Quercetin’s influence on exercise-induced changes in plasma cytokines and muscle and leukocyte cytokine mRNA

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Nieman DC, Henson DA, Davis JM, Murphy EA, Jenkins DP, Gross SJ, Carmichael MD, Quindry JC, Dumke CL, Utter AC, McAnulty SR, McAnulty LS, Triplett NT, Mayer EP. Quercetin’s influence on exercise-induced changes in plasma cytokines and muscle and leukocyte cytokine mRNA. J Appl Physiol 103: 1728–1735, 2007. The physiological effects of dietary flavonoids such as quercetin are of great current interest due to their antioxidant, anti-inflammatory, anti-pathogenic, cardioprotective, and anticarcinogenic activities (8, 15, 28). Quercetin (3,3,5,7-penta-hydroxyflavone) is the major bioflavonoid in the human diet and is sufficiently bioavailable to exert wide-ranging physiological effects in humans (8, 19). Quercetin is a powerful antioxidant, and in vitro data show that quercetin in aglycone form has an antioxidant potency that is approximately fivefold greater than vitamin C (2). Significant food sources include apples, onions, berries, leafy green vegetables, hot peppers, red grapes, and black tea. Several studies indicate that quercetin mediates some of its effects by inhibiting cyclooxygenase-2 (COX-2) and nuclear factor-kappaB (NF-κB) (2–5, 11, 18, 20, 22). NF-κB is activated by pro-inflammatory stimuli such as TNF-α and LPS and controls the expression of genes encoding pro-inflammatory cytokines and chemokines such as IL-1β, TNF-α, MCP-1, and macrophage inflammatory protein 1α/β (7, 12, 14, 16). In unstimulated cells, NF-κB is localized in the cytoplasm and complexed with the inhibitory protein I kappa B (IkB). Several stimuli including pro-inflammatory cytokines, reactive oxygen species, pathogen exposure, hormones, DNA damage, and physical stress activate protein kinases that phosphorylate IkB, causing its rapid degradation and translocation of NF-κB to the nucleus (16). In the nucleus, NF-κB binds to target genes, stimulating transcription of gene products such as IL-1β, TNF-α, and IL-6. Quercetin acts by stimulating factor, monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1β, tumor necrosis factor-α (TNF-α), and macrophage migration inhibitory factor (21, 24–29, 32). These cytokines are produced by multiple cell types both within and outside the immune system. Several studies indicate that IL-6, IL-1β, and TNF-α mRNA content is increased within postexercise muscle biopsy samples, with the greatest fold increases measured for IL-6 and IL-8 mRNA (20- to 30-fold change from rest when rest = 1) (10, 24, 25, 31). Blood leukocytes may produce IL-8, IL-10, and IL-1ra during sustained exercise (27).

Several types of nutritional supplements including N3-polyunsaturated fatty acids, glutamine, bovine colostrum, and various types of antioxidants such as vitamins C and E have been studied for their potential effect in countering exercise-induced increases in plasma cytokines (23). None have proven consistently effectual except for carbohydrate supplements. Carbohydrate decrease with placebo beverage intake attenuates exercise-induced increases in IL-6, IL-10, and IL-1ra, but not IL-8 (23–25). Carbohydrate may exert these effects through multiple mechanisms, including elevation in blood glucose and tissue glucose uptake, decreases in cytokine mRNA expression, reductions in pro-inflammatory signals, and an attenuation of IL-6 release from the working muscle tissue (23–25).

The physiological effects of dietary flavonoids such as quercetin are of great current interest due to their antioxidant, anti-inflammatory, anti-pathogenic, cardioprotective, and anticarcinogenic activities (8, 15, 28). Quercetin (3,3,5,7-penta-hydroxyflavone) is the major bioflavonoid in the human diet and is sufficiently bioavailable to exert wide-ranging physiological effects in humans (8, 19). Quercetin is a powerful antioxidant, and in vitro data show that quercetin in aglycone form has an antioxidant potency that is approximately fivefold greater than vitamin C (2). Significant food sources include apples, onions, berries, leafy green vegetables, hot peppers, red grapes, and black tea. Several studies indicate that quercetin mediates some of its effects by inhibiting cyclooxygenase-2 (COX-2) and nuclear factor-kappaB (NF-κB) (2–5, 11, 18, 20, 22). NF-κB is activated by pro-inflammatory stimuli such as TNF-α and LPS and controls the expression of genes encoding pro-inflammatory cytokines and chemokines such as IL-1β, TNF-α, MCP-1, and macrophage inflammatory protein 1α/β (7, 12, 14, 16). In unstimulated cells, NF-κB is localized in the cytoplasm and complexed with the inhibitory protein I kappa B (IkB). Several stimuli including pro-inflammatory cytokines, reactive oxygen species, pathogen exposure, hormones, DNA damage, and physical stress activate protein kinases that phosphorylate IkB, causing its rapid degradation and translocation of NF-κB to the nucleus (16). In the nucleus, NF-κB binds to target genes, stimulating transcription of gene products such as IL-1β, TNF-α, and IL-6. Quercetin acts by...
blocking the protein kinase-mediated IkB degradation, thereby preventing NF-κB activation (4, 11, 22). Prostaglandins are also involved in inflammation, and COX-2 is a key enzyme regulating prostaglandin production. COX-2 is an inducible enzyme, is abundant in activated macrophages and other cells at sites of inflammation, and converts the essential fatty acid arachidonic acid to prostaglandin. Quercetin inhibits COX-2 and may thus be one of the mechanisms responsible for quercetin’s anti-inflammatory effects (11). However, it should be emphasized that most of these data on quercetin’s inhibitory effects on NF-κB and COX-2 come from cell culture and animal studies.

The influence of quercetin ingestion on exercise-induced increases in cytokine mRNA expression, plasma cytokine levels, muscle NF-κB, and muscle COX-2 mRNA in human endurance athletes is unknown. One study showed that supplementation with mixed tocopherols, flavonoids including quercetin, and docosahexaenoate for 2 wk decreased plasma IL-6 levels following eccentric arm curl exercise (30). The authors related the IL-6-lowering effect to the anti-inflammatory properties of the combined supplement.

Given quercetin’s potential to block NF-κB and COX-2, and thereby reduce transcription of pro-inflammatory mediators including prostaglandins, cytokines, and chemokines, we hypothesized that quercetin compared with placebo ingestion would attenuate cytokine mRNA expression in muscle and blood leukocytes and lower plasma cytokine levels in endurance athletes engaging in repeated and sustained heavy exertion.

METHODS

Subjects. Forty trained male cyclists were recruited as experimental subjects through local and collegiate cycling clubs. Written, informed consent was obtained from each subject, and the experimental procedures were approved by the institutional review board of Appalachian State University.

Research design. Two to three weeks before the first test session, subjects reported to the ASU Human Performance Laboratory for orientation and measurement of cardiorespiratory fitness. Maximal oxygen uptake (V̇O₂max) was determined using a graded maximal orientation and measurement of cardiorespiratory fitness. Maximal subjects reported to the ASU Human Performance Laboratory for preventing NF-κB activation (4, 11, 22). Prostaglandins are

Subjects agreed to avoid the use of large-dose vitamin/mineral supplements (above 100% of recommended dietary allowances), nutritional supplements, ergogenic aids, herbs, and medications known to affect immune function for 3 wk before and during a 3-day period of intensified exercise. During orientation, a dietitian instructed the subjects to follow a diet moderate in carbohydrate (using a food list) during the 24-day study period. Subjects recorded food intake in a 3-day food record before the first exercise test session. The food records were analyzed using a computerized dietary assessment program (Food Processor, ESHA Research, Salem, OR).

The cyclists were randomized to quercetin (n = 20) or placebo (n = 20) groups. Under double-blind procedures, subjects received quercetin (1,000 mg/day) or placebo supplements for 3 wk before and during a 3-day period of intensified exercise. Tang powder (Kraft Foods, Northfield, IL) and 500 mg of pure quercetin powder (QU995, Quercegen Pharma, Newton, MA) were mixed with powdered food coloring and placed in plastic vials. Subjects mixed the contents of the vials with 8 oz. of water in green plastic bottles and consumed the beverage before their first and last meals of each day to achieve an intake of 1,000 mg quercetin/day. Subjects returned empty vials to the study dietitian to verify compliance with the supplementation regimen.

Subjects came to the laboratory for 3 consecutive days following the 3-wk quercetin or placebo supplementation period. Subjects cycled for 3 h at ~57% maximal work rate (in Watts). Subjects reported to the laboratory at 2:00 PM, not having ingested energy in any form after 12:30 PM. Blood samples were collected ~30 min preexercise and ~15 min postexercise for each exercise session. Subjects ingested 0.5–1.0 liter of water per hour during the 3-h cycling bout, with no other beverages or food ingested during the test sessions. Muscle biopsies were obtained before and after the first and third exercise sessions.

During the test sessions, experimental subjects cycled using their own bicycles on CompuTrainer Pro model 8001 trainers (RacerMate, Seattle, WA), with the exercise load set at ~57% maximal work rate. Metabolic measurements were made every 30 min of cycling using the MedGraphics CPX metabolic system to verify workload.

Blood samples. Blood samples were drawn from an antecubital vein with subjects in the supine position. Routine complete blood counts were performed by our clinical hematology laboratory and provided hemoglobin and hematocrit for determination of plasma volume change using the method of Dill and Costill (6). Other blood samples were centrifuged in sodium heparin or EDTA tubes, and plasma was aliquoted and then stored at −80°C before plasma quercetin and cytokine analysis.

Plasma quercetin. Total plasma quercetin (quercetin and its primary conjugates) was measured following solid-phase extraction via reverse-phase HPLC with ultraviolet detection as described by Quercegen Pharma (Newton, MA; personal communication). This procedure is similar to that previously published by Ishii et al. (13). Quercetin conjugates were hydrolyzed by incubating 250- to 500-μl plasma aliquots with 10 μl of 10% DTT solution, 50 μl of 0.58 M acetic acid, 50 μl of a mixture of β-glucuronidase and arylsulfatase, and crude extract from Helix pomatia (Roche Diagnostics, Mannheim, Germany) for 40 min at 37°C. After incubation, 500 μl of 0.01 M oxalic acid was added, and then each sample was vortexed and centrifuged for 5 min at 10,000 rpm. 1 ml of supernatants were then applied to solid-phase extraction cartridges (Oasis HLB 1cc (30 mg) SPE cartridge (Waters, Milford Massachusetts)) that were preconditioned with 1 ml of methanol (MeOH), 0.5 ml of 0.01 M oxalic acid, and 1 ml of distilled H₂O (dH₂O) and drawn through at a rate of 0.5 ml/min using a vacuum manifold (Waters Milford Massachusetts). Cartridges were then washed with 0.5 ml of MeOH × 2. Eluant was collected into 1.5-ml microcentrifuge tubes. Ten microliters of 10% DTT solution were added to the combined eluant, and the samples were then vortexed for 1 min and placed into a vacuum concentrator (Savant Speed Vac SC 110 Savant Instruments, Farmindale, NY) until MeOH was completely evaporated. The residue was reconstituted with 150 μl of MeOH/dH₂O (1:1). Fifty-microliter injections were used for HPLC analysis.

Chromatographic analysis was performed using a Waters Breeze system (Waters, Milford, MA) consisting of a Waters 1525 Binary HPLC pump, 2487 ultraviolet detector, and Symmetry C18 5 μm 4.6 × 150 mm column. The mobile phase, consisting of a mixture of acetonitrile with 0.1% HCOOH (0–15 min: 10–90%) and dH₂O with 0.1% HCOOH (0–15 min: 90–10%) with a gradient elution program at a flow rate of 1 ml/min.

Quantitation of the quercetin peak was based on the standard addition method using both plasma and MeOH with similar results. Both standards and samples were treated in an identical manner.

Plasma cytokine measurements. Total plasma concentrations of IL-1ra, IL-6, IL-8, IL-10, MCP-1, and TNF-α were determined using quantitative sandwich ELISA kits provided by R&D Systems (Min-
neapolis, MN). All samples and provided standards were analyzed in duplicate. High-sensitivity kits were used to analyze TNF-α and preexercise samples of IL-6 and IL-10. The minimum detectable concentration of IL-1ra was <22 pg/ml, IL-6 < 0.70 pg/ml, IL-6 (high sensitivity) < 0.039 pg/ml, IL-8 < 3.5 pg/ml, IL-10 < 3.9 pg/ml, IL-10 (high sensitivity) < 0.5 pg/ml, MCP-1 < 5.0 pg/ml, and TNF-α < 0.106 pg/ml. To improve sensitivity in the detection of IL-8, we employed SOFTmax analysis software (Molecular Devices, Sunnyvale, CA). When applicable, pre- and postexercise samples were analyzed on the same assay plate to decrease inter-kit assay variability.

**Skeletal muscle biopsies.** Skeletal muscle biopsies were acquired 30–60 min before and 20–40 min after the 3-h cycling bouts on days 1 and 3. The exact same procedures were utilized for all biopsies in accordance with previous studies (24, 25). On day 1, incisions were made pre- and postexercise in the same thigh ~3 cm apart. One day 3, muscle samples were collected from the opposite thigh, with leg order determined randomly. Local anesthica (1% Xylocaine) was injected subcutaneously and into the vastus lateralis. A muscle biopsy sample was then obtained using the percutaneous needle biopsy procedure modified to include suction (9). Muscle biopsy samples were divided into three pieces and immediately frozen in liquid nitrogen. Samples were stored at −80°C until subsequent analysis.

**NF-κB muscle analysis.** Frozen skeletal muscle tissue (~10 mg) was homogenized in 0.5 ml of lysis buffer (Active Motif, Carlsbad, CA) containing 10 mM DTT and a protease inhibitor cocktail using a polytron. The homogenized samples were incubated on ice for 30 min. Samples were transferred to prechilled microcentrifuge tubes and centrifuged at 10,000 RPM for 10 min at 4°C. The supernatant was transferred to a new prechilled microcentrifuge tube and centrifuged again to obtain a clarified lysate. Total soluble protein of the lysate was determined via bichoninic acid protein assay (Pierce, Rockford, IL). NF-κB was determined using a TransAM ELISA-based technique designed to determine the p65 subunit of NF-κB (Active Motif, Carlsbad, CA). Briefly, 30 µl of binding buffer containing DTT and herring sperm DNA was added to each well, followed by 20 µl of sample containing 2 µg. The plate was sealed and incubated for 1 h at room temperature with mild agitation (100 RPM on a plate shaker). After the incubation, the plate was washed three times with 200 µl of wash buffer. Primary NF-κB antibody (100 µl) was added to each well; the plate was covered and incubated for 1 h at room temperature. The wash step was repeated, and 100 µl of horseradish peroxidase antibody was added to each well followed by a 1-h incubation period. The plate was washed four times before 100 µl of developing solution was added to each well. The plate was incubated in the dark for 5 min, followed by addition of 100 µl of stop solution. The plate was read on a microplate reader within 5 min at 450 nm, with a reference wavelength of 655 nm.

**Leukocyte mRNA extraction and cDNA synthesis.** The QIAampRNA Blood Mini Kit Protocol (Qiagen, Valencia, CA; catalog no. 52304) was utilized to extract mRNA. From each subject, two 1.5-ml aliquots of whole blood collected in EDTA were purified for RNA. Briefly, erythrocytes were selectively lysed, and leukocytes were recovered by centrifugation. Samples were briefly centrifuged through a QIAshredder spin column, ethanol was added to adjust binding conditions, and the sample was applied to a QIAamp spin column. RNA was bound to the silica gel membrane during a brief centrifugation step. Contaminants were washed away, and total RNA was eluted in 30 µl of RNase-free water.

The extracted RNA (7.5 µl of sample) was dissolved in diethylpyrocarbonate-treated water and quantified spectrophotometrically at 260-nm wavelength. RNA was reverse transcribed into cDNA in a 50-µl reaction volume containing 19.25 µl of RNA in RNase-free water, 5 µl of 10× RT buffer, 11 µl of 25 mM MgCl2, 10 µl of deoxy-NTPs mixture, 2.5 µl of random hexamers, 1 µl of RNase inhibitor, and 1.25 µl of multiscribe reverse transcriptase (50 U/µl). Reverse transcription was performed at 25°C for 10 min, 37°C for 60 min, and 95°C for 5 min, followed by quick chilling on ice and stored at −20°C until subsequent amplification.

**Quantitative real-time PCR analysis.** Quantitative real-time polymerase chain reaction (RT-PCR) analysis was done per manufacturer’s instructions (Applied Biosystems) using TaqMan Gene Expression Assays. DNA amplification was carried out in 12.5 Taqman Universal PCR Master Mix (AmpliTaq Gold DNA Polymerase, Passive Reference 1, Buffer, dNTPs, AmpErase), 1 µl of cDNA, 9 µl of RNase-free water, and 1.25 µl of 18S primer (VIC) and 1.25 µl of primer (FAM) (for endogenous reference and target cytokine) in a final volume of 25 µl/well. Human control RNA (calibrator RNA) was also used and served as a calibrator for each plate. Samples were loaded in a MicroAmp 96-well reaction plate. Plates were run using ABI Sequence Detection System (Applied Biosystems, Foster City, CA). After 2 min at 50°C and 10 min at 95°C, plates were coamplified by 50 repeated cycles, of which one cycle consisted of a 15-s denaturing step at 95°C and a 1-s annealing/extension step at 60°C. Data were analyzed using ABI software using the cycle threshold (CT), which is the value calculated and based on the time (measured by PCR cycle number) at which the reporter fluorescent emission increases beyond a threshold level (based on the background fluorescence of the system) (28), and it reflects the cycle number at which the cDNA amplification is first detected. We have previously reported detailed methodology concerning the dual amplification technique (24, 25). Samples were run in duplicate, and the intra-assay and interassay coefficients of variation were determined to be 1.69 and 1.65% for the ΔCts, respectively.

**Muscle total RNA isolation and cDNA synthesis.** Procedures for RNA isolation were in accordance with previous publications (24, 25). Briefly, skeletal muscle tissue was homogenized under liquid nitrogen with a polytron, and total RNA was extracted using the guanidine thiocyanate method with TRIzol Reagent (Life Technologies, Gibco). The extracted RNA (2.5 µl of sample) was dissolved in diethylpyrocarbonate-treated water and quantified spectrophotometrically at 260-nm wavelength. RNA was reverse transcribed into cDNA in a 50-µl reaction volume containing 19.25 µl of RNA (1.5 µg) in RNase-free water, 5 µl of 10× RT buffer, 11 µl of 25 mM MgCl2, 10 µl of deoxy-NTPs mixture, 2.5 µl of random hexamers, 1 µl of RNase inhibitor, and 1.25 µl of multiscribe reverse transcriptase (50 U/µl). Reverse transcription was performed at 25°C for 10 min, 37°C for 60°C for 5 min, followed by quick chilling on ice and stored at −20°C until subsequent amplification.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Quercetin (n = 20)</th>
<th>Placebo (n = 20)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline measures</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, yr</td>
<td>26.1 ± 1.8</td>
<td>29.1 ± 2.4</td>
<td>0.321</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>74.7 ± 0.2</td>
<td>74.2 ± 1.4</td>
<td>0.846</td>
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<tr>
<td>VO2max, ml·kg⁻¹·min⁻¹</td>
<td>53.2 ± 1.2</td>
<td>54.7 ± 1.1</td>
<td>0.365</td>
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<td>Powermax, W</td>
<td>314 ± 9</td>
<td>320 ± 69</td>
<td>0.554</td>
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<tr>
<td>HRmax, beats/min</td>
<td>188 ± 1</td>
<td>190 ± 2</td>
<td>0.328</td>
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<tr>
<td>Performance measures</td>
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<tr>
<td>Mean power, W</td>
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<td>181 ± 4</td>
<td>0.626</td>
</tr>
<tr>
<td>Mean power, %Wattsmax</td>
<td>56.8 ± 0.2</td>
<td>56.5 ± 0.3</td>
<td>0.486</td>
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<tr>
<td>Mean heart rate, beats/min</td>
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<td>149 ± 3</td>
<td>0.897</td>
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<tr>
<td>Heart rate, %HRmax</td>
<td>79.4 ± 0.8</td>
<td>78.1 ± 1.0</td>
<td>0.300</td>
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<tr>
<td>Mean VO2, ml·kg⁻¹·min⁻¹</td>
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<td>36.9 ± 0.8</td>
<td>0.458</td>
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<td>Mean VO2, %VO2max</td>
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<td>67.6 ± 1.1</td>
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<td>Cadence, RPM</td>
<td>80.8 ± 2.2</td>
<td>83.7 ± 2.2</td>
<td>0.349</td>
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</table>

Values are means ± SE. VO2, volume of oxygen consumption; VO2max, maximal VO2; Powermax, maximal power; HRmax, maximal heart rate; Wattsmax, maximal power rate.
compared with placebo groups over a 3-day period.

Initial exclusion criteria consisted of FAM/H9004/H9004/... then calculated for each sample, and relative quantification was calculated and compared between quercetin and placebo groups using Student’s t-tests, with significance set at P ≤ 0.05.

RESULTS

Subject characteristics and performance data for the 40 cyclists randomized to quercetin and placebo groups are summarized in Table 1. No significant differences were found between groups for age or maximal performance measures. Subjects in the quercetin and placebo groups came into the study averaging 242 ± 27 and 270 ± 29 km/wk, and 1.5 ± 0.2 and 1.6 ± 0.2 h cycling per training bout, respectively. Thus the 3-day intensified exercise period (9 h of exercise) represented nearly a doubling of their normal exercise workload.

Box’s M suggested that the assumptions necessary for the univariate approach were not tenable, the multivariate approach to repeated-measures ANOVA was used (Pillai’s trace). When interaction effects were significant (P ≤ 0.05), pre- to postexercise changes were calculated and compared between quercetin and placebo groups using Student’s t-tests, with significance set at P ≤ 0.05.

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Subjects in quercetin and placebo groups were able to maintain a mean power output of ~57% \( \text{Watts}_{\text{max}} \) at an oxygen consumption of ~68% \( \text{VO}_{2\text{max}} \) and a cadence slightly above 80 RPM during the 9 h of exercise (Table 1).

Plasma volume change did not differ between groups during the three exercise bouts and averaged <1% due to ingestion of 0.5–1.0 liters of water per hour of exercise (data not shown). After 3 wk of supplementation, a 9.2-fold difference in plasma quercetin levels was measured between quercetin and placebo groups (Fig. 1). Three-day food records before the 3-day exercise period revealed no significant group differences in energy or macro-nutrient intake (data not shown), and for all subjects combined energy intake was 11.2 ± 0.5 MJ/day (2,684 ± 117 kcal/day), with carbohydrate representing 56.6 ± 2.5%, protein 16.2 ± 0.8%, and fat 27.2 ± 1.8%. A poststudy questionnaire revealed that nine subjects in each group guessed they were on quercetin compared with placebo group on days 1 and \( n = 20 \) groups during 3 consecutive days. Main time effect, \( P < 0.01; \) group × time interaction effect, \( P = 0.944 \).

Significant main time effects were measured for each of the six plasma cytokines in Table 2, but the pattern of increase during each 3-h cycling bout did not differ significantly between quercetin and placebo groups. The patterns of change for plasma IL-8 and TNF-\( \alpha \) tended to be lower in the quercetin compared with placebo group (\( P = 0.094 \) for both). The exercise-induced change in plasma IL-8 was 29% lower in the quercetin compared with placebo group on day 1 (\( P = 0.161 \)) but was not markedly different on days 2 and 3. The magnitude of the pre- to postexercise increases in plasma cytokine levels decreased over the 3-day period for all six cytokines (day × time effect, \( P < 0.05 \)).

Muscle NF-\( \kappa \)B content did not differ between quercetin and placebo groups and was unaltered by exercise (Fig. 2) (interaction effect, \( P = 0.662 \); time effect, \( P = 0.872 \)). Muscle COX-2 mRNA expression increased significantly postexercise on days 1 and 3 (\( P < 0.025 \)) but did not differ between quercetin and placebo groups (Fig. 3) (interaction effect, \( P = 0.944 \)). Significant main time effects were measured for postexercise increases in blood leukocyte IL-8, IL-10, and IL-1ra mRNA (\( P < 0.001 \) for each) (Figs. 4–6). The overall pattern of change over time was significantly lower for quercetin compared with placebo groups for leukocyte IL-8 and IL-10 but not IL-1ra mRNA (\( P = 0.019, 0.012, \) and 0.256, respectively). Postexercise leukocyte IL-8 mRNA averaged 2.8 ± 0.2 and 4.2 ± 0.2 (fold difference from rest) during the 3-day exercise period for quercetin and placebo groups, respectively (33% difference) (\( P = 0.004 \)). Leukocyte IL-10 mRNA expression increased postexercise on day 1 (36% less for the quercetin compared with placebo group) but was unaltered on days 2 and 3 (day × time effect, \( P < 0.001 \)).

Muscle cytokine mRNA expression increased significantly postexercise for IL-6, IL-8, IL-1\( \beta \), and TNF-\( \alpha \) (main time effects, \( P < 0.001 \) for each), but the pattern of change did not differ between quercetin and placebo groups (interaction effects, \( P = 0.595, 0.913, 0.910, \) and 0.930, respectively).
The magnitude of increase in muscle cytokine mRNA expression decreased across the 3-day period (day × time effects, $P < 0.001$ for each).

**DISCUSSION**

Quercetin (1,000 mg/day) compared with placebo ingestion by cyclists during a 24-day period did not alter muscle NF-κB content or exercise-induced increases in muscle COX-2 mRNA or mRNA expression for IL-6, IL-8, IL-1β, and TNF-α. Quercetin supplementation did attenuate increases in postexercise blood leukocyte IL-8 and IL-10 mRNA and tended to lower plasma levels of IL-8 and TNF-α.

Few studies have measured pre- and postexercise levels for plasma cytokine levels while also providing data on cytokine mRNA expression in muscle samples and blood leukocytes. Exercise-induced increases in these measures confirm previous findings in our laboratory and reports from other investigators (10, 24–27, 31). Of the various cytokines measured in this study, IL-8 changes with exercise were most strongly influenced by quercetin supplementation. IL-8 mRNA expression in blood leukocytes but not muscle biopsy samples was reduced 33% postexercise in the quercetin compared with placebo group, with a tendency toward lowered plasma IL-8 levels (29% lower on day 1).

IL-8 is produced by multiple cell types including endothelial cells, fat cells, mononuclear cells in blood, macrophages, mast cells, and cells in bone, skin, and muscle (10). IL-8 is an important mediator of inflammatory disorders and stimulates polymorphonuclear cell adherence, degranulation, and respiratory burst activity. IL-8 is rapidly induced by many stimuli including TNF-α, IL-1, and bacterial agents. In vitro data indicate that quercetin at concentrations within the range achieved by the subjects in this study inhibits TNF-α production and gene expression via modulation of NF-κB and IκB in human peripheral blood mononuclear cells (22). Other in vitro data with mast cells demonstrate that quercetin strongly inhibits secretion of TNF-α and IL-8 (15). These two in vitro studies, however, used quercetin in aglycone form, and application to the conjugated quercetin found in vivo is currently unknown.

Quercetin also significantly reduced blood leukocyte IL-10 mRNA expression after the first day of exercise but without any measurable effect on plasma IL-10 levels. In vitro data from bone marrow-derived macrophages indicate that quercetin stimulates the expression of IL-10, a finding consistent with quercetin’s anti-inflammatory effects (3). Thus our finding of an increased IL-10 mRNA expression in blood leukocytes is difficult to interpret, but this effect occurred only after the first day of exercise (3). Most cytokine measures in this study showed diminished postexercise levels by the third compared with the first day of exercise, suggesting adaptation via the hypothalamic-pituitary-adrenal axis, as discussed elsewhere (28). These data indicated that trained cyclists adapted quickly to the physiological stress of repeated bouts of intense and prolonged exercise, and by the second and third bout experienced lower levels of stress hormones and diminished immune perturbations.

Contrary to our hypothesis, neither exercise nor quercetin had an effect on muscle NF-κB content, and exercise-induced increases in muscle COX-2 mRNA were similar in quercetin and placebo groups. Prolonged and intensive exercise in-
creased both muscle and leukocyte mRNA cytokine expression and plasma cytokine levels, suggesting that these effects occurred without muscle NF-κB regulation. Steensberg et al. (31) also reported exercise-induced increases in muscle IL-6 and IL-8 mRNA without muscle NF-κB involvement. Primary signaling mechanisms and transcription factors for cytokine gene expression during exercise are poorly understood, but nitric oxide production, calcineurin nuclear factor of activated T cells, mitogen activated protein kinases, and other pathways are being explored (31). NF-κB regulates the expression of many target genes within cells of the immune system, including those encoding cytokines, immune receptors, cell adhesion molecules, apoptosis, and acute phase proteins (22). In vitro studies with a variety of cells, including macrophages, indicate that inhibition of COX-2 by quercetin may contribute to anti-inflammatory effects via mechanisms involving a decrease in NF-κB activation (11, 18). However, our data do not suggest a link between oral quercetin ingestion, muscle NF-κB content, and muscle COX-2 mRNA expression during intensified exercise training.

The physiology of NF-κB in cells outside the immune system is not well understood. Chronic NF-κB activation in muscle leads to muscle wasting as seen in cancer, AIDS, and bed rest (7, 16). Rodent studies indicate that the NF-κB signaling pathway is activated in skeletal muscle during exercise, with reactive oxygen species generated during exercise serving as critical messengers to activate upstream kinases of the NF-κB cascade (1, 12, 14, 16). A high alpha-tocopherol diet in rats blunts exercise-induced increases in muscle NF-κB and myeloperoxidase, indicating underlying redox-sensitive pathways (1). Studies with human subjects, however, do not support these findings. Durham et al. (7) showed a 50% decrease in NF-κB activity in thigh muscle samples taken from human subjects who engaged in 45 min of fatiguing leg squat and extension exercise. The decrease in NF-κB was transient with a full recovery by 1-h postexercise. Male subjects engaging in knee extensor exercise for 2 h did not experience an increase in NF-κB activity from thigh muscle biopsy samples (31). Plasma F_2-isoprostanes increased significantly in our subjects (data being presented elsewhere) after each exercise bout, indicating significant oxidative stress without muscle NF-κB activation. Thus currently available data do not support muscle NF-κB activation in humans during exercise or a regulatory role in muscle or blood cytokine mRNA expression. We used a whole-cell extract procedure to extract NF-κB from homogenized muscle tissue and measured the P65 subunit of NF-κB using an antibody ELISA method that is specific for the activated form of p65. Other investigators have measured DNA binding activity of NF-κB using an antibody Western blot (31). Thus some caution is urged in comparing results across studies. We did not measure NF-κB activation in blood peripheral blood mononuclear, but quercetin’s influence in decreasing blood leukocyte IL-8 and IL-10 mRNA expression suggests some level of immune cell NF-κB regulation during exercise.

In summary, 1,000 mg/day quercetin supplementation by trained cyclists over a 24-day period did not have widespread effects in countering exercise-induced increases in muscle cytokine or COX-2 mRNA expression or plasma cytokine levels across a 3-day intensified exercise period. Quercetin compared with placebo ingestion did diminish postexercise expression for leukocyte IL-8 and IL-10 mRNA, suggesting that elevated plasma quercetin levels exerted some effects within the blood compartment. Quercetin ingestion had no influence on any of our muscle outcome measures, including muscle NF-κB content and cytokine and COX-2 mRNA, suggesting that quercetin may not accumulate in muscle tissue, at least within the context of this study. Other data from this study published elsewhere indicate that the cyclists experienced a markedly lower incidence of upper respiratory tract infection symptoms during the 2-wk period following intensified exercise (28). Group differences in illness rates occurred despite no influence of quercetin on several measures of immune function. Thus quercetin may have reduced illness rates using direct anti-pathogenic pathways, as shown in vitro. Quercetin is conjugated with glucuronic acid, sulfate, and methyl groups in human plasma, and additional research is needed to discover which metabolites influence blood leukocyte IL-8 and IL-10 mRNA expression and exert anti-pathogenic activity.

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REFERENCES


