

## 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. First published August 23, 2007; doi:10.1152/jappphysiol.00707.2007.— Trained male cyclists ( $n = 40$ ) ingested quercetin (Q;  $n = 20$ ) (1,000 mg/day) or placebo (P;  $n = 20$ ) supplements under randomized, double-blinded methods for 3 wk before and during a 3-day period in which subjects cycled for 3 h/day at ~57% maximal work rate. Blood samples were collected before and after each exercise session and assayed for plasma IL-6, IL-10, IL-1ra, IL-8, TNF- $\alpha$ , and monocyte chemoattractant protein 1, and leukocyte IL-10, IL-8, and IL-1ra mRNA. Muscle biopsies were obtained before and after the first and third exercise sessions and assayed for NF- $\kappa$ B and cyclooxygenase-2 (COX-2), IL-6, IL-8, IL-1 $\beta$ , and TNF- $\alpha$  mRNA. Postexercise increases in plasma cytokines did not differ between groups, but the pattern of change over the 3-day exercise period tended to be lower in Q vs. P for IL-8 and TNF- $\alpha$  ( $P = 0.094$  for both). mRNA increased significantly postexercise for each cytokine measured in blood leukocyte and muscle samples. Leukocyte IL-8 and IL-10 mRNA were significantly reduced in Q vs. P (interaction effects,  $P = 0.019$  and 0.012, respectively) with no other leukocyte or muscle mRNA group differences. Muscle NF- $\kappa$ B did not increase postexercise and did not differ between Q and P. Muscle COX-2 mRNA increased significantly postexercise but did not differ between Q and P. In summary, 1 g/day quercetin supplementation by trained cyclists over a 24-day period diminished postexercise expression of leukocyte IL-8 and IL-10 mRNA, indicating that elevated plasma quercetin levels exerted some effects within the blood compartment. Quercetin did not, however, influence any of the muscle measures, including NF- $\kappa$ B content, cytokine mRNA, or COX-2 mRNA expression across a 3-day intensified exercise period.

nuclear factor kappa B; cyclooxygenase-2; immune; inflammation; flavonoids

PLASMA CONCENTRATIONS OF PRO- and anti-inflammatory cytokines increase during prolonged and heavy exertion including interleukin (IL)-6, IL-10, IL-8, IL-1ra, granulocyte colony-stimulating factor, monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1 $\beta$ , tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), 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-8, IL-1 $\beta$ , and TNF- $\alpha$  mRNA content is increased within postexercise muscle biopsy sam-

ples, 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 compared 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 antioxidative, anti-inflammatory, anti-pathogenic, cardioprotective, and anticarcinogenic activities (8, 15, 28). Quercetin (3,3',4',5,7-pentahydroxyflavone) 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- $\kappa$ B) (2–5, 11, 18, 20, 22). NF- $\kappa$ B is activated by pro-inflammatory stimuli such as TNF- $\alpha$  and LPS and controls the expression of genes encoding pro-inflammatory cytokines and chemokines such as IL-1 $\beta$ , TNF- $\alpha$ , MCP-1, and macrophage inflammatory protein 1 $\alpha$ / $\beta$  (7, 12, 14, 16). In unstimulated cells, NF- $\kappa$ B is localized in the cytoplasm and complexed with the inhibitory protein I kappa B (I $\kappa$ B). Several stimuli including pro-inflammatory cytokines, reactive oxygen species, pathogen exposure, hormones, DNA damage, and physical stress activate protein kinases that phosphorylate I $\kappa$ B, causing its rapid degradation and translocation of NF- $\kappa$ B to the nucleus (16). In the nucleus, NF- $\kappa$ B binds to target genes, stimulating transcription of inflammatory mediators such as IL-1 $\beta$ , TNF- $\alpha$ , and IL-6. Quercetin acts by

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blocking the protein kinase-mediated  $\kappa$ B degradation, thereby preventing NF- $\kappa$ B 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- $\kappa$ B 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- $\kappa$ B, 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- $\kappa$ B 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 ( $\dot{V}O_{2\max}$ ) was determined using a graded maximal protocol (25-W increase every 2 min starting at 150 W) with the subjects using their own bicycles on CompuTrainer Pro model 8001 trainers (RacerMate, Seattle, WA). Oxygen uptake and ventilation were measured using the MedGraphics CPX metabolic system (MedGraphics, St. Paul, MN). Heart rate was measured using a chest heart rate monitor (Polar Electro, Woodbury, NY). Basic demographic and training data were obtained through a questionnaire.

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  $\sim 57\%$  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  $\sim 30$  min preexercise and  $\sim 15$  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  $\sim 57\%$  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  $-80^\circ\text{C}$  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- $\mu\text{l}$  plasma aliquots with 10  $\mu\text{l}$  of 10% DTT solution, 50  $\mu\text{l}$  of 0.58 M acetic acid, 50  $\mu\text{l}$  of a mixture of  $\beta$ -glucuronidase and arylsulfatase, and crude extract from *Helix pomatia* (Roche Diagnostics, Mannheim, Germany) for 40 min at  $37^\circ\text{C}$ . After incubation, 500  $\mu\text{l}$  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  $\text{H}_2\text{O}$  ( $\text{dH}_2\text{O}$ ) 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  $\text{MeOH} \times 2$ . 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, Farmingdale, NY) until MeOH was completely evaporated. The residue was reconstituted with 150  $\mu\text{l}$  of  $\text{MeOH}/\text{dH}_2\text{O}$  (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  $\mu\text{m}$   $4.6 \times 150$  mm column. The mobile phase, consisting of a mixture of acetonitrile with 0.1% HCOOH (0–15 min: 10–90%) and  $\text{dH}_2\text{O}$  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- $\alpha$  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- $\alpha$  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- $\alpha$  < 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 biopsy samples 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 anesthesia (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  $-80^{\circ}\text{C}$  until subsequent analysis.

**NF- $\kappa$ B 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  $4^{\circ}\text{C}$ . 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 bicinchoninic acid protein assay (Pierce, Rockford, IL). NF- $\kappa$ B was determined using a TransAM ELISA-based technique designed to determine the p65 subunit of NF- $\kappa$ B (Active Motif, Carlsbad, CA). Briefly, 30  $\mu$ l of binding buffer containing DTT and herring sperm DNA was added to each well, followed by 20  $\mu$ l of sample containing 2  $\mu$ g. 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  $\mu$ l of wash buffer. Primary NF- $\kappa$ B antibody (100  $\mu$ l) was added to each well; the plate was covered and incubated for 1 h at room temperature. The wash step was repeated, and 100  $\mu$ l of horseradish peroxidase antibody was added to each well followed by a 1-h incubation period. The plate was washed four times before 100  $\mu$ l of developing solution was added to each well. The plate was incubated in the dark for 5 min, followed by addition of 100  $\mu$ l 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  $\mu$ l of RNase-free water.

The extracted RNA (7.5  $\mu$ l of sample) was dissolved in diethylpyrocarbonate-treated water and quantified spectrophotometrically at 260-nm wavelength. RNA was reverse transcribed into cDNA in a 50- $\mu$ l reaction volume containing 19.25  $\mu$ l of RNA in RNase-free water, 5  $\mu$ l of  $10\times$  RT buffer, 11  $\mu$ l of 25 mM  $\text{MgCl}_2$ , 10  $\mu$ l of deoxy-NTPs mixture, 2.5  $\mu$ l of random hexamers, 1  $\mu$ l of RNase inhibitor, and 1.25  $\mu$ l of multiscribe reverse transcriptase (50 U/ $\mu$ l).

Reverse transcription was performed at  $25^{\circ}\text{C}$  for 10 min,  $37^{\circ}\text{C}$  for 60 min, and  $95^{\circ}\text{C}$  for 5 min, followed by quick chilling on ice and stored at  $-20^{\circ}\text{C}$  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 (AmpliAq Gold DNA Polymerase, Passive Reference 1, Buffer, dNTPs, AmpErase), 1  $\mu$ l of cDNA, 9  $\mu$ l of RNase-free water, and 1.25  $\mu$ l of 18S primer (VIC) and 1.25  $\mu$ l of primer (FAM) (for endogenous reference and target cytokine) in a final volume of 25  $\mu$ l/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  $50^{\circ}\text{C}$  and 10 min at  $95^{\circ}\text{C}$ , plates were coamplified by 50 repeated cycles, of which one cycle consisted of a 15-s denaturing step at  $95^{\circ}\text{C}$  and a 1-min annealing/extending step at  $60^{\circ}\text{C}$ . Data were analyzed by 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  $\Delta$ CTs, 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  $\mu$ l of sample) was dissolved in diethylpyrocarbonate-treated water and quantified spectrophotometrically at 260-nm wavelength. RNA was reverse transcribed into cDNA in a 50- $\mu$ l reaction volume containing 19.25  $\mu$ l of RNA (1.5  $\mu$ g) in RNase-free water, 5  $\mu$ l of  $10\times$  RT buffer, 11  $\mu$ l of 25 mM  $\text{MgCl}_2$ , 10  $\mu$ l of deoxyNTPs mixture, 2.5  $\mu$ l of random hexamers, 1  $\mu$ l of RNase inhibitor, and 1.25  $\mu$ l of multiscribe reverse transcriptase (50 U/ $\mu$ l). Reverse transcription was performed at  $25^{\circ}\text{C}$  for 10 min,  $37^{\circ}\text{C}$  for 60

Table 1. Subject characteristics measured at baseline and performance data averaged during 3-h cycling bouts over 3 days

Variable	Cyclists		P Value
	Quercetin (n = 20)	Placebo (n = 20)	
Baseline measures			
Age, yr	26.1 $\pm$ 1.8	29.1 $\pm$ 2.4	0.321
Weight, kg	74.7 $\pm$ 0.2	74.2 $\pm$ 1.4	0.846
$\dot{V}O_{2\text{max}}$ , ml $\cdot$ kg $^{-1}$ $\cdot$ min $^{-1}$	53.2 $\pm$ 1.2	54.7 $\pm$ 1.1	0.365
Power $_{\text{max}}$ , W	314 $\pm$ 9	320 $\pm$ 69	0.554
HR $_{\text{max}}$ , beats/min	188 $\pm$ 1	190 $\pm$ 2	0.328
Performance measures			
Mean power, W	178 $\pm$ 5	181 $\pm$ 4	0.626
Power, %Watts $_{\text{max}}$	56.8 $\pm$ 0.2	56.5 $\pm$ 0.3	0.486
Mean heart rate, beats/min	149 $\pm$ 2	149 $\pm$ 3	0.897
Heart rate, %HR $_{\text{max}}$	79.4 $\pm$ 0.8	78.1 $\pm$ 1.0	0.300
Mean $\dot{V}O_2$ , ml $\cdot$ kg $^{-1}$ $\cdot$ min $^{-1}$	36.0 $\pm$ 0.9	36.9 $\pm$ 0.8	0.458
$\dot{V}O_2$ , % $\dot{V}O_{2\text{max}}$	67.8 $\pm$ 0.8	67.6 $\pm$ 1.1	0.919
Cadence, RPM	80.8 $\pm$ 2.2	83.7 $\pm$ 2.2	0.349

Values are means  $\pm$  SE.  $\dot{V}O_2$ , volume of oxygen consumption;  $\dot{V}O_{2\text{max}}$ , maximal  $\dot{V}O_2$ ; Power $_{\text{max}}$ , maximal power; HR $_{\text{max}}$ , maximal heart rate; Watts $_{\text{max}}$ , maximal power rate.



Table 2. Pre- to postexercise (3-h cycling bouts at  $\sim 57\%$   $Watts_{max}$ ) changes in plasma cytokine levels in quercetin compared with placebo groups over a 3-day period

Variable	Quercetin ( $n = 20$ )		Placebo ( $n = 20$ )		P Values: Time; 2 $\times$ 6 Interaction
	Preexercise	Postexercise	Preexercise	Postexercise	
IL-6, pg/ml					
Day 1	1.1 $\pm$ 0.3	35.7 $\pm$ 4.5	0.6 $\pm$ 0.1	28.3 $\pm$ 6.0	<0.001; 0.279
Day 2	1.4 $\pm$ 0.4	23.1 $\pm$ 4.7	1.2 $\pm$ 0.4	14.1 $\pm$ 1.9	Day P < 0.001
Day 3	1.4 $\pm$ 0.4	15.7 $\pm$ 1.9	0.7 $\pm$ 0.1	11.0 $\pm$ 1.7	
IL-10 (pg/ml)					
Day 1	4.6 $\pm$ 0.7	80.9 $\pm$ 19.0	5.0 $\pm$ 1.0	95.0 $\pm$ 23.9	<0.001; 0.924
Day 2	5.0 $\pm$ 0.7	22.8 $\pm$ 4.0	6.0 $\pm$ 1.0	23.3 $\pm$ 4.2	Day P < 0.001
Day 3	4.5 $\pm$ 0.6	25.8 $\pm$ 5.4	5.2 $\pm$ 1.0	19.7 $\pm$ 5.3	
IL-8 (pg/ml)					
Day 1	4.2 $\pm$ 1.0	10.7 $\pm$ 1.4	5.4 $\pm$ 1.0	14.6 $\pm$ 1.5	<0.001; 0.094
Day 2	4.3 $\pm$ 0.9	8.9 $\pm$ 1.3	5.6 $\pm$ 1.1	10.6 $\pm$ 0.8	Day P < 0.001
Day 3	4.1 $\pm$ 1.0	8.6 $\pm$ 1.2	5.4 $\pm$ 0.4	8.2 $\pm$ 1.1	
IL-1ra (pg/ml)					
Day 1	135 $\pm$ 16	871 $\pm$ 373	135 $\pm$ 10	1016 $\pm$ 446	<0.001; 0.755
Day 2	174 $\pm$ 21	768 $\pm$ 366	180 $\pm$ 22	376 $\pm$ 52	Day P = 0.051
Day 3	152 $\pm$ 18	484 $\pm$ 130	150 $\pm$ 13	305 $\pm$ 38	
TNF- $\alpha$ (pg/ml)					
Day 1	1.09 $\pm$ 0.10	1.26 $\pm$ 0.10	1.08 $\pm$ 0.09	1.31 $\pm$ 0.07	<0.001; 0.094
Day 2	0.99 $\pm$ 0.07	1.21 $\pm$ 0.07	1.11 $\pm$ 0.08	1.39 $\pm$ 0.10	Day P < 0.001
Day 3	0.97 $\pm$ 0.06	1.06 $\pm$ 0.08	1.15 $\pm$ 0.08	1.20 $\pm$ 0.09	
MCP-1 (pg/ml)					
Day 1	123 $\pm$ 7	310 $\pm$ 27	127 $\pm$ 9	291 $\pm$ 23	<0.001; 0.680
Day 2	131 $\pm$ 11	247 $\pm$ 16	129 $\pm$ 15	231 $\pm$ 16	Day P < 0.001
Day 3	122 $\pm$ 6	237 $\pm$ 14	132 $\pm$ 11	231 $\pm$ 16	

Values are means  $\pm$  SE.

min, and 95°C for 5 min, followed by quick chilling on ice and stored at  $-20^{\circ}\text{C}$  until subsequent amplification.

**Calculations for relative quantification.** Quantification of mRNA expression for leukocyte IL-8, IL-10, and IL-1ra, and muscle COX-2, IL-6, IL-8, TNF- $\alpha$  and IL-1 $\beta$  was calculated using the  $\Delta\Delta\text{CT}$  method as described by Livak and Schmittgen (17). This method uses a single sample, the calibrator sample, for comparison of every unknown sample's gene expression. This method of analysis and quantification has been shown to give similar results as the standard curve method (33). Briefly,  $\Delta\text{CT}$  [CT(FAM) - CT(VIC)] was calculated for each sample and calibrator.  $\Delta\Delta\text{CT}$  [ $\Delta\text{CT}(\text{calibrator}) - \Delta\text{CT}(\text{sample})$ ] was then calculated for each sample, and relative quantification was calculated as  $2^{\Delta\Delta\text{CT}}$ . Initial exclusion criteria consisted of FAM CT  $\geq 40$  and VIC CT  $\geq 23$ .

**Statistical analysis.** Data are expressed as means  $\pm$  SE. Data in Table 1 were compared between groups using Student's *t*-tests. Data in Table 2 and Figs. 1 through 10 were analyzed using a 2 (groups)  $\times$  4 (muscle) or 6 (plasma) (time points) repeated-measures ANOVA. For certain variables of interest, an overall "day" effect was calculated that compared changes across the 3 days of measurement. When

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 ( $P \leq 0.05$ ), pre- to postexercise changes were calculated and compared between quercetin and placebo groups using Student's *t*-tests, with significance set at  $P \leq 0.05$ .

## 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  $242 \pm 27$  and  $270 \pm 29$  km/wk, and  $1.5 \pm 0.2$  and  $1.6 \pm 0.2$  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.

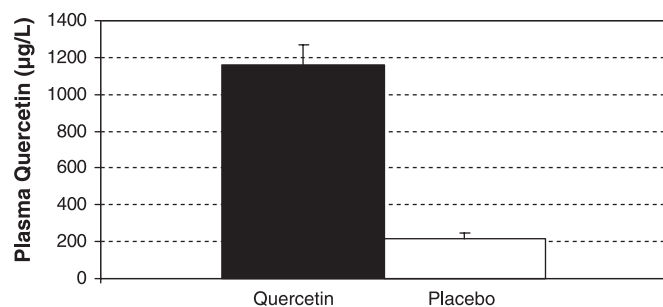


Fig. 1. Plasma quercetin levels after 3-wk supplementation and before the 3-day intensified exercise period in quercetin ( $n = 20$ ) and placebo ( $n = 20$ ) groups ( $P = 0.002$ ).

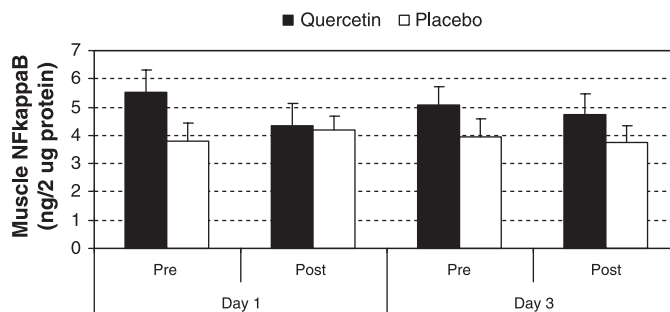


Fig. 2. Muscle NF- $\kappa$ B content pre- and post-3 h of cycling at 57% maximal work rate ( $Watts_{max}$ ) in quercetin ( $n = 20$ ) and placebo ( $n = 20$ ) groups on days 1 and 3. Main time effect,  $P = 0.872$ ; group  $\times$  time interaction effect,  $P = 0.662$ .

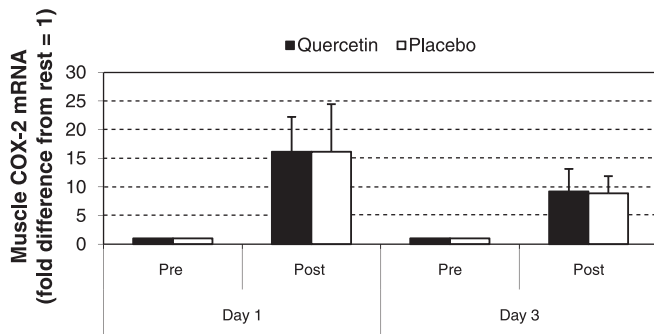


Fig. 3. Muscle COX-2 mRNA pre- and post-3 h of cycling at 57%  $\text{Watts}_{\text{max}}$  in quercetin ( $n = 20$ ) and placebo ( $n = 20$ ) groups on *days 1* and *3*. Main time effect,  $P < 0.05$ ; group  $\times$  time interaction effect,  $P = 0.944$ .

Subjects in quercetin and placebo groups were able to maintain a mean power output of  $\sim 57\%$   $\text{Watts}_{\text{max}}$  at an oxygen consumption of  $\sim 68\%$   $\dot{V}O_{2\text{max}}$  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 ( $P < 0.001$ ) (Fig. 1).

Three-day food records before the 3-day exercise period revealed no significant group differences in energy or macronutrient intake (data not shown), and for all subjects combined energy intake was  $11.2 \pm 0.5$  MJ/day ( $2,684 \pm 117$  kcal/day), with carbohydrate representing  $56.6 \pm 2.5\%$ , protein  $16.2 \pm 0.8\%$ , and fat  $27.2 \pm 1.8\%$ . A poststudy questionnaire revealed that nine subjects in each group guessed they were on quercetin, four in each group guessed placebo, and seven in each group were uncertain (thus no significant group differences).

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

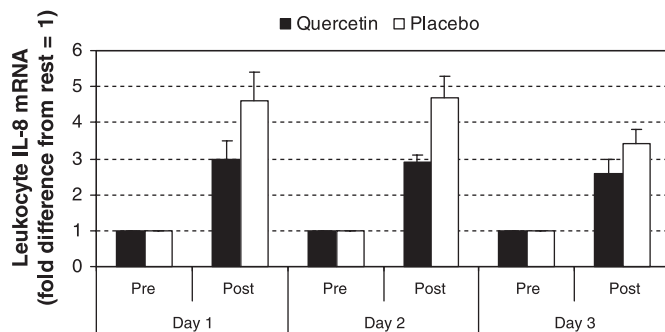


Fig. 4. Blood leukocyte IL-8 mRNA pre- and post-3 h of cycling at 57%  $\text{Watts}_{\text{max}}$  in quercetin ( $n = 20$ ) and placebo ( $n = 20$ ) groups during 3 consecutive days. Main time effect,  $P < 0.001$ ; group  $\times$  time interaction effect,  $P = 0.019$ .  $P$  values for group comparisons of pre- to postexercise changes were 0.089, 0.010, and 0.148 for *days 1–3*, respectively.

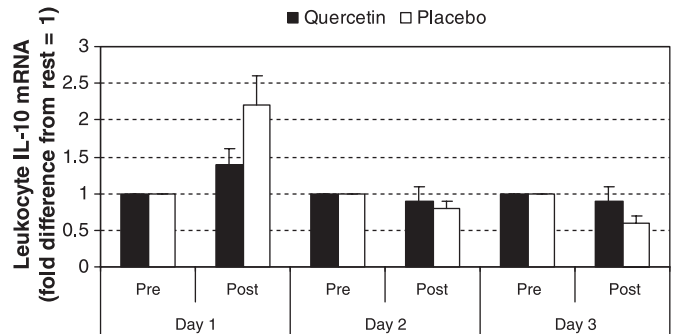


Fig. 5. Blood leukocyte IL-10 mRNA pre- and post-3 h of cycling at 57%  $\text{Watts}_{\text{max}}$  in quercetin ( $n = 20$ ) and placebo ( $n = 20$ ) groups during 3 consecutive days. Main time effect,  $P < 0.001$ ; group  $\times$  time interaction effect,  $P = 0.012$ .  $P$  values for group comparisons of pre- to postexercise changes were 0.074, 0.855, and 0.298 for *days 1–3*, respectively.

of the pre- to postexercise increases in plasma cytokine levels decreased over the 3-day period for all six cytokines (day  $\times$  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  $2.8 \pm 0.2$  and  $4.2 \pm 0.2$  (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  $\times$  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)

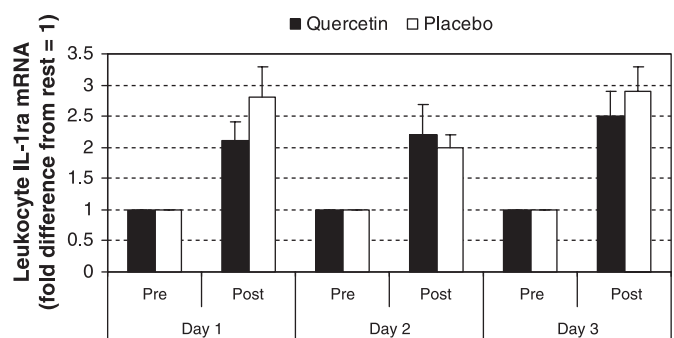


Fig. 6. Blood leukocyte IL-1ra mRNA pre- and post-3 h of cycling at 57%  $\text{Watts}_{\text{max}}$  in quercetin ( $n = 20$ ) and placebo ( $n = 20$ ) groups during 3 consecutive days. Main time effect,  $P < 0.001$ ; group  $\times$  time interaction effect,  $P = 0.558$ .

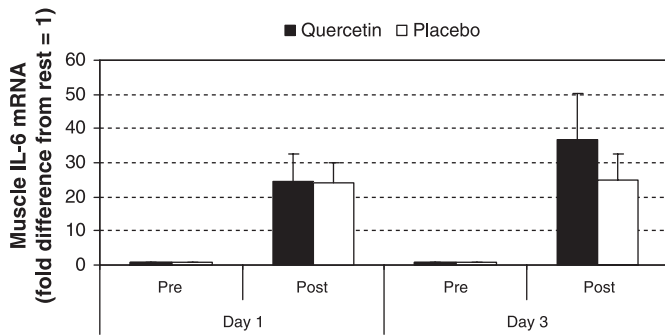


Fig. 7. Muscle IL-6 mRNA content pre- and post-3 h of cycling at 57%  $Watt_{max}$  in quercetin ( $n = 20$ ) and placebo ( $n = 20$ ) groups on *days 1* and *3*. Main time effect,  $P < 0.001$ ; group  $\times$  time interaction effect,  $P = 0.595$ .

(Figs. 7–10). The magnitude of increase in muscle cytokine mRNA expression decreased across the 3-day period (day  $\times$  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- $\kappa$ B content or exercise-induced increases in muscle COX-2 mRNA or mRNA expression for IL-6, IL-8, IL-1 $\beta$ , and TNF- $\alpha$ . 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- $\alpha$ .

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

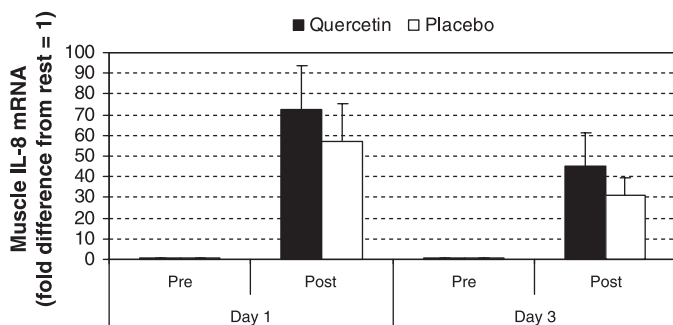


Fig. 8. Muscle IL-8 mRNA content pre- and post-3 h of cycling at 57%  $Watt_{max}$  in quercetin ( $n = 20$ ) and placebo ( $n = 20$ ) groups on *days 1* and *3*. Main time effect,  $P < 0.001$ ; group  $\times$  time interaction effect,  $P = 0.913$ .

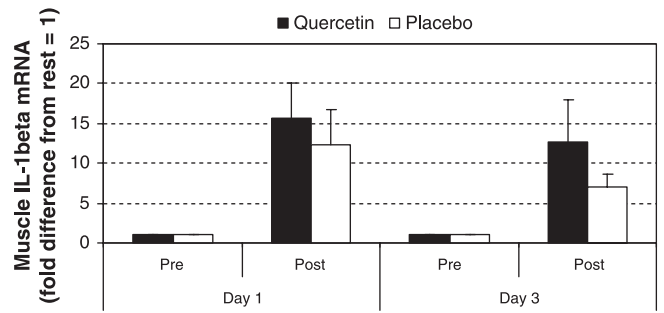


Fig. 9. Muscle IL-1 $\beta$  mRNA content pre- and post-3 h of cycling at 57%  $Watt_{max}$  in quercetin ( $n = 20$ ) and placebo ( $n = 20$ ) groups on *days 1* and *3*. Main time effect,  $P < 0.001$ ; group  $\times$  time interaction effect,  $P = 0.910$ .

including TNF- $\alpha$ , 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- $\alpha$  production and gene expression via modulation of NF- $\kappa$ B and I $\kappa$ B in human peripheral blood mononuclear cells (22). Other in vitro data with mast cells demonstrate that quercetin strongly inhibits secretion of TNF- $\alpha$  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- $\kappa$ B content, and exercise-induced increases in muscle COX-2 mRNA were similar in quercetin and placebo groups. Prolonged and intensive exercise in-

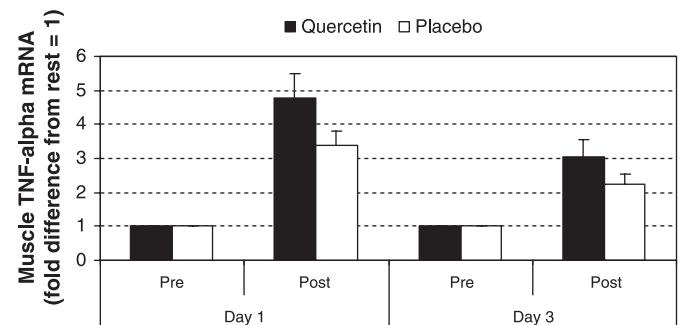


Fig. 10. Muscle TNF- $\alpha$  mRNA content pre- and post-3 h of cycling at 57%  $Watt_{max}$  in quercetin ( $n = 20$ ) and placebo ( $n = 20$ ) groups on *days 1* and *3*. Main time effect,  $P < 0.001$ ; group  $\times$  time interaction effect,  $P = 0.930$ .

creased both muscle and leukocyte mRNA cytokine expression and plasma cytokine levels, suggesting that these effects occurred without muscle NF- $\kappa$ B regulation. Steensberg et al. (31) also reported exercise-induced increases in muscle IL-6 and IL-8 mRNA without muscle NF- $\kappa$ B 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- $\kappa$ B 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- $\kappa$ B activation (11, 18). However, our data do not suggest a linkage between oral quercetin ingestion, muscle NF- $\kappa$ B content, and muscle COX-2 mRNA expression during intensified exercise training.

The physiology of NF- $\kappa$ B in cells outside the immune system is not well understood. Chronic NF- $\kappa$ B activation in muscle leads to muscle wasting as seen in cancer, AIDS, and bed rest (7, 16). Rodent studies indicate that the NF- $\kappa$ B 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- $\kappa$ B cascade (1, 12, 14, 16). A high alpha-tocopherol diet in rats blunts exercise-induced increases in muscle NF- $\kappa$ B 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- $\kappa$ B 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- $\kappa$ B 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- $\kappa$ B activity from thigh muscle biopsy samples (31). Plasma F<sub>2</sub>-isoprostanes increased significantly in our subjects (data being presented elsewhere) after each exercise bout, indicating significant oxidative stress without muscle NF- $\kappa$ B activation. Thus currently available data do not support muscle NF- $\kappa$ B 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- $\kappa$ B from homogenized muscle tissue and measured the P65 subunit of NF- $\kappa$ B using an antibody ELISA method that is specific for the activated form of p65. Other investigators have measured DNA binding activity of NF- $\kappa$ B from muscle cell nuclear extracts (7, 14, 16) or muscle content of I $\kappa$ B $\alpha$  using Western blot (31). Thus some caution is urged in comparing results across studies. We did not measure NF- $\kappa$ B 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- $\kappa$ B 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- $\kappa$ B 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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