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# Short-term blackcurrant extract consumption modulates exercise-induced oxidative stress and lipopolysaccharide-stimulated inflammatory responses

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**Lyall KA, Hurst SM, Cooney J, Jensen D, Lo K, Hurst RD, Stevenson LM.** Short-term blackcurrant extract consumption modulates exercise-induced oxidative stress and lipopolysaccharide-stimulated inflammatory responses. *Am J Physiol Regul Integr Comp Physiol* 297: R70–R81, 2009. First published April 29, 2009; doi:10.1152/ajpregu.90740.2008.—Exercise-induced oxidative stress is instrumental in achieving the health benefits from regular exercise. Therefore, inappropriate use of fruit-derived products (commonly applied as prophylactic antioxidants) may counteract the positive effects of exercise. Using human exercise and cellular models we found that 1) blackcurrant supplementation suppressed exercise-induced oxidative stress, e.g., plasma carbonyls ( $0.9 \pm 0.1$  vs.  $0.6 \pm 0.1$  nmol/mg protein, placebo vs. blackcurrant), and 2) preincubation of THP-1 cells with an anthocyanin-rich blackcurrant extract inhibited LPS-stimulated cytokine secretion [TNF- $\alpha$  ( $16,453 \pm 322$  vs.  $10,941 \pm 82$  pg/ml, control vs. extract,  $P < 0.05$ ) and IL-6 ( $476 \pm 14$  vs.  $326 \pm 32$  pg/ml, control vs. extract,  $P < 0.05$ )] and NF- $\kappa$ B activation. In addition to its antioxidant and anti-inflammatory properties, we found that postexercise plasma collected after blackcurrant supplementation enhanced the differential temporal LPS-stimulated inflammatory response in THP-1 cells, resulting in an early suppression of TNF- $\alpha$  ( $1,741 \pm 32$  vs.  $1,312 \pm 42$  pg/ml, placebo vs. blackcurrant,  $P < 0.05$ ) and IL-6 ( $44 \pm 5$  vs.  $36 \pm 3$  pg/ml, placebo vs. blackcurrant,  $P < 0.05$ ) secretion after 24 h. Furthermore, by using an oxidative stress cell model, we found that preincubation of THP-1 cells with hydrogen peroxide ( $H_2O_2$ ) prior to extract exposure caused a greater suppression of LPS-stimulated cytokine secretion after 24 h, which was not evident when cells were simultaneously incubated with  $H_2O_2$  and the extract. In summary, our findings support the concept that consumption of blackcurrant anthocyanins alleviate oxidative stress, and may, if given at the appropriate amount and time, complement the ability of exercise to enhance immune responsiveness to potential pathogens.

acute inflammation; fruit anthocyanins; moderate exercise

PARTICIPATING IN REGULAR PHYSICAL exercise has a number of health benefits (14). Exercise-induced oxidative stress (11, 48, 50), either via reactive oxygen species (ROS)-dependent (2, 14) or -independent (23) mechanisms, appears to be an important modulator in a number of adaptive responses to exercise: upregulation of endogenous antioxidant systems (42, 18), modulation of muscle function (17), improved efficacy of influenza immunization (15), and enhanced immune surveillance (14). Characterization of how exercise modulates the immune system in untrained individuals as well as athletes shows how the type, intensity, and duration of exercise affects the immune responses to pathogenic agents, such as bacterial endotoxin LPS (9, 12, 16). In addition to regular exercise, the ingestion of

antioxidant supplements or foods high in antioxidants and vitamins, such as C and E, have become commonplace in efforts to maintain health and prevent chronic oxidative stress-associated ailments (5). While antioxidant supplementation has been shown to alleviate exercise-induced oxidative stress and benefit athletes undergoing long-term strenuous training by reducing oxidative stress-related injuries and illnesses (48, 51), exercise studies using untrained healthy individuals show that antioxidant supplementation may counteract the healthy benefits of regular exercise (8). It is therefore possible that the removal of exercise-induced oxidative stress by antioxidant supplementation (and possible anti-inflammatory agents) may also remove any putative enhancement of the innate and adaptive immune system.

Exploring the health properties of fruit and vegetable products during exercise has become the focus of recent research into functional foods. Emerging evidence indicates that fruits contain important flavonoids that underlie both antioxidant (6, 29, 35) and immune modulatory (21, 26, 53) mechanisms that could alleviate oxidative stress as well as enhance innate and adaptive immunity. However, it is still debatable whether fruit-derived supplements containing these antioxidant and/or anti-inflammatory properties complement or counteract the adaptive health benefits achieved by regular exercise. Blackcurrants (BCs) are labeled a “superfruit” with a number of health benefits, especially the alleviation of chronic oxidative stress-related conditions (28). Although these health benefits are attributed a high antioxidant status, recent feeding and cellular studies indicate that BC flavonoids and anthocyanins exhibit a range of health benefits, including antioxidant (35, 36) and anti-inflammatory (21, 26) properties, although mechanisms underlying these processes are not well understood. We selected an “off the shelf” BC extract that primarily consisted of anthocyanins (and a low amount of vitamin C) to explore the hypothesis that fruit-derived supplements, which exhibit both antioxidant and anti-inflammatory properties, complement the putative health benefits of regular exercise. Using exercise and cellular models, we found that ingestion of an anthocyanin-rich BC extract exhibited antioxidant and anti-inflammatory properties and enhanced the immune responsiveness to the bacterial endotoxin LPS achieved by regular exercise. Our findings, therefore, support the concept that fruit-derived supplements/foods may be of value for individuals undergoing regular exercise for training purposes or for those wishing to maintain general health and fitness.

## METHODS

### BC Extract Analysis

For this study we used a commercial BC powdered extract: Bioactive Blackcurrant (Lifestream International, Auckland, NZ) that was produced from New Zealand BCs and extracted by Vitec New

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Zealand (Nelson, NZ). We also compared the composition of this commercial extract with an anthocyanin-rich BC (BC-A; cultivar Ben Ard) extract kindly donated by Dr. T. McGhie, The New Zealand Institute for Plant and Food Research. Mass spectrometry (MS) was employed to clarify the flavonoids composition of the extracts. Separation of BC flavonoid compounds followed standard methodology previously described (1, 30). Briefly, anthocyanins were identified by liquid chromatography-mass spectrometry using an linear ion trap fitted with an electrospray ionization interface (ThermoQuest; Finnigan, San Jose, CA) coupled to an Ettan multidimensional liquid chromatography (GE Healthcare BioSciences) and Surveyor photodiode array (PDA) detector. Separation of anthocyanin compounds was carried out using a Synergi Hydro-RP 4  $\mu\text{m}$  80Å, column (Phenomenex, Torrance, CA) and detected at an absorbance of 520 nm. Anthocyanin structure was determined using controlled glycosylation to provide molecular weight information about the aglycone core and glycosyl subunits. Separation and identification of other BC flavonoid compounds was performed by liquid chromatography-mass spectrometry using a LCQ Deca 3D ion trap mass spectrophotometer fitted with an Ettan multidimensional interface (ThermoQuest) and coupled to a Surveyor high pressure liquid chromatography and photodiode array detector. Compound separation was achieved using a Gemini 3- $\mu\text{m}$  C18 100Å, (Phenomenex) and detection at an absorbance between 200 and 600 nm. Identification of BC flavonoid compounds was confirmed by comparing the fragmentation pattern with standard flavonoid compounds.

#### Exercise Study Protocols

**Subject selection.** This exercise study was approved by The Northern Ethical Regional Committee (Region Y), Hamilton, NZ (No. NTY/07/10/107), and the trial was registered with the Australian and New Zealand Clinical Trials Registry (No. ACTRN126080003263392). Individuals were recruited from within The New Zealand Institute for Plant and Food Research. Suitability for participation in the trial was based upon a health questionnaire that was devised and assessed by an occupational health organization (Waikato Occupational Health Consultancy, Hamilton, NZ). Subjects were excluded from the study if they had known fruit allergies, a previous physical injury, a respiratory disorder, a chronic disease, high blood pressure, or clotting disorders or were pregnant or planning to become pregnant. Ten healthy individuals comprising five males and five females aged between 37 and 63 yr old (average  $48 \pm 2.5$  yr) who, although displaying different fitness levels and routinely undergoing some type of daily physical moderate exercise, were selected to participate in this study. All subjects were provided written informed consent. The participants also completed a diet (3 days), health, and exercise diary during the period of the study. They were also asked to refrain from all antioxidant supplementation and berryfruit products, but otherwise, to maintain normal dietary patterns.

**Nutritional supplementation.** The commercial BC extract was encapsulated in gelatin. Trial participants were given a total of four capsules (two before and two after exercise) that contained concentrated freeze-dried whole BC fruit extract approximately equivalent to 48 g of BCs. The total anthocyanin and vitamin C content given to each individual (4 capsules) were 240 mg and  $< 1$  mg, respectively, calculated from the manufacturer's extract information and confirmed by our chemical analysis. A sugar placebo (also encapsulated in gelatin) similar to that used in other feeding/exercise trials (3, 44) was selected to exclude any possible acute increases in plasma antioxidant capability being due to sugar metabolism (29).

**Exercise protocol.** In this study we selected a 30-min row, using a Concept 2 rower, with an individual's rowing intensity maintained at 80%  $\dot{V}O_{2\text{max}}$ , providing a cardiac exercise utilizing both arm and leg muscles. The study participants displayed different physical characteristics (Table 1) and did not routinely row. To take this into account, two familiarization sessions were carried out a week before the start of the study.

Table 1. *Subject characteristics*

| Variable                                | Subject ( $n = 10$ ) | Range       |
|-----------------------------------------|----------------------|-------------|
| Age, yr                                 | $48 \pm 2.5$         | 37–63       |
| Weight, kg                              | $75.2 \pm 2.4$       | 63–86       |
| Resting heart rate                      | $58 \pm 2.0$         | 46–66       |
| Body mass index, $\text{kg}/\text{m}^2$ | $25 \pm 0.5$         | 22–29       |
| Body fat, %                             | $19 \pm 1.9$         | 10–30       |
| Body fat mass, kg                       | $14.7 \pm 1.6$       | 8–24        |
| Lean body mass, kg                      | $60.4 \pm 2.7$       | 47–71       |
| Soft lean mass, kg                      | $56.6 \pm 2.6$       | 44–67       |
| Basal metabolic rate, kcal              | $1,532 \pm 47$       | 1,258–1,735 |
| Total body water, kg                    | $47.5 \pm 4.2$       | 35–79       |
| Fitness score (score out of 100)        | $82.2 \pm 1.8$       | 77–87       |

Results are means  $\pm$  SE;  $n$ , number of subjects. Analysis of body characteristics was performed using a bioimpedance machine (Body Composition Analyzer Inbody version 3.0; Biospace, New Zealand). The fitness score is the overall fit score.

**Trial format.** The trial was conducted over a 3-wk period with a week washout and as a double-blind crossover study where neither researchers nor study participants knew what supplement was being taken. This format was chosen so each subject could act, as a self control to eliminate any biological variability. Participants conducted exercise sessions at the same time of day (morning) and a brief warm-up session (stretches). In addition, all participants were asked to rest and fast for at least 2 h prior to initiation of the exercise trial. Immediately before starting the 30-min row, subjects were given two opaque gelatin capsules (containing either the BC extract or the placebo) with water. During the exercise, a heart monitor (model AXN700 heart monitor, Polar Electro, Auckland, NZ) and a coach were employed to ensure individuals maintained personalized 80%  $\dot{V}O_{2\text{max}}$ . At the end of the exercise, subjects were given another two capsules. After a week out, the subjects repeated the trial.

**Blood sampling.** Venous blood samples were collected into sodium heparin prior to taking the initial two supplement capsules and exercise, then immediately following the 30-min row but before the subject took the next two capsules. Further blood sampling was taken at 1-, 2-, and 24-h postexercise. Collected blood was either immediately used in the peripheral blood assay or centrifuged (300 g, 10 min, room temperature), and the plasma either used immediately to assess oxidative stress indices or frozen and stored at  $-80^\circ\text{C}$  in 100  $\mu\text{l}$  aliquots.

#### Plasma Oxidative Stress Parameters

**Plasma protein carbonyl levels.** Plasma protein carbonyls were measured using a protocol described by Morabito et al., (37). Briefly, plasma (100  $\mu\text{l}$ ) was added to an equal volume of 2,4-dinitrophenylhydrazine (DNPH; Sigma-Aldrich, Auckland, NZ) in 2 M HCl (control = plasma/HCl in the absence of DNPH) and incubated in the dark for 1 h. Protein was precipitated with 50% trichloroacetate (Sigma-Aldrich) and washed with equal amounts of ethanol/ethylacetate, resuspended in 1 ml of 6 M guanidine (Merck NZ, Palmerston North, NZ), and the absorbance of the final suspension measured at 360 nm in a UV-visible 1601 spectrophotometer (Shimadzu, Kyoto, Japan). Carbonyl levels were calculated using the absorbance difference between test and control using the molar absorption coefficient ( $\epsilon$ ): 22,000 M/cm. Plasma protein levels were measured using the Bradford method (4) and commercial Bradford reagent (Bio-Rad Laboratories, Auckland, NZ). Results are expressed as nanomoles of protein carbonyls per milligram total protein.

**Plasma ROS generating capability.** Since exercise induces a variety of ROS (11), we used a modified carboxy-dihydro-2',7'-dichloro-hydrofluorescein diacetate (carboxy- $\text{H}_2\text{DCFDA}$ ) kinetic assay described by Wang and Joseph (52) to assess ROS-generating capability. Preliminary experiments found that the addition of 0.25  $\mu\text{M}$  hydrogen

peroxide ( $H_2O_2$ ) detected subtle increases in exercise-induced ROS-generating capability in plasma. Although this approach does not cover all inducible oxidative generating activity that may alter cellular function (11), it does provide an indicator of the plasma oxidative generating capability induced by exercise. The assay consisted of hydrolyzing 10  $\mu$ M carboxy- $H_2$ DCFDA (Molecular Probes, Invitrogen NZ, Auckland, NZ) into the product dichlorohydrofluorescein (DCF), which when oxidized is fluorescent. Hydrolysis of carboxy- $H_2$ DCFDA was performed by incubation with an equal volume of methanol and 0.5 M potassium hydroxide for 1 h at room temperature. The product, DCF (5  $\mu$ l) was added to diluted plasma (1:10 in PBS, pH 7.4) or PBS control, and then 0.25  $\mu$ M  $H_2O_2$  was added and the changes in fluorescence intensity (FI) measured over 5 min at 22°C using a fluorescence plate reader (BMG FluorStar Optima; Alphatech Systems, Auckland, NZ) with excitation and emission wavelengths of 485 and 520 nm, respectively. Data were calculated as the difference in FI ( $\Delta FI_{5min} - \Delta FI_{0min}$ ) minus the corresponding control ( $H_2O_2$ /PBS only) and presented as the change in relative FI after 5 min ( $\Delta RFI_{5min}$ ).

**Plasma creatine kinase activity.** Plasma (100  $\mu$ l) was assessed for plasma creatine kinase (CK) activity using automated Hitachi protocols optimized by a clinical diagnostic laboratory (LabPlus, Auckland, NZ). Results are expressed as CK activity in units per liter of plasma.

#### LPS-Stimulated Cytokine Generation in Peripheral Blood and THP-1 cells

**Peripheral blood experiments.** Peripheral blood collected (as described above) prior to and immediately after the 30-min row was diluted (1:1) with RPMI medium (containing 10 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 25  $\mu$ g/ml amphotericin B) and stimulated with LPS [purified from *Escherichia coli* 0111.B4 (InvivoGen, San Diego, CA) and shown primarily to target the TLR4 receptor (10)]; 500 ng/ml (for TNF- $\alpha$  production) or 1 ng/ml (for IL-6 production) in a 37°C, 95% humidified atmosphere, 5%  $CO_2$  incubator for 24 h. The blood was then centrifuged at 300 g for 10 min, and the supernatant collected and frozen at -80°C until measurement of TNF- $\alpha$  or IL-6 by specific ELISAs (Duoset ELISA kits; R&D Systems).

**THP-1 cell experiments.** Monocytic THP-1 cells (cat. no. TIB202; American Type Culture Collection, Manassas, VA, c/o Cryosite, Lane Cove NSW, Australia) were grown in RPMI medium containing 10 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 25  $\mu$ g/ml amphotericin B, and 10% FBS, unless otherwise stated. All cell culture materials were purchased from Invitrogen, and cells were incubated in standard tissue culture conditions i.e., 37°C in a 95% humidified atmosphere at 5%  $CO_2$ .

#### Determination of LPS-Stimulated Cytokine Generation by THP-1 Cells

**Effect of plasma.** THP-1 cells ( $3 \times 10^6$  cells/ml) were suspended in RPMI media (no FBS) containing plasma (final concentration 25%) collected prior to and immediately postexercise. The cells were then incubated for 30 min, washed, resuspended in RPMI media ( $3 \times 10^6$  cells/ml) and then stimulated with LPS (50 ng/ml) for 3, 6, or 24 h. At the end of each time point, the supernatant was separated from cells by centrifuging at 300 g for 5 min at room temperature and frozen (-80°C) for subsequent TNF- $\alpha$  and IL-6 measurements.

**Direct effect of BC-A extract.** THP-1 cells ( $3 \times 10^6$  cells/ml) in RPMI media were preincubated with either culture medium, 5 or 50 ng/ml BC-A extract (diluted in PBS) for 30 min followed by LPS (500 ng/ml) stimulation for 1, 3, 6, 12, or 24 h. At the end of each time point, the supernatant was separated from the cells, as described above and frozen (-80°C) for subsequent cytokine determination.

**Effect of BC-A extract on  $H_2O_2$ -induced oxidative stressed cells.** Preliminary kinetic experiments using THP-1 cells ( $3 \times 10^6$  cells/ml) loaded with carboxy- $H_2$ DCFDA revealed that 10  $\mu$ M  $H_2O_2$  caused an in-

crease in ROS (measured by a change in FI) over a 30-min period with minimal cell cytotoxicity (data not shown). In addition, we found that simultaneous addition of 5 ng/ml BC-A extract with 10  $\mu$ M  $H_2O_2$  inhibited this increase in ROS generation. Using this cell model, we explored the interactions between  $H_2O_2$ -induced ROS and BC-A extract on LPS-stimulated cytokine generation THP-1 cells. THP-1 cells were suspended in RPMI (no FBS) at a concentration of  $3 \times 10^6$  cells/ml and preincubated with culture media alone,  $H_2O_2$  (10  $\mu$ M) alone, BC-A extract (5 ng/ml) alone, or  $H_2O_2$  (10  $\mu$ M) plus BC-A extract (5 ng/ml) for 30 min and then washed twice (centrifuging at 300 g, 5 min at room temperature) with RPMI and resuspended at  $3 \times 10^6$  cells/ml. The cells were then either 1) stimulated with LPS (100 ng/ml) for 24 h, after which the cell suspension was centrifuged and the supernatants collected for subsequent cytokine determination or 2) incubated for a further 30 min with either culture media alone or the BC-A extract (5 ng/ml), washed, and then stimulated with LPS (100 ng/ml) for 24 h. Cell supernatants were also collected as described above for cytokine.

#### Assessment of LPS-Induced NF- $\kappa$ B Signaling Events in THP-1 Cells

Using a similar approach as others (22, 54), we suspended the THP-1 cells ( $3 \times 10^6$  cells/ml) in RPMI (no FBS) and incubated them in the presence or absence of BC-A extract (1–100 ng/ml) for 30 min. The cells were then washed once and then stimulated with LPS (500 ng/ml) for 60 min [for detection of phosphorylated NF- $\kappa$ B<sub>p65</sub> (pNF- $\kappa$ B<sub>p65</sub>) nuclear translocation] or 40 min (for measurement of cellular I $\kappa$ B $\alpha$  degradation). Nuclear extracts were prepared as previously described by Schreiber et al. (43). Whole cell extracts were prepared by resuspending cells in ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, proteinase inhibitor cocktail, 1 mM  $Na_3VO_4$ , 1 mM NaF), incubating on ice for 15 min, centrifuging (12,000 g, 5 min, 4°C), and then the collected supernatant was frozen at -80°C. Nuclear extracts (30  $\mu$ g) and whole cell extracts (60  $\mu$ g) were separated on 8% or 10% SDS-polyacrylamide gel, respectively, transferred to imobilon membrane (Bio-Rad Laboratories) by semidry blotting, and blocked overnight at 4°C in Tris-buffered saline, pH 7.4 (TBS) containing 5% skimmed powdered milk. Membranes were incubated in TBS containing either 1  $\mu$ g/ml anti-human pNF- $\kappa$ B<sub>p65</sub> rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA), 1  $\mu$ g/ml anti-human I $\kappa$ B $\alpha$  monoclonal antibody (Santa Cruz Biotechnology) or 1:2,000 dilution of actin monoclonal antibody (clone AC-40; Sigma-Aldrich, NZ) for 2 h at room temperature, washed 3 times (10 min) with TBS, containing 0.5% Tween-20. Membranes were then incubated in TBS/Tween-20 containing the appropriate secondary antibody (1:1,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG or 1:3,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG; Bio-Rad Laboratories) for 45 min and then washed 5 times with TBS/Tween-20 buffer (10 min). The membrane-bound antibody was then detected by chemiluminescence (ECL kit; Pierce, Global Science, Auckland, NZ) on Kodak BioMax XAR film (Radiographic Supplies, Christchurch, NZ). Semiquantitative analysis of immune reactivity was measured using ImageJ version 1.41o software (National Institutes of Health). Results are expressed as 1) nuclear translocation of phosphorylated NF- $\kappa$ B<sub>p65</sub> as a ratio of the optical density value for pNF- $\kappa$ B<sub>p65</sub> cells detected in cells stimulated with LPS alone and 2) degradation of I $\kappa$ B $\alpha$  as a ratio of the optical value for cellular I $\kappa$ B $\alpha$  and respective actin levels measured in the same whole cell extract.

#### Statistical analysis

Results are expressed as means  $\pm$  SE for at least 10 (exercise-induced oxidative stress), eight (experiments using peripheral blood or plasma and THP-1 cells), and four (experiments exploring BC extract cellular processes) observations. Statistical significance for the com-

parison of two groups was assessed by using a paired Student's *t*-test. Multiple comparisons were assessed by a two-way ANOVA. Where appropriate, the original data were transformed to achieve normality and constant variance in the residuals. Statistical significance for all indices was set at  $P < 0.05$  with a confidence level of 95%.

## RESULTS

### Qualitative Analysis of the BC Extracts

Qualitative analysis of the BC extracts by liquid chromatography, electrospray ionization, and mass spectrometry were combined with Surveyor photodiode array detector and were consistent with the BC flavonoids reported by others (1, 30). Specifically, anthocyanins were the major class of phenolic compounds present within the commercial BC extract, accounting for 89% of the total phenolics present (Fig. 1A), and displayed a similar profile to the BC-A extract (Fig. 1B). The anthocyanin profile for both extracts showed four main anthocyanins; 1) delphinidin-3-*O*-glucoside, 2) delphinidin-3-*O*-rutinoside, 3) cyanidin-3-*O*-glucoside, and 4) cyanidin-3-*O*-rutinoside. Furthermore, the sensitivity of this type of analysis revealed the presence of three minor anthocyanins: 1) petunidin-3-*O*-rutinoside, 2) pelargonidin-3-*O*-rutinoside, and 3) peonidin-3-*O*-rutinoside. In addition, further MS analysis of the commercial BC extract (remaining 11%) identified (+) catechin, (-) epicatechin, and glycoside derivatives of myricetin, quercetin, and kaempferol.

### Subject's Analysis

Physical and subjective analysis of individuals (Table 1) revealed that all participants undertook moderate exercise at least 3 times a week and showed an average fitness score of

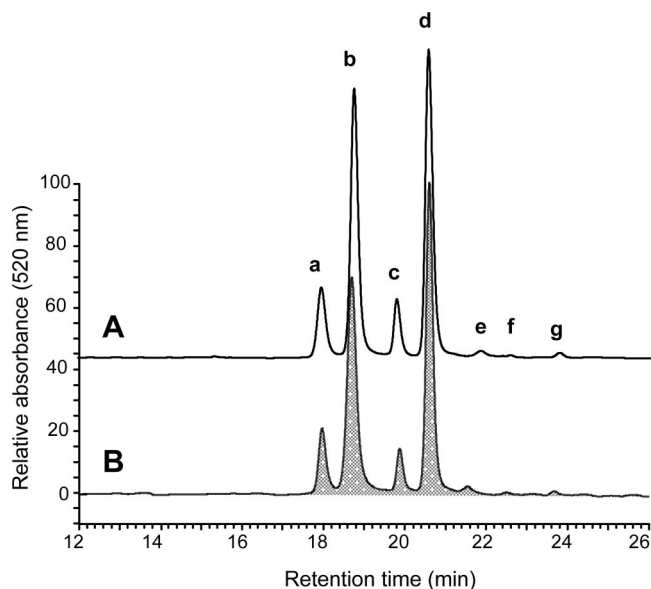


Fig. 1. HPLC profiles (520 nm) of the major anthocyanins in the blackcurrant (BC) extracts. Commercial BC (A) and anthocyanin-rich BC (BC-A; B) extracts were analyzed using liquid chromatography-electrospray ionization-mass spectrometry combined with photodiode array detection. The anthocyanins profile for the BC extract was similar to that displayed by the BC-A extract and identified 7 distinct anthocyanins derivatives, delphinidin-3-*O*-glucoside (a), delphinidin-3-*O*-rutinoside (b), cyanidin-3-*O*-glucoside (c), cyanidin-3-*O*-rutinoside (d), petunidin-3-*O*-rutinoside (e), pelargonidin-3-*O*-rutinoside (f), and peonidin-3-*O*-rutinoside (g).

$82 \pm 2$  (out of 100). Familiarization sessions to optimize the exercise intensity required to induce oxidative stress (determined using the same biochemical indices used in this study) showed that a 30-min row at a personalized  $\dot{V}O_{2\max}$  of 80% [estimated using individual physical characteristics (Table 1), and the Karvonen scale (25)] induced a reproducible transient oxidative stress. Subjective analysis revealed that the 30-min row caused no obvious muscle soreness or aching. Exercise and health diaries showed that all participants continued with their normal exercise routine (except on exercise trial days) with no reported illnesses or injuries. Basic analysis of the 3-day food diaries, by a registered sport nutritionist (School of Sport & Exercise, Waikato Institute of Technology, Waikato, NZ) showed that although subjects refrained from taking antioxidant supplements and foods that specifically contained berryfruit, their diet consisted of a variety of foods, including fruits and vegetables, which did contain natural antioxidants (including anthocyanins). Furthermore, ingestion of the commercial BC extract (~48 g of BCs, 240 mg anthocyanin) or placebo prior to and immediately after the 30-min row had no adverse effect on the participant's well-being or ability to perform the exercise.

### BC Extract Supplementation Attenuated Exercise-Induced Oxidative Stress

Ingestion of the sugar placebo showed an exercise-induced oxidative stress profile similar to that observed in familiarization sessions (data not shown). As shown in Fig. 2A, the 30-min row in the placebo group evoked a 1.4-fold increase ( $P < 0.05$ ) in plasma carbonyl levels immediately after the exercise ( $0.6 \pm 0.1$  vs.  $0.9 \pm 0.1$  nmol/mg protein, pre- vs. postexercise), which returned to within preexercise levels by 2 h ( $0.6 \pm 0.1$  nmol/mg protein). In contrast, consumption of the BC extract prevented ( $P < 0.05$ ) an increase of exercise-induced carbonyls ( $0.3 \pm 0.1$  vs.  $0.9 \pm 0.1$  nmol/mg protein, BC extract vs. placebo). A similar pattern was observed when using a modified DCF assay (used an indicator of changes in ROS generating capability see METHODS; Fig. 2B). We found that plasma collected after the 30-min row (placebo group) caused a transient fourfold increase ( $P < 0.05$ ) in FI ( $6,134 \pm 1,887$  vs.  $24,643 \pm 8,458 \Delta FI_{5\min}$ ) pre- vs. postexercise. BC supplementation prevented ( $P < 0.05$ ) this increase ( $5,288 \pm 1,756$  vs.  $2,699 \pm 1,061 \Delta FI_{5\min}$ , pre- vs. postexercise). In terms of plasma CK activity (Fig. 2C), the placebo group showed a 1.3-fold increase ( $P < 0.05$ ) in CK activity immediately after the 30-min row [ $74.7 \pm 14.0$  vs.  $94.2 \pm 16.9$  U/l, pre- vs. postexercise (0.5 h)] of which, in most subjects, was still elevated 24 h postexercise. Ingestion of the BC extract blunted the temporal increase in CK activity generated by exercise and was significantly different ( $P < 0.05$ ) from placebo group values 24 h postexercise.

### BC Supplementation Modulated LPS-Stimulated Inflammatory Responses

Exercise-induced oxidative stress has been shown to inhibit bacterial endotoxin-stimulated inflammatory cytokines (41, 47). Since ingestion of BC extract appears to attenuate exercise-induced oxidative stress, we explored what effect BC extract supplementation may have on this process. We

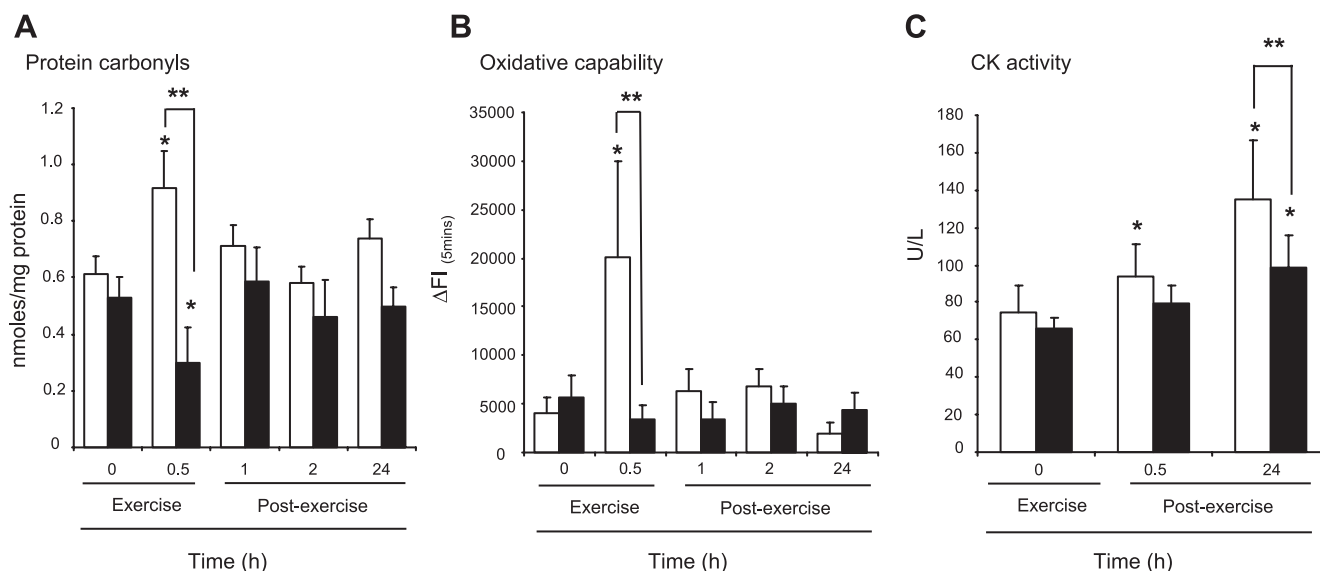


Fig. 2. BC extract supplementation suppressed oxidative stress parameters. Healthy untrained individuals were given a placebo (white bars) or BC extract (black bars) prior to and immediately after a 30-min row ( $80\% \dot{V}O_{2\max}$ ). Blood samples were taken prior to and after exercise (0–24 h), and the plasma isolated. **A:** protein carbonyl levels were measured using a colorimetric assay (nmol/mg total protein). **B:** hydrolyzed carboxy-dihydro-2',7'-dichloro-4,6-diamidino-2-methyl-6-phenylindole (carboxy  $H_2DCFDA$ ) formed the reactive oxygen species (ROS)-sensitive dichloro-4,6-diamidino-2-methyl-6-phenylindole (DCF) product that together with  $0.25 \mu M$  hydrogen peroxide ( $H_2O_2$ ) was used to assess the potential ROS generating capability of plasma prior to and after (0–24 h) exercise. Data were calculated as change in relative FI after 5 min ( $\Delta FI_{5min}$ ). **C:** creatine kinase (CK) activity was assessed before (0), immediately after (0.5 h), and 24 h postexercise by a clinical diagnostic laboratory (LabPlus, Auckland, NZ). Data were expressed as units per liter of plasma. Results are expressed as means  $\pm$  SE,  $n = 10$ . \* $P < 0.05$  statistical difference (paired Student's *t*-test) between preexercise and postexercise time points for both placebo and BC extract groups. \*\* $P < 0.05$  statistical difference (paired Student's *t*-test) between placebo and BC extract groups.

initially examined immune responsiveness of pre- and postexercise peripheral blood leukocytes and isolated plasma.

**Peripheral blood cultures.** Results of peripheral blood cultures are shown in Fig. 3A. A marginal but significant ( $P < 0.05$ ) suppression in TNF- $\alpha$  secretion ( $2,671 \pm 316$  vs.  $2,087 \pm 317$  pg/ml, pre- vs. postexercise) was detected in the placebo group, whereas a 40% suppression ( $2,341 \pm 226$  vs.  $1,457 \pm 180$  pg/ml, pre- vs. postexercise,  $P < 0.05$ ) was observed in the BC group. Comparison of placebo and BC groups revealed no significant difference in preexercise LPS-stimulated TNF- $\alpha$  secretion, whereas a greater ( $P < 0.05$ ) reduction in postexercise TNF- $\alpha$  secretion was observed in the BC group. Results of LPS-stimulated IL-6 secretion are shown in Fig. 3A. In the placebo group, exercise caused a decrease in IL-6 secretion ( $575 \pm 47$  vs.  $453 \pm 53$  pg/ml, pre- vs. postexercise), whereas a 30% suppression was observed in the BC group ( $480 \pm 29$  vs.  $312 \pm 36$  pg/ml, pre- vs. postexercise). Comparison between placebo and BC group results showed a significant ( $P < 0.05$ ) difference between postexercise IL-6 levels. Furthermore, no statistical difference was observed in spontaneous TNF- $\alpha$  or IL-6 secretion from pre- or postexercise peripheral blood leukocytes in either the placebo or BC groups (data not shown).

**Isolated plasma experiments.** We also used a monocytic cell line, THP-1, which is commonly used to evaluate monocyte function (7, 54) to explore the immune modulation of postexercise plasma on peripheral cell function. Incubation of pre- or postexercise plasma (final concentration 25%) with THP-1 cells had no effect on the spontaneous TNF- $\alpha$  and IL-6 production (data not shown); however, in the presence of LPS, a differential temporal pattern of LPS-stimulated cytokine secretion was observed (Fig. 3B). LPS exposure of THP-1 cells for

3 h caused a significant ( $P < 0.05$ ) increase in TNF- $\alpha$  secretion from cells preincubated with postexercise plasma after placebo ingestion ( $1,821 \pm 24$  vs.  $1,984 \pm 37$  pg/ml, pre- vs. postexercise). This increase was significantly ( $P < 0.05$ ) augmented after BC supplementation ( $1,892 \pm 69$  vs.  $2,517 \pm 140$  pg/ml, pre- vs. postexercise). LPS stimulation for 6 h showed a marginal increase in TNF- $\alpha$  secretion from cells preincubated with postexercise plasma after placebo supplementation ( $2,227 \pm 95$  vs.  $2,374 \pm 103$  pg/ml, pre- vs. postexercise). In contrast, preincubation of THP-1 cells with postexercise plasma after BC ingestion caused a significant ( $P < 0.05$ ) suppression in TNF- $\alpha$  secretion ( $2,253 \pm 114$  vs.  $1,829 \pm 70$  pg/ml, pre- vs. postexercise), which was also significantly ( $P < 0.05$ ) different from levels measured after ingestion of the placebo. A longer LPS incubation time (24 h) revealed a significant ( $P < 0.05$ ) reduction in TNF- $\alpha$  secretion from cells preincubated with postexercise plasma from both supplementation groups. Furthermore, exercise induced suppression of TNF- $\alpha$  secretion after BC supplementation was significantly ( $P < 0.05$ ) greater than that observed after ingestion of the placebo. LPS stimulation (3 h) of THP-1 cells preincubated with postexercise plasma after placebo ingestion caused a marginal increase in IL-6 secretion ( $34 \pm 2$  vs.  $37 \pm 4$  pg/ml, pre- vs. postexercise). This was significantly ( $P < 0.05$ ) augmented after BC extract supplementation ( $31 \pm 6$  vs.  $49 \pm 4$  pg/ml, pre- vs. postexercise). Interestingly, a 6-h LPS stimulation of cells preincubated postexercise plasma after placebo ingestion caused a significant ( $P < 0.05$ ) increase in IL-6 secretion ( $40 \pm 2$  vs.  $48 \pm 4$  pg/ml, pre- vs. postexercise), whereas a significant ( $P < 0.05$ ) suppression was observed after BC extract supplementation ( $46 \pm 3$  vs.  $33 \pm 5$  pg/ml, pre- vs. postexercise). In contrast, a longer LPS stimu-

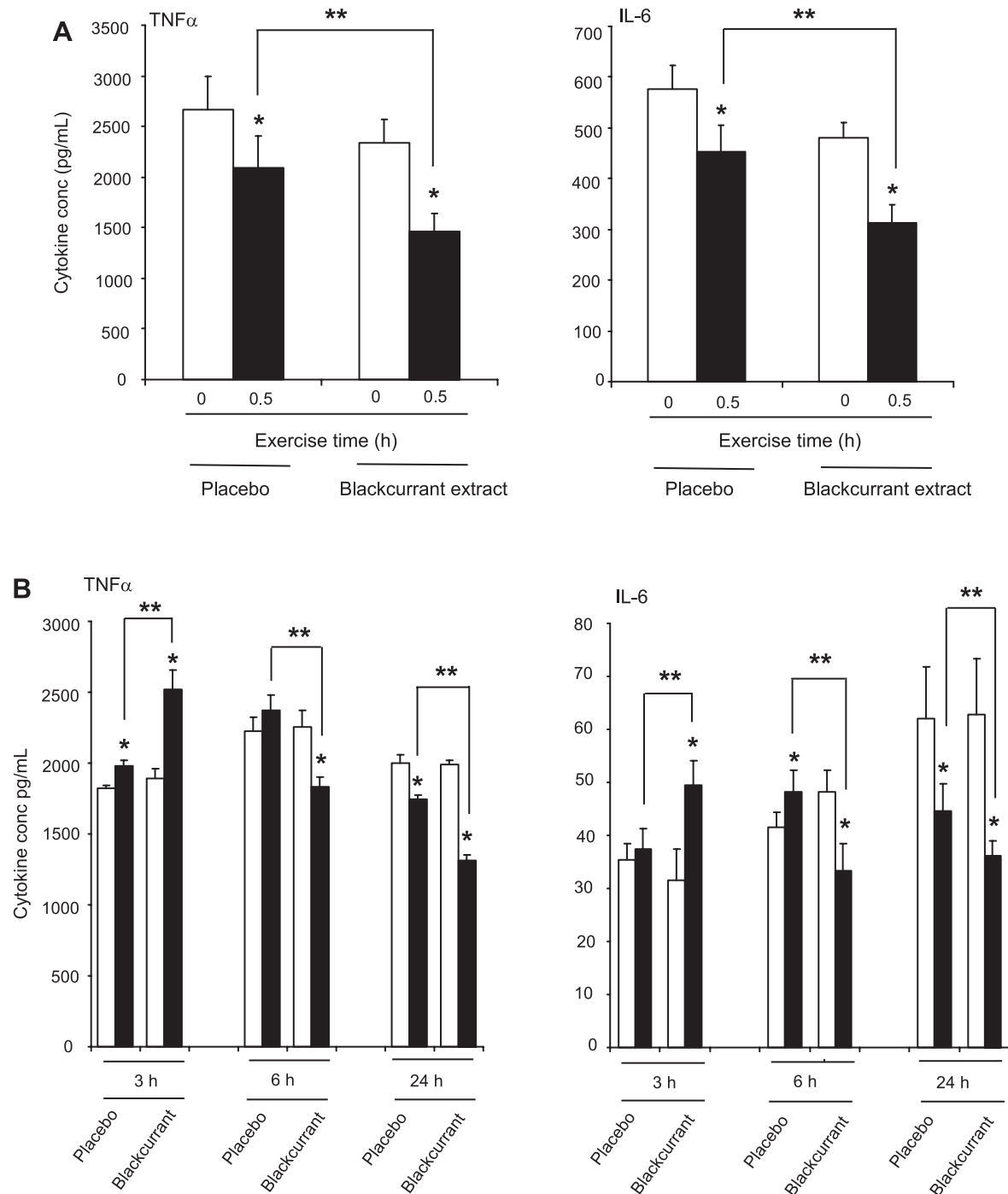


Fig. 3. Moderate exercise modulated LPS-stimulated cytokine expression. *A*: peripheral blood collected prior to (white bars) and immediately postexercise (0.5 h; black bars) was diluted 1:1 with RPMI medium and incubated with LPS (500 ng/ml) for 24 h. Supernatant was collected and measured for TNF- $\alpha$  and IL-6 by ELISA. Results are means  $\pm$  SE,  $n = 8$ . \* $P < 0.05$  statistically significant (paired Student's  $t$ -test) between preexercise (0) and postexercise (0.5 h) values within both placebo and BC extract groups. \*\* $P < 0.05$  statistical significance (paired Student's  $t$ -test) between placebo and BC groups. *B*: monocytic THP-1 cells were incubated in RPMI media (no FBS) containing diluted plasma (final concentration 25%) isolated from blood collected preexercise (white bars) and immediately postexercise (0.5 h; black bars) from placebo or BC extract groups. Cells were preincubated for 30 min with the plasma and then stimulated with LPS (50 ng/ml) for 3, 6, or 24 h, followed by the isolated supernatant being measured for either TNF- $\alpha$  or IL-6. Results are means  $\pm$  SE,  $n = 8$ . \* $P < 0.05$  statistical significance (paired Student's  $t$ -test) between preexercise (0) and postexercise (0.5 h) LPS-stimulated TNF- $\alpha$  and IL-6 values using plasma from either placebo or BC extract groups. \*\* $P < 0.05$  statistical difference (paired Student's  $t$ -test) between placebo and BC extract groups.

lation (24 h) of THP-1 cells preincubated with postexercise plasma from both supplementation groups caused a significant ( $P < 0.05$ ) reduction in IL-6 secretion, which was significantly ( $P < 0.05$ ) greater after BC supplementation.

#### BC Anthocyanins Modulate Inflammatory Events

Since the commercial BC extract primarily consisted of anthocyanins (89%), we focused on the effect of BC-derived

anthocyanins (using a BC-A extract) on LPS-stimulated inflammatory processes in THP-1 cells and explored how the putative timing of BC-A supplementation during exercise may influence these events.

**LPS-stimulated cytokine secretion.** Preincubation of THP-1 cells with the BC-A extract (5 or 50 ng/ml) for 30 min prior to LPS stimulation induced a time- and concentration-dependent suppression of TNF- $\alpha$  secretion (Fig. 4). A significant ( $P < 0.05$ ) suppression in TNF- $\alpha$  secretion was first observed after 6-h LPS stimulation ( $16,705 \pm 745$  vs.  $12,780 \pm 1,623$  or  $9,108 \pm 1,226$  pg/ml, control vs. BC-A extract 5 or 50 ng/ml). This suppressive effect was still evident after 24-h LPS stimulation. In contrast, a concentration-dependent suppression of IL-6 secretion by the BC-A extract was not observed until after a 12-h LPS stimulation ( $178 \pm 13$  vs.  $139 \pm 19$  or  $113 \pm 8$  pg/ml, control vs. BC-A extract 5 or 50 ng/ml), which, like the suppression of stimulated TNF- $\alpha$  secretion, was still evident after a 24-h LPS stimulation.

**LPS-stimulated NF- $\kappa$ B signaling.** Activation of the transcription factor, NF- $\kappa$ B, involves a series of coordinate cellular events (19). We specifically examined the effect of the BC-A extract on nuclear translocation of pNF- $\kappa$ B<sub>p65</sub> and cellular degradation of I $\kappa$ B $\alpha$  inhibitor following LPS stimulation Fig. 5. Semiquantitative analysis of immunoblots revealed that a 30-min preincubation of cells prior to LPS stimulation with the BC-A extract induced a concentration-dependent reduction in the amount of pNF- $\kappa$ B<sub>p65</sub> detected in nuclear extracts (Fig. 5A). When results were expressed as a ratio of LPS-stimulated pNF- $\kappa$ B<sub>p65</sub> levels, a significant ( $P < 0.05$ ) reduction in nuclear extract levels was first detected using 5 ng/ml of BC-A extract ( $0.78 \pm 0.16$ ). A BC-A extract concentration of 100 ng/ml caused a reduction in LPS-stimulated pNF- $\kappa$ B<sub>p65</sub> levels ( $0.44 \pm 0.1$ ), which were similar to that detected in unstimulated nuclear cell extracts ( $0.35 \pm 0.1$ ). Furthermore, preincubation of the BC-A extract had no effect on the total cellular amount of NF- $\kappa$ B<sub>p65</sub> detected

by immunoblot (data not shown). When cellular I $\kappa$ B $\alpha$  levels were expressed as a ratio of corresponding actin levels, LPS stimulation caused a significant reduction on cellular I $\kappa$ B $\alpha$  ( $1.8 \pm 0.4$  vs.  $0.7 \pm 0.1$ , I $\kappa$ B $\alpha$ -to-actin ratio, unstimulated vs. LPS-stimulated) (Fig. 5B). Preincubation of BC-A extract caused a concentration-dependent attenuation in LPS-induced cellular I $\kappa$ B $\alpha$  degradation, which became significant when 5 ng/ml of the BC-A extract ( $0.84 \pm 0.1$ , I $\kappa$ B $\alpha$ -to-actin ratio) was used, whereas a high concentration of BC-A extract (100 ng/ml) displayed I $\kappa$ B $\alpha$  levels similar to unstimulated cells ( $1.6 \pm 0.2$ , I $\kappa$ B $\alpha$ -to-actin ratio).

**ROS and BC-A extract modulation of LPS-stimulated cytokine secretion.** We used a cellular model to simulate an increase in ROS to examine its effect, and the timing of BC-A extract exposure, on LPS-stimulated inflammatory responses. Addition of 10  $\mu$ M H<sub>2</sub>O<sub>2</sub> to carboxy H<sub>2</sub>DCFDA-loaded THP-1 cells caused a  $\sim 2.5$ -fold increase in FI (ROS generation);  $2,876 \pm 597$  vs.  $8,052 \pm 288$   $\Delta$ FI<sub>30min</sub>, baseline vs. H<sub>2</sub>O<sub>2</sub>. We found that concomitant addition of 5 ng/ml of the BC-A extract with H<sub>2</sub>O<sub>2</sub> to the carboxy H<sub>2</sub>DCFDA-loaded THP-1 cells inhibited ROS generation by  $\sim 85\%$  ( $3,654 \pm 592$ ). Since this BC-A extract concentration (5 ng/ml) showed a marginal suppression of TNF- $\alpha$  and IL-6 secretion in THP-1 cells after 24-h LPS stimulation (Fig. 4), it was utilized to explore how the timing in the administration of the BC-A supplement may modulate LPS-induced inflammatory cytokine secretion (Fig. 6). Incubation of THP-1 cells with H<sub>2</sub>O<sub>2</sub> (10  $\mu$ M) for 30 min followed by stimulation with LPS (100 ng/ml) for 24 h caused a significant ( $P < 0.05$ ) suppression in LPS-stimulated TNF- $\alpha$  ( $16,483 \pm 466$  vs.  $8,277 \pm 715$  pg/ml, control vs. H<sub>2</sub>O<sub>2</sub>) and IL-6 ( $318 \pm 12$  vs.  $238 \pm 14$  pg/ml, control vs. H<sub>2</sub>O<sub>2</sub>,  $P < 0.05$ ) secretion. Addition of the BC-A extract (5 ng/ml) to THP-1 cells that had been pretreated (30 min) with 10  $\mu$ M H<sub>2</sub>O<sub>2</sub> caused a significant ( $P < 0.05$ ) suppression in LPS-stimulated TNF- $\alpha$  ( $6,012 \pm 316$  pg/ml) and IL-6 ( $199 \pm 12$  pg/ml) levels that was signif-

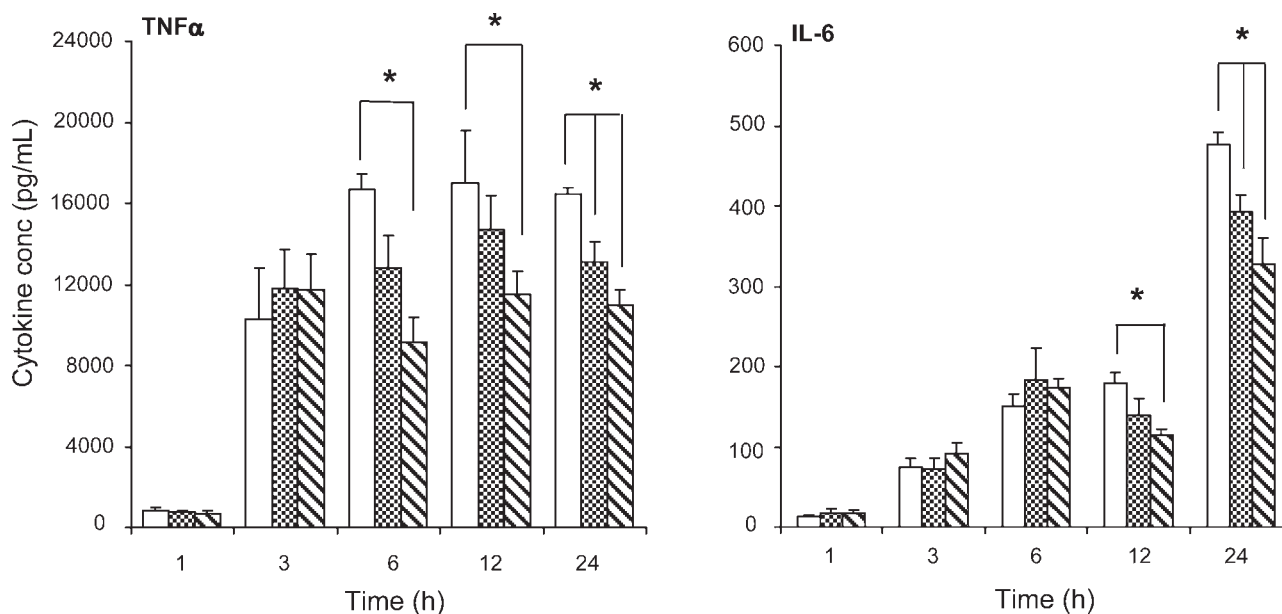


Fig. 4. BC-A extract induced a time-dependent suppression of LPS-stimulated cytokine generation in THP-1 cells. THP-1 cells were incubated with RPMI media alone (white bars) or in the presence of 5 (checked bars), or 50 ng/ml (hatched bars) BC-A extract for 30 min prior to LPS (500 ng/ml) stimulation for 1–24 h. At each time point the supernatant was separated from the cells by centrifugation and using appropriate ELISAs measured for either TNF- $\alpha$  or IL-6. Concentration. Results are means  $\pm$  SE,  $n = 6$ , \* $P < 0.05$  statistical difference (paired Student's  $t$ -test) from control (media alone) at a specified time point.



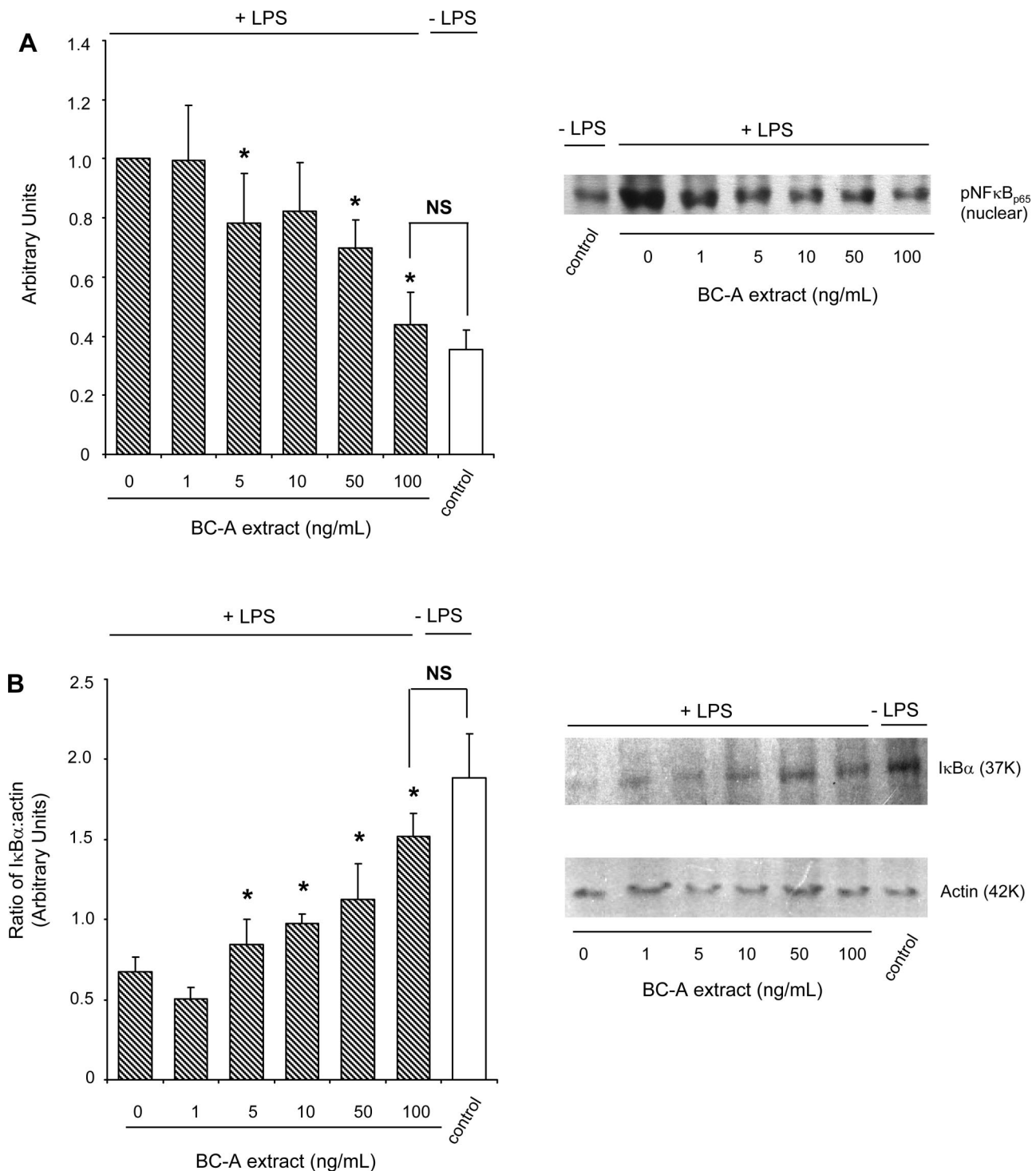


Fig. 5. Anthocyanin-rich BC (BC-A) extract attenuated LPS-induced NF- $\kappa$ B signaling in THP-1 cells. **A**: THP-1 cells were incubated with BC-A extract (0–100 ng/ml) for 30 min, washed, and then incubated with either RPMI media alone (white bars) or 500 ng/ml LPS (hatched bars) for a further 60 min. Nuclear extracts were prepared and used to assess phosphorylated NF- $\kappa$ B<sub>p65</sub> levels by Western blots. Using densitometry analysis (ImageJ version 1.41o; National Institutes of Health), data were calculated as a ratio of phosphorylated NF- $\kappa$ B<sub>p65</sub> levels measured in nuclear preparation from cells stimulated with LPS alone. Results are expressed as means  $\pm$  SE of 5 separate experiments. \* $P$  < 0.05 statistical difference (paired Student's  $t$ -test) between LPS and LPS in the presence of BC-A extract. NS means no statistical significance (paired Student's  $t$ -test) between THP-1 cells incubated with RPMI alone and those incubated with LPS in the presence of BC-A extract. **B**: THP-1 cells were incubated with BC-A extract (0–100 ng/ml) for 30 min, washed, and then incubated with either RPMI alone (white bars) or 500 ng/ml LPS (hatched bars) for a further 40 min. Whole cell extracts were prepared and I $\kappa$ B $\alpha$  and actin levels were determined by Western blot analysis. Data were calculated as a ratio between I $\kappa$ B $\alpha$  and actin levels using densitometry analysis. Results are means  $\pm$  SE of 4 separate experiments. \* $P$  < 0.05 statistical difference (paired Student's  $t$ -test) between LPS and LPS in the presence of BC-A extract.

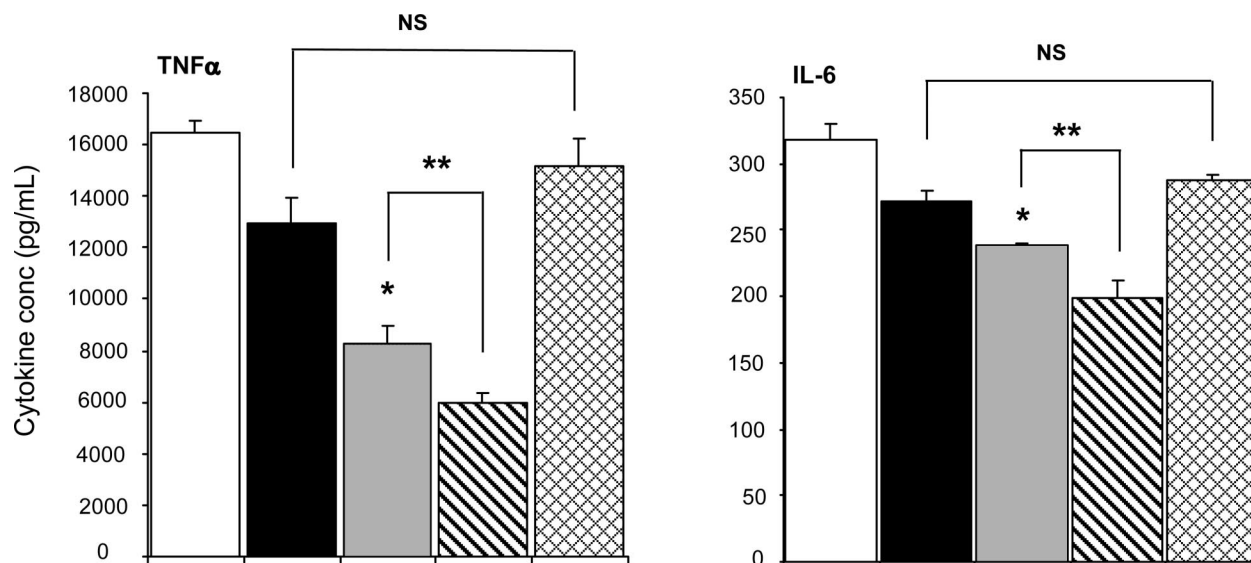


Fig. 6. BC-A extract modulated LPS-stimulated cytokine generation in THP-1 cells exposed to H<sub>2</sub>O<sub>2</sub>. A: THP-1 cells were incubated for 30 min with RPMI media alone (white bars), 5 ng/ml BC-A extract alone (black bars), 10  $\mu$ M H<sub>2</sub>O<sub>2</sub> alone (gray bars), 10  $\mu$ M H<sub>2</sub>O<sub>2</sub> followed by a further 30-min incubation with 5 ng/ml BC-A (hatched bars) or 10  $\mu$ M H<sub>2</sub>O<sub>2</sub> plus 5 ng/ml BC-A extract simultaneously (crossed bars). Cells were then stimulated with LPS (100 ng/ml) for 24 h, supernatant collected and measured for TNF- $\alpha$  or IL-6 generation using appropriate ELISA. Data were calculated as pg/ml. Results are expressed as means  $\pm$  SE,  $n = 6$ . \* $P < 0.05$  and \*\* $P < 0.05$  statistical difference (paired Student's  $t$ -test) between cells treated with H<sub>2</sub>O<sub>2</sub> alone vs. control and cells treated with H<sub>2</sub>O<sub>2</sub> alone vs. H<sub>2</sub>O<sub>2</sub> followed by BC-A extract, respectively. NS represents no statistical significance between cells incubated with BC-A extracts alone and cells simultaneously incubated with H<sub>2</sub>O<sub>2</sub> plus BC-A extract.

icantly ( $P < 0.05$ ) greater than that caused by H<sub>2</sub>O<sub>2</sub> alone. However, when THP-1 cells were preincubated with H<sub>2</sub>O<sub>2</sub> prior to LPS stimulation in the presence of the BC-A extract (at a concentration that prevented H<sub>2</sub>O<sub>2</sub>-induced ROS) a significant ( $P < 0.05$ ) attenuation of the suppressive effect of H<sub>2</sub>O<sub>2</sub> on TNF- $\alpha$  ( $15,141 \pm 1,121$  pg/ml) and IL-6 ( $288 \pm 13$  pg/ml) secretion was observed. These levels were similar to those detected in cells treated with the BC-A extract alone:  $12,969 \pm 466$  and  $271 \pm 7$  pg/ml, TNF- $\alpha$  and IL-6 levels, respectively.

## DISCUSSION

Dietary antioxidant supplements are commonly used to limit exercise-induced oxidative stress (40, 44, 49); however, their use may not always complement the health benefits gained from regular exercise (8). Using human exercise and cellular models, we explored how a BC extract, exhibiting both antioxidant and anti-inflammatory properties, if ingested at appropriate amounts and at the appropriate time, may enhance exercise-induced health benefits. An important consideration in this process is the time availability of the potential BC bioactives, i.e., anthocyanins. Some fruit- and vegetable-derived anthocyanins are absorbed by the stomach and small intestine and distributed in blood as intact glycosylated forms (31). Bioavailability studies indicate that berryfruit anthocyanins (in their glycosylated forms) are rapidly detected in the plasma after ingestion (31, 32, 34), and hence in our study the consumption of the BC extract (containing a total of  $\sim 240$  mg anthocyanins) immediately prior to and after the 30-min row may have transiently elevated plasma glycosylated anthocyanins and their metabolites and thus subsequent bioavailability. However, anthocyanins are also quickly degraded and excreted (39), indicating that any biological action may be quite short lived. Given the brief nature of the exercise-induced oxidative stress in our study, the temporary rise in plasma anthocyanins

may have been significant in this regard. It is also possible that long-term consumption of these compounds may activate adaptive antioxidant and immune modulatory mechanisms. Indeed, consumption of berryfruit juices over several weeks significantly increased plasma antioxidant capacity in elderly individuals and modulated immune responsiveness (35, 38). We detected no change in exercise-induced indices in the placebo groups during our 3-wk crossover study, and hence, while the participant's food diaries revealed some consumption of foods that contain anthocyanins as part of their daily nutrition, effects of this consumption was of no significance.

Anthocyanins (major constituents of the BC extract utilized) are strong antioxidants as attested by a vast array of in vivo and in vitro studies (20, 21, 24, 29) and therefore may contribute to the overall antioxidant properties of BCs. We found that ingestion of the BC extract reduced transient increases in plasma oxidative generating capability and protein carbonyls generated by the 30-min row. A reduction in plasma CK activity (an indicator of micro and macro muscle damage) after BC supplementation was also observed, however, was not distinguishable from the CK activity from the placebo group until 24 h postexercise. Although subjective questioning failed to reveal muscle soreness (upper and lower limbs) an increase in CK activity was not unexpected as rowing was not routinely used by the study's participants. Moreover, it is feasible that the temporal differential inhibition of exercise-induced oxidative stress indices may be a consequence of the limited antioxidant action of anthocyanins, where it is able to quickly neutralize any increase or tissue extracellular fluid in plasma mediators of oxidative stress but may be unable to prevent increases in exercise-induced oxidative stress within the muscle tissue itself resulting in some damage and release of CK. Ongoing studies within this group using ex vivo mouse soleus muscle tissue and electrical stimulation show modification of

force production following exposure to some fruit extracts (45). An alternate mediator of the reduction in exercise-induced oxidative stress caused by BC extract ingestion is an increase in plasma uric acid. Elevated plasma uric acid has been related to an increase in plasma antioxidant capacity following the consumption of some fruit sugars (29). However, an antioxidant effect was not observed in our sugar placebo group supporting the antioxidant action of the anthocyanins of the BC extract. Similar results have been observed in human feeding trials where the anthocyanins content in plant (13) and fruit (35, 36) extracts increased measures of plasma antioxidant capacity, while a sugar placebo had no detectable action. These findings, in combination with the high amount of anthocyanins detected within in the commercial BC extract, leads us to conclude that anthocyanins acted as effective antioxidants in reducing the oxidative stress induced by the 30-min row.

Exercise-induced oxidative stress (via ROS-dependent or -independent mechanisms) results in an acute inflammatory response (3, 50). Here we focused on how the BC extract supplementation and exercise modulated the ability of LPS to stimulate an acute inflammatory response. In *ex vivo* experiments, using peripheral leukocytes and THP-1 cells supplemented with pre- or postexercise plasma, we found, like others (12, 16, 41, 47), that exercise suppressed TNF- $\alpha$  and IL-6 secretion after a 24-h LPS stimulation. However, shorter LPS exposure times (3 or 6 h) revealed that exercise appears to enhance TNF- $\alpha$  and IL-6 secretion. Since exercise-induced oxidative stress and bacterial endotoxin LPS may augment cellular events that result in NF- $\kappa$ B activation (23, 27, 33), it is feasible that exercise-induced transient oxidative stress enhances the temporal LPS-stimulated acute inflammatory response, resulting in an earlier resolution time. This may also account for the differential temporal cytokines secretion pattern observed after exercise in this study and by others (47). Furthermore, it is feasible that exercise-induction of an acute inflammation may be an important step in the activation of appropriate adaptive immune responses (14), which is in contrast to the chronic subclinical inflammation observed in some athletes undergoing long-term strenuous training regimens, which results in immune suppression (9).

Ingestion of plant-derived anthocyanins has also been shown to modulate immune function (21, 26). Berryfruit-rich anthocyanin extracts have been shown to possess anti-inflammatory properties (53) that may involve NF- $\kappa$ B inhibition (24). We found that exposure of the BC-A extract to THP-1 cells caused a concentration- and time-dependent inhibition of LPS-stimulated TNF- $\alpha$  and IL-6 secretion in THP-1 cells. In addition, preliminary exploration into the putative signaling mechanisms revealed that preincubation of cells with the BC-A extract reduced cellular events involved in the activation of the transcription factor, NF- $\kappa$ B. However, we found that ingestion of the commercial BC extract actually augmented ability of exercise to modulate the temporal cytokine secretion profile that resulted in a suppression of TNF- $\alpha$  and IL-6 secretion after 24-h LPS stimulation (Fig. 3). Since our results and others (47) suggest that regular exercise may improve the resolution time of acute inflammation stimulated by bacterial endotoxins, a possible explanation for our findings, in light of BC extract's antioxidant and anti-inflammatory properties, is that the bio-availability of BC components was subsequent to exercise-induced oxidative stress and serves to further augment the

resolution time of a LPS-stimulated acute inflammatory response, thereby possibly accounting for the enhanced suppression of TNF- $\alpha$  and IL-6 secretion observed after 24-h LPS stimulation.

To explore this possibility, we used an H<sub>2</sub>O<sub>2</sub>-induced oxidative stress cell model and found that addition of the BC-A extract (at a concentration that inhibited H<sub>2</sub>O<sub>2</sub>-induced ROS but only marginally attenuated LPS-stimulated TNF- $\alpha$  and IL-6 secretion) to THP-1 cells pretreated with H<sub>2</sub>O<sub>2</sub> enhanced the suppression of LPS-induced TNF- $\alpha$  and IL-6 production observed after a 24-h incubation. This action was not evident when the THP-1 cells were incubated simultaneously with the BC fruit extract and H<sub>2</sub>O<sub>2</sub>. These results tentatively suggest that appropriate timing of the BC extract may complement exercise-induced immune responsiveness; however, further experiments are needed to clarify how the BC extract supplementation and exercise modulates the temporal LPS-stimulated inflammatory response. Furthermore, since other plant-derived flavonoids may induce an oxidative stress (46), we also cannot exclude the possibility that BC components may enhance the temporal LPS-stimulated acute inflammatory response.

#### *Perspectives and significance*

Regular exercise is essential to maintain health and reduce susceptibility to common ailments (14). Exercise-induced transient oxidative stress (via ROS-dependent or -independent mechanisms) is an important element in the activation of appropriate adaptive events that may underpin the improved immune function achieved from regular exercise (14, 17, 18, 50). Therefore, excessive or inappropriate timing of supplements (e.g., antioxidant, anti-inflammatory) that completely mitigate oxidative stress during exercise may prevent these adaptive events. Although the BC extract used in this study demonstrated both antioxidant and anti-inflammatory properties, our findings support the concept that fruits and vegetables rich in anthocyanins, if consumed in the appropriate amounts and at the optimum time, have the ability to augment the health benefits of regular exercise. It is therefore feasible that more knowledge on plant-derived flavonoids and their mode of action could lead to functional food products that could be tailored to suit the needs of different groups of potential consumers from those undergoing regular moderate exercise for maintaining health and fitness to those undergoing more intensive exercise regimens for training purposes.

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