide treatment (Figure 3B). Hydrogen peroxide-treated cells that did not receive QRC pre-treatment were not organized into discrete colonies, cells retained a more fibroblastic morphology, and alkaline phosphatase staining was less intense compared to controls. If cells were

pretreated with QRC before incubation with 300 µM hydrogen peroxide, colony formation, cell morphology, and alkaline phosphatase staining were similar to controls.

Figure 4 shows the effect of QRC pretreatment and hydrogen peroxide on the expression of osteoblast phenotype gene expression. Gene expression in cells pretreated with QRC and then changed to control media (0 µM hydrogen peroxide) was similar to 0 µM control. Hydrogen peroxide without QRC pretreatment suppressed Runx2, ALP, and BSP at the 300 µM dose. There was also a marked dose-dependent suppression of OCN in cells treated with hydrogen peroxide without QRC pretreatment. This hydrogen peroxide-induced suppression was mitigated if cells were first pre-treated with 20 µM QRC, particularly for OCN.

Quercetin Pretreatment Blocks Induction of the Antioxidant Response by Hydrogen Peroxide

Figure 5 shows the effect of QRC pretreatment and hydrogen peroxide on the expression of antioxidant response genes (Figure 5A) and proteins (Figure 5B). The 12h pretreatment with QRC did not result in detectable alterations in expression of GCLC genes or proteins after 4-days of incubation in media containing 0 µM hydrogen peroxide. However, incubation for 4 days with a 300 µM dose of hydrogen peroxide in cells that were not pretreated with QRC resulted in slight up-regulation of GCLC mRNA and proteins, which was mitigated if cells were first pretreated with QRC. A similar pattern was observed for HO-1 mRNA and protein expression. Although HO-1 gene expression was statistically significant in cells that received QRC pretreatment alone, there were no marked alterations in this treatment group at the protein level when

compared to control. Hydrogen peroxide treatment alone up-regulated HO-1 mRNA levels nearly 2-fold, but this up-regulation was mitigated by QRC pretreatment.

To examine the acute effect of QRC pretreatment and hydrogen peroxide alterations of the antioxidant response, HO-1 and GCLC proteins were assessed directly after QRC pretreatment and the first 24h of incubation with 0 or 300 μM hydrogen peroxide (Figure 6). Treating cells for 12h with 20 μM QRC and then switching to control media (0 μM hydrogen peroxide) resulted in up-regulation of HO-1 and GCLC proteins compared to cells that were not pretreated (0 μM QRC). Up-regulation was sustained up to 12h after QRC was removed and media switched to control conditions. Treating cells for 12h with 0 μM QRC and then switching to media containing 300 μM hydrogen peroxide robustly up-regulated HO-1 protein expression in cells after 3, 6, and 12h. This up-regulation was partially blocked, however, if cells were first pretreated with 20 μM QRC, particularly at 6 and 12h. Hydrogen peroxide treatment did not alter GCLC protein levels similarly to HO-1, since hydrogen peroxide appeared to block QRC-induced up-regulation of GCLC proteins, such that levels were comparable to control at each time point.

Nrf2 and ERK1/2 protein levels were also assessed in order to identify potential pathways involved in the QRC/hydrogen peroxide effects on osteoblast antioxidant response (Figure 7). Nrf2 protein levels were not altered at any time point or any treatment condition. At the end of the 12h pretreatment phosphorylated ERK1/2 was down-regulated in cells pretreated with 20 μM compared to 0 μM control. If cells were switched to media containing 0 μM hydrogen peroxide, phosphorylated ERK1/2 was up-

regulated after 1.5h, and returned to baseline levels within 3h in cells pretreated with 0uM QRC, but up-regulation was sustained up to 3h in cells that were pre-treated with 20 µM QRC. If cells were pre-treated with 0 µM QRC followed by 300 µM hydrogen peroxide phosphorylated ERK1/2 was up-regulated and sustained up to 12h, while in cells pretreated with 20 µM QRC followed by 300 µM hydrogen peroxide, phosphorylated ERK1/2 was down-regulated after 1.5h and 3h.

Discussion

This study demonstrates a protective effect of the flavonol, QRC, on development of the osteoblast phenotype when cells are cultured in a suppressive, oxidative stress environment. Hydrogen peroxide treatment for four days, starting at confluence, suppressed the number of alkaline phosphatase positive colonies and markedly altered cellular morphology. Additionally, the most specific gene marker of osteoblasts, OCN, was robustly down-regulated by hydrogen peroxide, while less-specific gene markers expressed by immature osteoprogenitors (BSP, ALP, COLL1a, Runx2) were less robustly suppressed and only at the 300 µM dose. This has also been shown in rabbit bone marrow stromal and calvarial osteoblasts, where a 4-day treatment with 100-200 µM hydrogen peroxide resulted in lower alkaline phosphatase staining, lower ALP activity, and fewer osteoblast-like colonies [12]. Together, these data indicate that 300 µM hydrogen peroxide prevents or delays progression of differentiation pathways that are necessary to produce mature osteoblasts in culture. In contrast, cells pre-treated for 12h with 20 µM QRC before 300 µM hydrogen peroxide treatment, had more alkaline phosphatase staining, higher expression of osteoblast phenotypic gene markers, and cell

morphology that was more similar to vehicle controls (0 μM QRC treatment followed by 0 μM hydrogen peroxide).

There are several lines of evidence that suggest that hydrogen peroxide, or other oxidative stress conditions, suppress osteoblastic differentiation by diverting signaling proteins and energy resources away from pathways that promote osteogenic development and towards pathways that promote cell survival and redox homeostasis. The Wnt/TCF signaling pathway is essential for osteoblast differentiation, but during oxidative stress, the FoxO transcription factors have been shown to directly compete for β-catenin, a protein required in both signaling pathways [16, 17], which results in down-regulating osteoblast differentiation. Activation of the Nrf2-mediated antioxidant response has also been shown to block osteoblast differentiation by preventing recruitment of transcription factor Runx2 to the osteocalcin promoter [18]. Furthermore, calvarial cells isolated from Nrf2 knockout mice have higher expression levels of osteoblast phenotypic gene markers and mineralized surface area [19], suggesting that the antioxidant response is in direct competition with osteogenic signaling and transcription pathways. Our data support the theory that osteoblast differentiation and antioxidant response pathways must be balanced for normal differentiation to occur. This theory is demonstrated by the observation that QRC partially blocked hydrogen peroxide-induced expression of antioxidant response proteins, while simultaneously ameliorating hydrogen peroxide-induced suppression of the osteoblast phenotype. This finding suggests that QRC pretreatment “primed” the cells for the subsequent oxidative insult, and that resources could be allocated towards normal differentiation and function, thus preserving phenotype.

Although both QRC and hydrogen peroxide altered levels of HO-1 and GCLC proteins, the acute, 20 μM dose of QRC did not suppress osteoblast phenotype, and protected cells from the suppressive effects of hydrogen peroxide. Cells activate HO-1 and GCLC gene transcription during oxidative stress, suggesting that QRC actually behaves as a pro-oxidant in these culture conditions, and only acts as an antioxidant relative to a subsequent oxidative stress event. This observation is consistent with other studies that show harmful effects of QRC when used at higher doses or when co-treated simultaneously with an oxidative stress-producing reagent [10,20,21]. The acute, relatively low dose of QRC used in this study appears to produce a low grade antioxidant response that does not significantly block osteoblast differentiation, but primes cells for a subsequent oxidative insult. The exact functional outcome of QRC-mediated alterations in HO-1 and GCLC remain unknown without further experiments, but it seems likely that QRC pre-treatment produces a cellular environment that is better equipped to scavenge free radicals, and may also inhibit the signaling pathways that mediate hydrogen peroxide-induced activation of the antioxidant response.

Although QRC and hydrogen peroxide up-regulated two Nrf2 target genes, we did not detect accumulation of Nrf2 proteins in the cell lysates after treatment with either QRC or hydrogen peroxide. The specific pathways involved in regulating Nrf2 activation are complex and involve dimerization with other transcription factors, as well as post-translational modification of Nrf2 and its cytoplasmic sequestering protein, Keap 1 [22], so further experiments are needed to completely rule out involvement of Nrf2. We did detect QRC induced alterations in phosphorylated ERK1/2 levels. The 12h

pretreatment of QRC down-regulated phosphorylated ERK1/2, but levels were up-regulated after changing to control media (0 μM hydrogen peroxide), and cells that received 20 μM QRC had sustained, higher levels of phosphorylated ERK1/2 up to 3h after the media change compared to cells that were pretreated with 0 μM QRC. Cells that received 300 μM hydrogen peroxide also showed up-regulated levels of phosphorylated ERK1/2, but this up-regulation was transiently suppressed in cells pretreated with 20 μM QRC. These data suggest that ERK1/2 signaling may be involved in the mechanism behind QRC's protective effects. ERK1/2 is indispensable for activating many transcription factors, and has been shown to be essential for osteoblast differentiation [23] but further investigation is needed in order to establish the exact role or requirement of ERK1/2 in these outcomes.

These studies reinforce the idea that oxidative stress and activation of the antioxidant response is linked to suppression of the osteoblast phenotype. Several studies in human populations have shown a positive association between flavonoid rich diets and bone density [24,25], and as previously mentioned, QRC is also protective of bone health in rodents. Although it is unlikely that the dose of QRC used in this study is directly applicable to the *in vivo* bone environment, a role for plant-based dietary antioxidants in bone health cannot be ruled out. *In vitro*, osteoblast differentiation progresses steadily throughout a 2 week period, but *in vivo*, bone formation in remodeling packets lasts for months, and occurs asynchronously at various places in the skeleton throughout life [26]. Therefore, exposure to mixtures of dietary antioxidants *in vivo* after consuming flavonoid rich foods may result in cumulative positive effects on osteoblasts that difficult to detect

in the *in vitro* setting. These studies provide insight into the antioxidant response of osteoblasts in the presence of oxidative stress and suggest that the osteoblast antioxidant pathway may be a viable target for intervention.

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Figure Legends

Figure 4.1. Treatment during early differentiation (D5-9) with up to 300 µM hydrogen peroxide suppresses osteoblast phenotypic development. (A) Representative wells of alkaline phosphatase stained cultures after treatment with hydrogen peroxide up to 300 µM on D5, D9 and D13. (B) Real-time PCR amplification of osteoblast phenotypic gene marker mRNA after treatment with up to 300 µM hydrogen peroxide from D5-D9 of culture. Means +/- SEM, n= 3 separate wells of RNA from the same experiment. Significance between groups was assessed by one-way ANOVA with Tukey posthoc tests. Means that do not share a common letter are significantly different ($p < 0.05$).

Figure 4.2. Acute treatment with 20 µM quercetin aglycone during early differentiation (D5-7) does not reduce alkaline phosphatase staining. Representative wells of alkaline phosphatase stained cultures after treatment with up to 40 µM quercetin for 48h.

Figure 4.3. Pretreatment with 20 µM quercetin aglycone prevents hydrogen peroxide-induced suppression of alkaline phosphatase staining and preserves normal cell morphology. (A) Representative wells of alkaline phosphatase stained cultures after treatment with 0 or 20 µM quercetin for 12h followed by 4-day (D5-9) treatment with 300 µM hydrogen peroxide. (B) Colonies of osteoblast-like cells stained for alkaline phosphatase under 100X bright-field magnification. Similar results were observed in 3 independent studies.

Figure 4.4. Pretreatment with 20 µM quercetin aglycone partially blocks hydrogen peroxide-induced suppression of osteoblast phenotypic gene marker mRNA. Real-time PCR amplification of osteoblast phenotypic gene marker mRNA after 12h pretreatment

with 0 or 20 µM quercetin aglycone followed by 4-day (D5-9) treatment with up to 300 µM hydrogen peroxide. Means +/- SEM, n= 3 separate wells of RNA from the same experiment. Univariate analysis revealed no interaction ($p > 0.05$) between hydrogen peroxide and quercetin treatment for any gene except alkaline phosphatase (ALP). Significance between quercetin and vehicle control was assessed by student's t-tests within each time point. Similar results were observed in 2 independent studies.

Figure 4.5. Pretreatment with 20 µM quercetin aglycone partially blocks up-regulation of HO-1 and GCLC mRNA and proteins after 4 days of hydrogen peroxide treatment. (A) Real-time PCR amplification of gene expression and (B) Western blotting of proteins of HO-1, GCLC and B-actin loading control after 12h pretreatment with 0 or 20 µM quercetin aglycone followed by 4-day (D5-9) treatment with up to 300 µM hydrogen peroxide. Means +/- SEM, n= 3 separate wells of RNA from the same experiment. Univariate analysis revealed no interaction ($p > 0.05$) between hydrogen peroxide and quercetin treatment for either gene. Significance between quercetin and vehicle control was assessed by student's t-tests within each time point. Similar results were observed in at least 2 independent studies.

Figure 4.6. Pretreatment with 20 µM quercetin aglycone partially blocks hydrogen peroxide-induced up-regulation of HO-1 and GCLC proteins within 3h of hydrogen peroxide treatment. Western blotting of HO-1, GCLC, and B-actin loading control after 12h pretreatment with 0 or 20 µM quercetin aglycone followed by up to 24h treatment with 300 µM hydrogen peroxide. Similar results were observed in 2 independent studies.

Figure 4.7. Pretreatment with 20 μM quercetin aglycone alters hydrogen peroxide-induced changes in phosphorylated ERK1/2, but not Nrf2 proteins. Western blotting of Nrf2, phosphorylated ERK1/2, total ERK1/2, and B-actin or GAPDH as loading controls after 12h pretreatment with 0 or 20 μM quercetin aglycone followed by up to 24h treatment with 300 μM hydrogen peroxide. Similar results were observed in 2 independent studies.

Figures

Figure 4.1. Treatment during early differentiation (D5-9) with up to 300 µM hydrogen peroxide suppresses osteoblast phenotypic development.

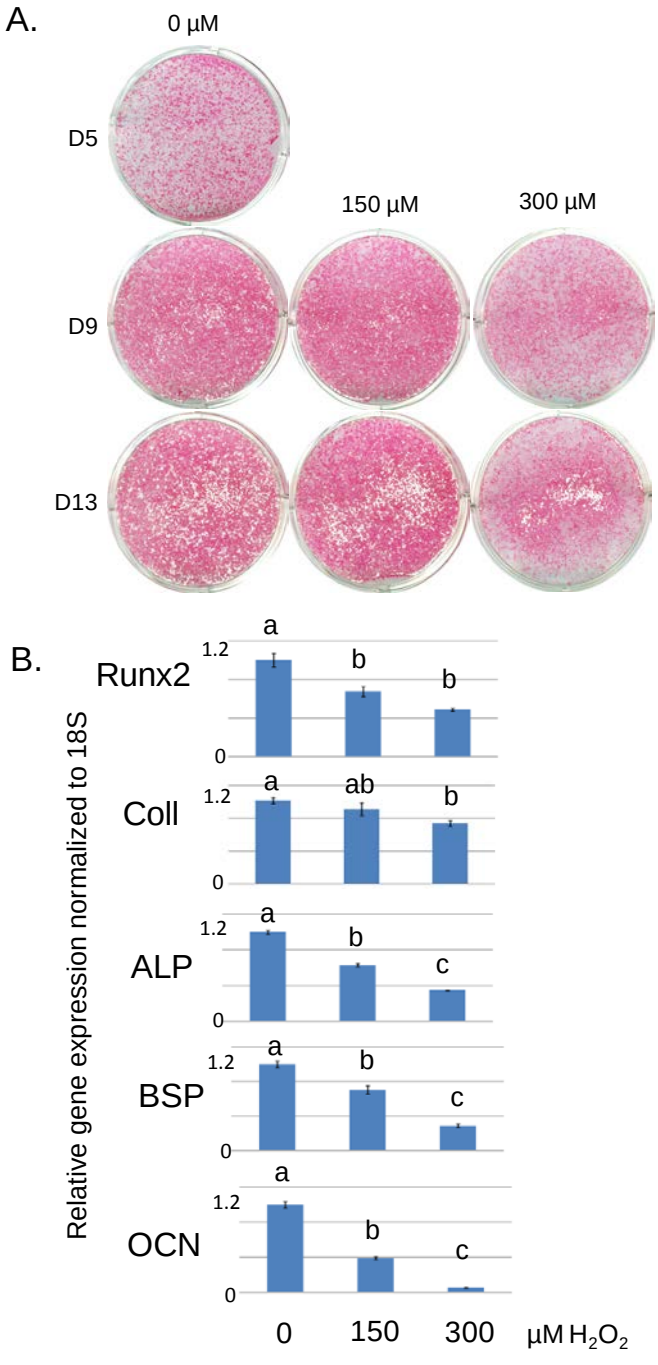


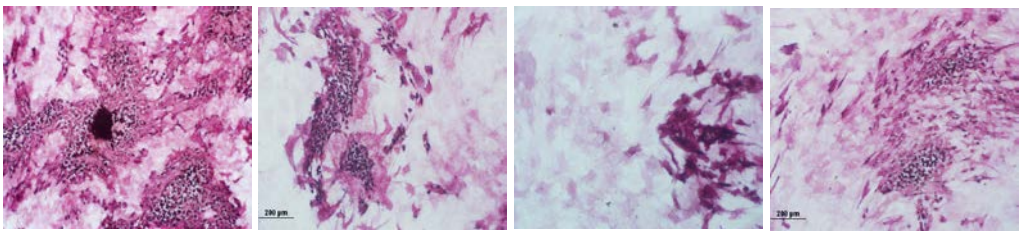
Figure 4.2. Acute treatment with 20 µM quercetin aglycone during early differentiation (D5-7) does not reduce alkaline phosphatase staining. Representative wells of alkaline phosphatase stained cultures after treatment with up to 40 µM quercetin for 48h.

Figure 4.3. Pretreatment with 20 µM quercetin aglycone prevents hydrogen peroxide-induced suppression of alkaline phosphatase staining and preserves normal cell morphology.

A.

-	+	-	+	20 µM QRC
-	-	+	+	300 µM H_2O_2

B.



-	+	-	+	20 µM QRC
-	-	+	+	300 µM H_2O_2

Figure 4.4. Pretreatment with 20 µM quercetin aglycone partially blocks hydrogen peroxide-induced suppression of osteoblast phenotypic gene marker mRNA.

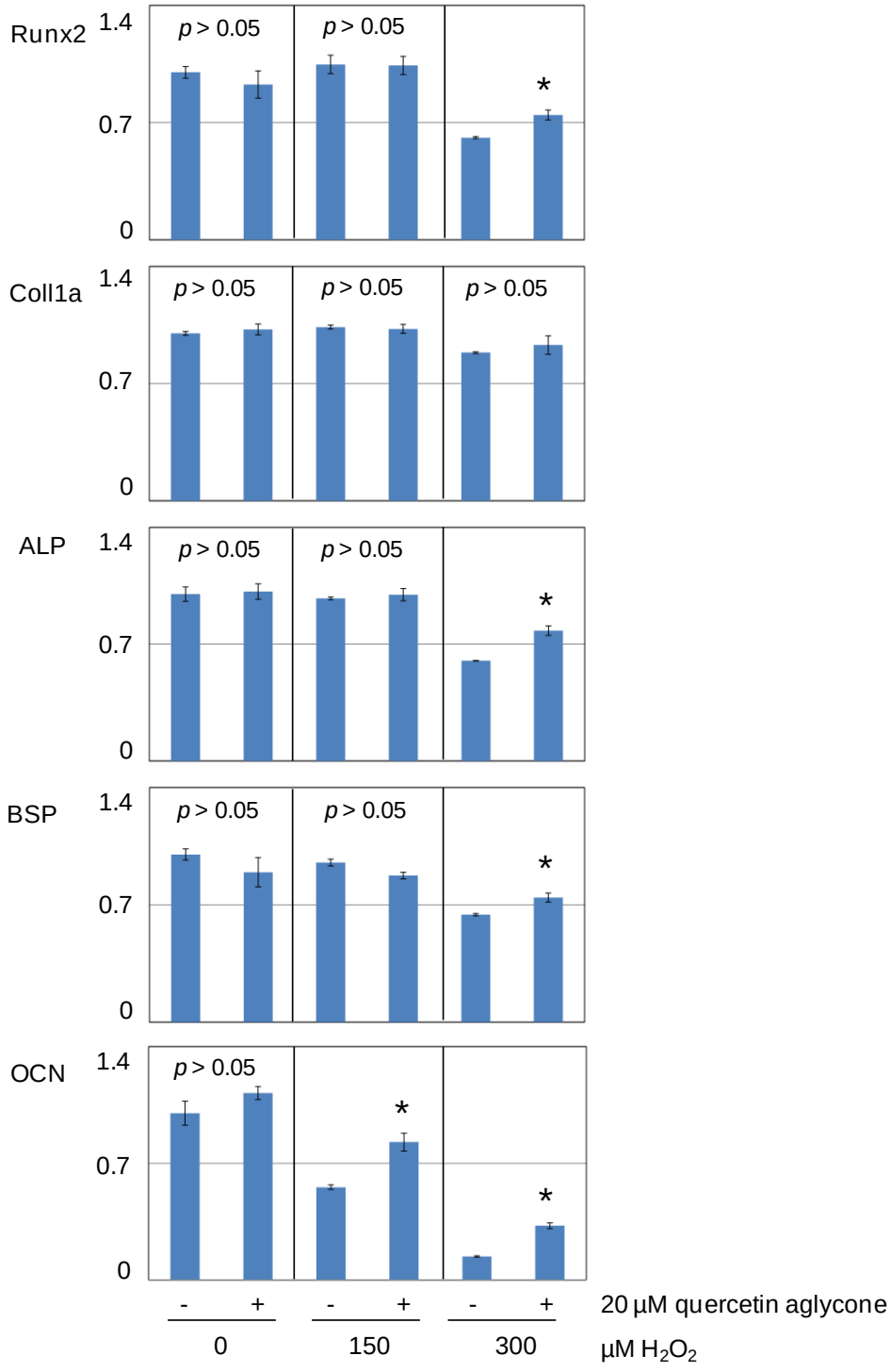
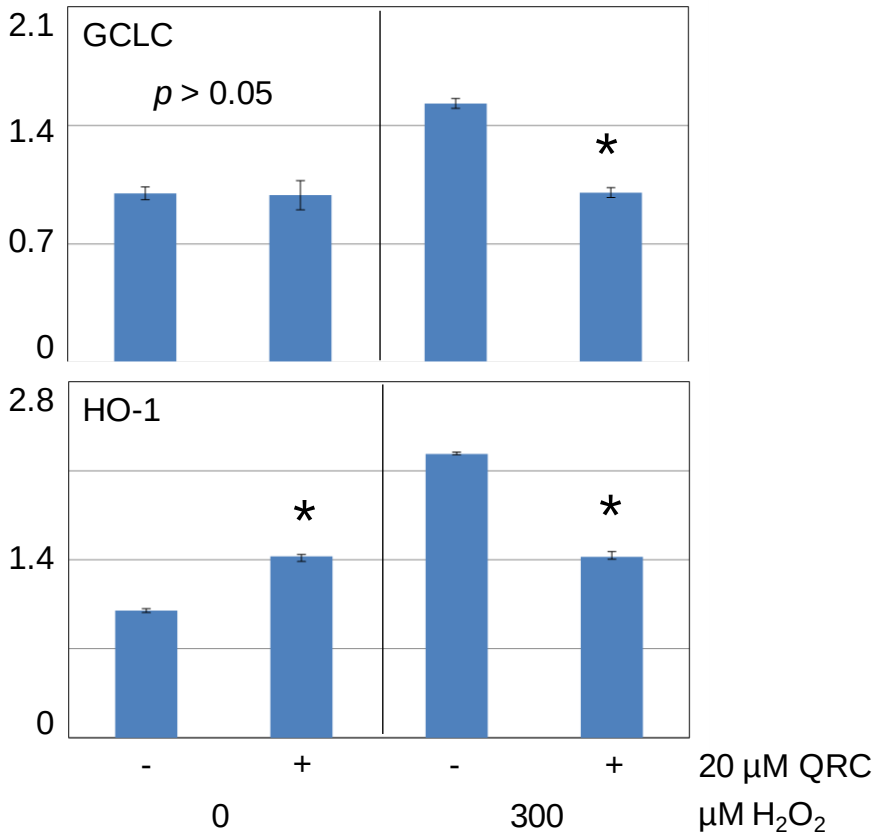


Figure 4.5. Pretreatment with 20 µM quercetin aglycone partially blocks up-regulation of HO-1 and GCLC mRNA and proteins after 4 days of hydrogen peroxide treatment.

A.



B.

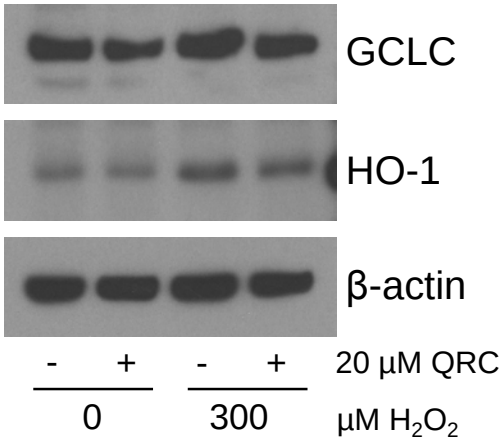


Figure 4.6. Pretreatment with 20 µM quercetin aglycone partially blocks hydrogen peroxide-induced up-regulation of HO-1 and GCLC proteins within 3h of hydrogen peroxide treatment.

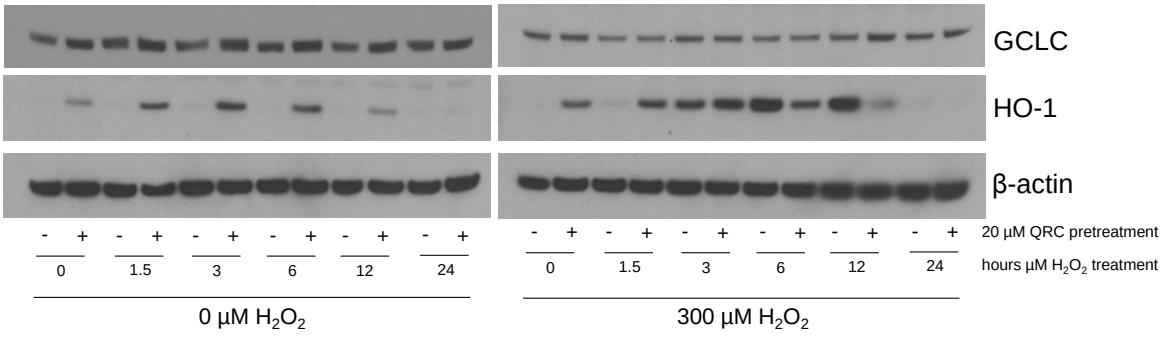
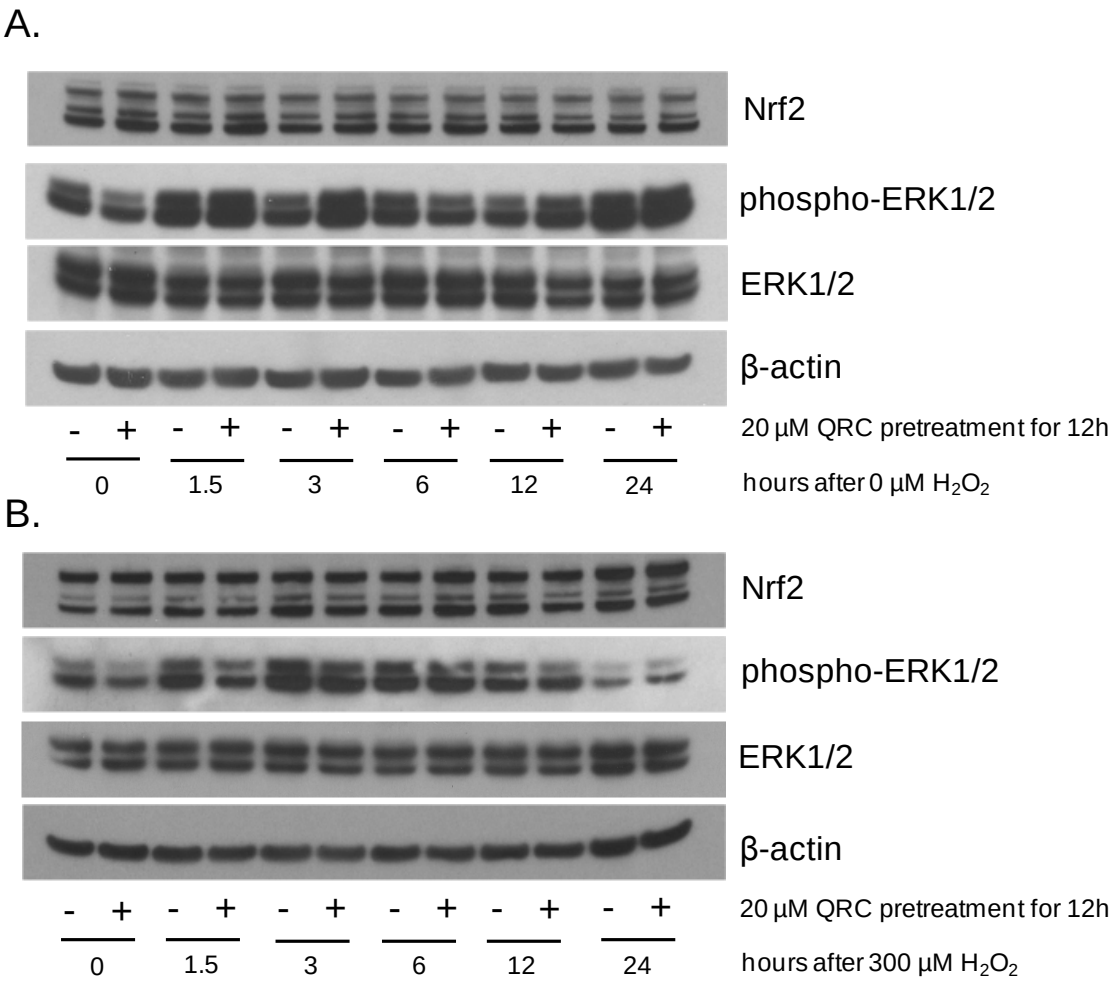


Figure 4.7. Pretreatment with 20 µM quercetin aglycone alters hydrogen peroxide-induced changes in phosphorylated ERK1/2, but not Nrf2 proteins.



CHAPTER V

EPILOGUE

Osteoporosis is a major public health concern that results in debilitating and expensive fragility fractures. Because of the link between oxidative stress in osteoblasts and bone loss, preventive or therapeutic strategies targeting the osteoblast antioxidant response may improve bone health and prevent or reverse osteoporosis. These studies have shown that quercetin metabolites up-regulate the antioxidant response of osteoblasts and this appears to "prime" cells for a subsequent oxidative stress event, which partially blocks hydrogen peroxide-induced up-regulation of the antioxidant response, while simultaneously preserving the development of the osteoblast phenotype. These observations provide more evidence supporting a link between oxidative stress and dysregulation of osteoblast differentiation and function, as well as offer new insights into the osteoblast antioxidant response and the cellular effects of quercetin metabolites.

While these studies have contributed novel observations of the osteoblast antioxidant response and phenotypic development in the presence of quercetin and in an oxidative stress environment, future studies are still needed to further elucidate mechanisms. These studies have identified targets for this future research. Two antioxidant response proteins, heme oxygenase-1 (HO-1) and γ-glutamate cysteine ligase (GCLC) were induced by quercetin, but the functional outcome of is still unknown, especially in the context of protecting the osteoblast phenotype during oxidative stress.

HO-1 was most robustly up-regulated by both quercetin and hydrogen peroxide. Furthermore, quercetin pretreatment blocked the expression of hydrogen peroxide-induced up-regulation of HO-1, which suggests that the function of this protein is important to the osteoblast antioxidant response and may have important roles in protecting the osteoblast phenotype. Higher levels of HO-1protein presumably increase heme catabolism and subsequently increase cellular levels of biliverdin, carbon monoxide, and iron. Experiments to block HO-1 activity with inhibitors, such as zinc protophorphyrin, as well as experiments to target the metabolic byproducts would help establish the exact role of HO-1 activity in osteoblasts when they are exposed to quercetin and hydrogen peroxide and understand the extent that it is necessary for protecting the osteoblast phenotype.

GCLC was also induced by quercetin, but this effect was delayed compared to HO-1. Since GCLC is the rate-limiting enzyme in glutathione synthesis, it would be expected that up-regulated GCLC would lead to higher levels of glutathione. Although alterations in total glutathione were not detected, quercetin may alter other aspects of glutathione metabolism in osteoblasts that were not assessed in these studies. Future experiments would establish alterations in the ratios of oxidized to reduce glutathione, as well as the activities and expression of enzymes, such as glutathione peroxidase and glutathione reductase, that regulate the redox state of this thiol.

In addition to functional outcomes of HO-1 and GCLC, the up-stream alterations in cell signaling pathways and transcriptional factors that mediate the effects of QRC and hydrogen peroxide bear further investigation. Unexpectedly, these studies showed that

up-regulation of HO-1 and GCLC were not associated with cellular accumulation of Nrf2, which is thought to be classically involved in up-regulating antioxidant response genes. Since this study did not examine Nrf2 transcriptional activity or post-transcriptional modifications to Nrf2 or its sequestering protein, Keap1, the necessity of Nrf2 in up-regulating HO-1 and GCLC cannot be ruled out without further studies. Robust alterations in phosphorylated ERK1/2 were also observed in these studies. Quercetin pre-treatment appeared to delay hydrogen peroxide-induced ERK1/2 phosphorylation, but resulted in up-regulation of phosphorylated ERK1/2 when cells were incubated in control media. These differential effects suggest that ERK1/2 signaling is involved in the antioxidant response and/or the preservation of the osteoblast phenotype in these conditions, but it is not yet clear of the exact role ERK1/2 plays in producing the outcomes observed here. In the first study, NFκB was down-regulated by quercetin treatment alone, suggesting that quercetin may have anti-inflammatory effects. Although alterations of NFκB were not observed in the second study after treatment with hydrogen peroxide, the suppression of this transcription factor may play a role in the response of hydrogen peroxide to quercetin that depends on alterations in NFκB target genes that are altered after the 12h pretreatment.

These studies also offer important insights into the behavior of quercetin itself. Although generally classified as an antioxidant, quercetin appears to produce both pro- and antioxidant effects on cells. In fact, these studies indicate that quercetin may only act as an antioxidant in the context of a subsequent oxidative stress event, since quercetin treatment alone activated the antioxidant response. Doses higher that 20 μM resulted in

down-regulation of the osteoblast phenotype, which suggests that in order to have protective effects on osteoblast differentiation outcomes, the dose must be low, or of short duration. These studies primarily used 20 μM quercetin aglycone to induce HO-1 and GCLC, but the cells did respond to lower doses of quercetin, and to the methylated metabolite, isorhamnetin. Since the physiological levels of quercetin metabolites are lower it might be useful to design studies which expand the dose response of quercetin used in the pretreatment phase to include even lower doses and assess the effects of hydrogen peroxide. Although this may not produce detectable alterations in protein levels of HO-1 and GCLC after the 12h pretreatment, there may be effects on the preservation of osteoblast phenotypic development.

Although epidemiological findings and animal studies support the hypothesis that quercetin metabolites from dietary sources have a protective effect on bone health, the mechanisms by which this effect occurs remain difficult to establish. This difficulty is due, at least in part, to the inherent challenges of translating physiological conditions to the *in vitro* setting. First, quercetin is extensively modified by gut and liver enzymes, and a mixture of enzymatically modified metabolites is present in blood plasma. The current studies have attempted to show a more inclusive picture of the effect of dietary quercetin at the cellular level, but the limited commercial availability of enzymatically altered metabolites (we only know of the three used in these studies) makes it difficult to design cell studies that fully capture the *in vivo* setting. Second, since primary cells growing *in vitro* have been separated from their tissue microenvironments, it is impossible to completely account for all the factors that might influence treatment outcomes. The cell

culture conditions may create an environment that makes the effect of lower doses of quercetin more difficult to detect, thus necessitating higher doses than those that have been recorded in blood plasma after consuming quercetin. Finally, fetal rat calvarial osteoblasts undergo differentiation within a 2-week period after reaching confluence, which is in contrast to bone formation in *remodeling* adult bone, which takes months to complete and occurs at different stages in discrete packets all over the skeleton. Additionally, bone *modeling* occurs in young, growing individuals leading to peak bone mass early in adulthood, and high peak bone mass is protective of osteoporosis later in life. Thus, it is difficult to fully replicate a lifetime of consuming quercetin or quercetin-rich foods in the culture dish. These limitations, however, do not preclude a direct role of quercetin metabolites as a preventive or therapeutic strategy, whether used pharmacologically, or at doses that are achievable through dietary intake.