Bcl-2 in endothelial cells is increased by vitamin E and α-lipoic acid supplementation but not exercise training

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Abstract

Atherosclerotic plaque contains apoptotic endothelial cells with oxidative stress implicated in this process. Vitamin E and α-lipoic acid are a potent antioxidant combination with the potential to prevent endothelial apoptosis. Regular exercise is known to increase myocardial protection, however, little research has investigated the effects of exercise on the endothelium. The purpose of these studies was to investigate the effects of antioxidant supplementation and/or exercise training on proteins that regulate apoptosis in endothelial cells. Male rats received a control or antioxidant-supplemented diet (vitamin E and α-lipoic acid) and were assigned to sedentary or exercise-trained groups for 14 weeks. Left ventricular endothelial cells (LVECs) were isolated and levels of the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax were measured. Antioxidant supplementation caused a fourfold increase in Bcl-2 (P < 0.05) with no change in Bax (P > 0.05). Bcl-2:Bax was increased sixfold with antioxidant supplementation compared to non-supplemented animals (P < 0.05). Exercise training had no significant effect on Bcl-2, Bax or Bcl-2:Bax either alone or combined with antioxidant supplementation (P > 0.05) compared to non-supplemented animals. However, Bax was significantly lower (P < 0.05) in the supplemented trained group compared to non-supplemented trained animals. Cultured bovine endothelial cells incubated for 24 h with vitamin E and/or α-lipoic acid showed the combination of the two antioxidants increased Bcl-2 to a greater extent than cells incubated with the vehicle alone. In summary, vitamin E and α-lipoic acid increase endothelial cell Bcl-2, which may provide increased protection against apoptosis.

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1. Introduction

Preservation of the structural integrity of the endothelium is crucial to maintain vascular function and prevent plaque and thrombus formation. Apoptosis, or programmed cell death, is known to occur in vascular endothelial cells with cellular debris of endothelial origin identified in atherosclerotic plaques [1]. Members of the Bcl-2 family of proteins are important regulators of apoptosis with individual members that can suppress (e.g. Bcl-2) or promote (e.g. Bax) programmed cell death. Overexpression of Bcl-2 confers increased cellular protection [2] and increased intracellular levels of Bcl-2 have been associated with decreased myocardial ischemia–reperfusion injury in vivo [3]. Furthermore, Bcl-2 also has antioxidant capabilities [4–6], thus increased cellular levels of Bcl-2 may provide dual protection against atherosclerosis.

 Reactive oxygen species (ROS) induce apoptosis in vitro and this is characterised by intracellular translocation of Bcl-2 and Bax [7]. Few studies have examined the link between antioxidant supplementation and apoptotic pathways and this study appears to be the first to investigate this relationship in the coronary endothelium in vivo.

α-Lipoic acid is a coenzyme involved in oxidative decarboxylation of keto acids. The thiol compound has an antioxidant capacity with the added ability to recycle vitamin E [8,9]. Our group and others have shown that combined supplementation of vitamin E and α-lipoic acid is a potent antioxidant combination [9,10].

While regular exercise is known to decrease the incidence of cardiovascular disease, it is still unclear whether this rela-
tionship is a result of a reduction in risk factors or improvements in cardioprotective mechanisms [11]. Acute exercise of moderate to high intensity increases ROS production whereas exercise training attenuates this rise and increases cardioprotection by a number of pathways including upregulation of myocardial antioxidant enzymes [12,13] and heat shock protein 70 (HSP70) [12,14,15]. However, the effect of exercise on apoptotic pathways within the endothelial cell is not known [16].

The purpose of these studies was to examine the effects of vitamin E and α-lipoic acid supplementation and/or exercise training on levels of Bcl-2 and Bax in the coronary endothelium. Regular exercise is known to cause an acute increase in ROS production, the exposure to which subsequently upregulates antioxidant defences and ultimately, myocardial protection. We hypothesised that exercise training would elevate endothelial Bcl-2 levels through a ROS-mediated pathway that would be attenuated by dietary antioxidant supplementation with little or no concurrent change in Bax. However, we found that vitamin E and α-lipoic acid supplementation rather than exercise training increased endothelial levels of Bcl-2. Subsequent experiments found that Bcl-2 was increased in cultured endothelial cells in vitro, however, no such change was evident in the myocardium of antioxidant-supplemented animals.

2. Materials and methods

2.1. Study 1 (in vivo)

2.1.1. Experimental animals, antioxidant supplementation and exercise training

Young male Wistar rats, aged 3 weeks (Central Animal Breeding House, The University of Queensland, Australia), were housed two to three per cage, maintained on a 12/12 h light/dark cycle and provided with standard rat chow and tap water ad libitum. Animals were assigned to one of four experimental groups: sedentary (n = 30), endurance exercise-trained for 14 weeks (n = 14), antioxidant-supplemented for 14 weeks (n = 23) or combined endurance trained and antioxidant-supplemented for 14 weeks (n = 14). Animals that received the antioxidant diet were fed the same rat chow as the non-supplemented groups with 1000 IU vitamin E/kg diet (β-α-tocopheryl succinate, Covitol 1185, Cognis, Melbourne, Australia) and 1.6 g α-lipoic acid/kg diet (Lipotec, Cognis) added. Prior to commencement of the training protocol, all animals underwent 5 d of familiarisation on an eight lane motorised treadmill consisting of standing and low-speed walking. Animals were then assigned to either the sedentary or exercise groups based on their willingness to run. This selection procedure for training animals was considered appropriate given that health status and muscle physiological properties do not generally differ among young adult rats with a willingness to run and those animals reluctant to exercise [17]. All animals in the exercise group underwent 14 weeks endurance programme modified from a previously described protocol [18] that provides training at approximately 75% maximal oxygen consumption (VO₂max). At week 1, rats ran at 25 m/min, 0% grade for 30 min/d. The speed, grade and duration were then increased progressively such that by week 10, rats were exercising at 30 m/min, 18% grade for 90 min/d and this was maintained until week 14. Rats exercised 4 d/week with each training period consisting of a 5 min warm up with a gradual increase to the desired running speed. This protocol has previously been shown to increase myocardial content of HSP72 and decrease myocardial lipid peroxidation following ischemia–reperfusion [12,15]. Mild electric shocks and compressed air were used sparingly to motivate the animals to run. Fatigue was defined as three shocks per animal per session, at which time the rat was immediately removed from the treadmill. This experiment was approved by The University of Queensland Animal Ethics Committee in accordance with National Health and Medical Research Council guidelines.

2.1.2. Isolation of left ventricular endothelial cells

Within 24–48 h following the final training session, animals were weighed, sacrificed via an intraperitoneal injection of sodium pentobarbital (100 mg/kg) and a thoracotomy was performed under anaesthesia. The heart was removed and immediately placed in ice-cold Krebs buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl₂·6H₂O, 1 mM NaH₂PO₄, 2.6 mM CaCl₂·2H₂O, 25 mM NaHCO₃, 11.1 mM glucose, 0.03 mM EDTA, pH 7.4). Left ventricular endothelial cell (LVEC) isolation was achieved by protease digestion according to the methods of Nishida et al. [19] with modification. Briefly, the aorta was cannulated with polyethylene tubing and the heart perfused with Krebs buffer. The heart was cleaned of epicardium, external fat and blood vessels and then weighed. The atria, right ventricle, septum and valvular material were removed and the left ventricle was finely minced with scalpels and washed with Krebs buffer. The crude homogenate was digested with 1 mg/ml collagenase (Type II, Sigma, St. Louis, MO) and 1 U/ml elastase (Sigma) dissolved in warm Krebs buffer and incubated on an orbital platform at 37 °C for 1 h. Disaggregation of the tissues was promoted by trituration of the solution through a 5 ml pipette after 30 min. Following incubation, each sample was filtered through a 100 µm nylon mesh (Millipore, Bedford, MA) to remove large undigested pieces and the filtrate was diluted with antioxidant buffer (50 mM Tris–HCl, 154 mM NaCl, 5 mM EDTA, 1 mM DTT, pH 7.6) and centrifuged at 1000 × g for 10 min. The pellet was washed three times in Ca²⁺ and Mg²⁺ free Hanks buffer (137 mM NaCl, 5.4 mM KCl, 5.5 mM glucose, 440 µM KH₂PO₄, 177 µM Na₂HPO₄, pH 7.4), resuspended in lysis buffer (50 mM Tris–HCl, pH 7.6, 1 mM EDTA, 2 mM leupeptin, 1 µ/l/ml aprotinin) and stored at −80 °C until assay. In our hands, the final cell yield contains approximately 80% endothelial cells with the remaining 20% consisting of predominantly leukocytes with minimal myocardial tissue or debris.
2.1.3. Myocardial tissue preparation

A smaller group of animals that consumed either a control diet (n = 7) or an antioxidant diet (n = 8) were also sacrificed and the heart removed and cleaned as described above. The left ventricular myocardium was frozen in liquid nitrogen and stored at −80 °C until analysis.

2.2. Study 2 (in vitro)

2.2.1. Cell culture and antioxidant treatment

To examine the individual and combined effects of vitamin E and α-lipoic acid, bovine aortic endothelial cells (BAEC) were maintained in Dulbecco’s modified Eagle Medium (DMEM) containing 10% bovine calf serum, 10 U/ml penicillin and 10 µg/ml streptomycin and incubated at 37 °C. Cells were grown to approximately 90% confluence and equal amounts of cells were plated in 10 cm culture plates containing a 10 mm diameter coverslip. Cells were used at the 7th to 8th passages. BAEC were incubated with vehicle, 1 mM α-lipoic acid (Ala), 50 µM α-tocopherol (VitE) or a combination of 1 mM α-lipoic acid and 50 µM α-tocopherol (Ala + VitE) for 24 h at 37 °C. Antioxidants were dissolved in ethanol and dimethylsulphoxide (DMSO) and prepared to final concentrations in serum-free DMEM containing 1000 U/ml penicillin and 1000 µg/ml streptomycin. Final concentrations of ethanol and DMSO were 0.05% and 0.35%, respectively (vehicle). Cells were lysed in RIPA buffer (50 mM Tris–Cl, pH 7.5, 150 mM NaCl, 25 mM NaF, 0.5 M EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% IGEPAL CA-630) containing 10 µg/ml aprotinin and 10 µg/ml leupeptin and scraped from the plate. Cells were dismembranated by drawing the cell solution through a 27G needle. Cell debris was removed using centrifugation at 15,000 × g for 15 min at 4 °C and the supernatant was collected and stored at −80 °C until analysis.

2.3. Immunodetection

Proteins from LVEC were precipitated using the chloroform/methanol method of Wessel and Flugge [20]. All protein concentrations were determined by the bicinchoninic acid assay (Pierce, Rockville, IL). Proteins were electrophoresed on 15% SDS-polyacrylamide gels at 200 V for 45 min and proteins transferred to PVDF membranes (Millipore) at 100 V for 2.5 h. The membranes were blocked for 1 h in blocking buffer (5% nonfat milk in Tris–buffered saline containing 0.05% Tween 20) and incubated with primary antibodies for either Bcl-2, Bax or HSP70 where appropriate (1:400, Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer overnight at 4 °C. After washing, membranes were incubated for 1 h with secondary anti-rabbit-IgG whole antibody conjugated to alkaline phosphatase (1:2000, Sigma) in blocking buffer. Actin (1:8000, Sigma) was used as an internal control. Blots were developed using a BCIP/NBT colour development substrate (Promega, Madison, WI) and analysed using Scion Image for Windows (Beta 4.0.2, Scion Corporation, Frederick, MD).

2.4. Antioxidants

Concentrations of vitamin E (α-tocopherol) were determined in LVEC by reverse-phase high performance liquid chromatography (HPLC) using the liquid–liquid extraction method of Taibi and Nicotra [21]. Briefly, proteins were precipitated and lipids extracted in a single step by incubation with an ethanol/chloroform mixture (3:1 v/v). After separation of the precipitated protein, 50 µl supernatant was injected onto a LiChrophor C18 column (250 × 4 mm, 5 µm; Merck, Darmstadt, Germany) with a flow rate of 1 ml/min and 9 MPa backpressure and analysed using fluorometric detection. Stock solutions of dl-α-tocopherol (Fluka, Buchs, Switzerland) were used as external standards. In our hands, the coefficient of variation for this assay is <2%.

LVEC concentrations of α-lipoic acid were not measured as preliminary data in our laboratory indicated that the compound was undetectable in both control and supplemented animals as α-lipoic acid is rapidly converted to various metabolites also with antioxidant properties [8,22]. This finding is consistent with previous studies [23].

2.5. Lipid peroxidation

LVEC levels of malondialdehyde (MDA) were determined by HPLC according to the method of Sim et al. [24]. Lysed samples were hydrolysed in 1.3 mM NaOH to a final concentration of 1 mM NaOH, incubated for 60 min at 60 °C and cooled on ice for 5 min. Proteins were precipitated by the addition of 35% perchloric acid, cooled on ice for 5 min and centrifuged for 5 min at 3500 × g. The supernatant was added to 2,3-dinitrophenylhydrazine (DNPH) and incubated for 10 min at room temperature in the dark. The aqueous phase was extracted with hexane and evaporated with the dry extract reconstituted in mobile phase containing 45% acetonitrile and 0.2% glacial acetic acid. MDA concentrations were determined at 310 nm using HPLC (Shimazu) with a LiChrophor C18 column (250 × 4 mm, 5 µm, 1 ml/min flow rate, 9.8 MPa backpressure; Merck). MDA aliquots of appropriate concentrations were used as external standards. The coefficient of variation for this assay is <5% in our hands.

2.6. Statistical analysis

Values reported are mean ± S.E.M. Data from Section 2.1 were analysed using a one way ANOVA and Tukey’s post hoc analysis was performed where appropriate with significance established at P < 0.05. In vitro data from Section 2.2 represents three independent experiments.

3. Results

3.1. In vivo study

Body weight was not significantly different between groups prior to the study (Table 1), however, animals in the exercise
training groups had a significantly lower body weight ($P < 0.05$) than sedentary animals at the completion of the training protocol. Heart weight was not significantly different between the sedentary and trained groups following the 14 week interventions ($P > 0.05$) however, the heart weight-to-body weight ratio was significantly greater in the trained groups ($P < 0.05$).

Fig. 1 shows that LVEC Bcl-2 levels were fourfold higher ($P < 0.05$) in the sedentary antioxidant-supplemented animals compared to non-supplemented controls whereas there was no significant difference ($P > 0.05$) in Bax. The elevation in Bcl-2 caused a significant ($P < 0.05$) sixfold increase in the Bcl-2:Bax ratio in the antioxidant-supplemented sedentary group compared to non-supplemented controls. No significant differences ($P > 0.05$) were seen in the exercise training groups in either Bcl-2, Bax or Bcl-2:Bax compared to sedentary animals. Animals that received a combination of exercise training and antioxidant supplementation did not show a significant difference ($P > 0.05$) in Bcl-2 expression whereas Bax expression was reduced (0.6-fold, $P < 0.05$) compared to non-supplemented, trained rats. There were no significant differences in Bcl-2:Bax ($P > 0.05$).

Bcl-2 and Bax proteins were examined in myocardial tissue to determine if the responses seen in endothelial cells was cell-specific. Fig. 2 shows that there were no significant differences ($P > 0.05$) in myocardial Bcl-2, Bax or Bcl-2:Bax between control diet and antioxidant-supplemented animals.

Vitamin E (a-tocopherol) in LVEC was significantly increased ($P < 0.05$) by 26% in antioxidant-supplemented animals compared to the sedentary, non-supplemented group (Table 2). In exercise-trained rats, LVEC vitamin E was increased by 16% with antioxidant supplementation compared to non-supplemented runners although this difference was not statistically significant ($P > 0.05$). LVEC MDA levels were 30% higher in the antioxidant-supplemented sedentary group than non-supplemented sedentary animals ($P < 0.05$). Exercise training alone significantly increased the levels of MDA in LVEC by 30% compared to sedentary animals ($P < 0.05$). This increase was attenuated by the combination of exercise training and antioxidant supplementation. Vitamin E and MDA levels were examined in myocardial tissue and no differences ($P > 0.05$) were found between non-supplemented and antioxidant-supplemented animals.

### 3.2. In vitro study

The increase in Bcl-2 with the combined antioxidant supplementation was further investigated using cultured BAEC incubated for 24 h with a-lipoic acid and/or vitamin E. Fig. 3 is representative of three independent experiments and shows that Bcl-2 was increased with vitamin E with a greater increase, evident with a combination of both a-lipoic acid and vitamin E.

### 4. Discussion

This is the first study to examine the effects of antioxidant supplementation and endurance exercise training on Bcl-2 and Bax in endothelial cells. Contrary to our hypothesis, the results indicate that Bcl-2 and consequently Bcl-2:Bax are significantly increased in endothelial cells following supplementation with vitamin E and a-lipoic acid but not exercise training, regardless of diet. These results were confirmed in vitro. Finally, as the antioxidant supplements had no effect on myocardial Bcl-2 the response appears to be endothelial cell-specific.

A key finding of this study is that dietary supplementation of vitamin E and a-lipoic acid in rats significantly increased endothelial levels of the anti-apoptotic Bcl-2 with no significant changes in the pro-apoptotic Bax, hence increasing Bcl-2:Bax. The significance of these changes on subsequent cellular protection is beyond the scope of this study, however, Rajesh et al. [3] have shown that infusion of the antioxidant MCI-186 increases myocardial levels of Bcl-2 and decreases ischemia–reperfusion injury in vivo. Similarly, Yang et al. [2] demonstrated that overexpression of Bcl-2 was associated with increased cellular protection in human acute myeloid leukaemia cells in vitro. Thus, it is possible that the increase in Bcl-2 with antioxidant supplementation seen in our study may increase cellular protection.

The mechanism underlying Bcl-2 upregulation with vitamin E and a-lipoic acid supplementation in the current study is unclear, however, one potential explanation may be found in the increased endothelial MDA in the sedentary supplemented animals. This finding indicates that the antioxidant supplements created a prooxidant intracellular environment and vitamin E has been reported to act in a prooxidant role both in vitro and in vivo [25,26]. As Bcl-2 has been shown to function as an intracellular antioxidant [4–6], the increased oxidative stress may have signalled for increased Bcl-2 synthesis.

Endurance exercise training had no effect on endothelial Bcl-2 and surprisingly the addition of exercise to antioxidant-supplemented animals blunted the increase in Bcl-2 as seen...
in supplemented sedentary animals. One possible explanation for these findings is that the increased oxidative stress due to exercise training overwhelmed the antioxidant sparing abilities of vitamin E and α-lipoic acid and resulted in Bcl-2 assisting with antioxidant functions in the endothelial cell. However, this is in contrast to the mechanism proposed to explain the increase in Bcl-2 found in antioxidant-supplemented animals. It is more likely that the changes seen in Bcl-2 in this study are associated with the endothelial levels of α-tocopherol both in vivo and in vitro. Indeed, vitamin E deficiency downregulates the Bcl2-L1 gene that encodes...
Bcl-Xₐ, an anti-apoptotic member of the Bcl-2 family [27]. Therefore, it is possible that increases in vitamin E might promote Bcl-2 expression. As there was no consistency in oxidative stress and vitamin E content in the current study, it is probable that this effect was due to the non-antioxidant properties of vitamin E and current work in our laboratory is investigating this mechanism.

Vitamin E and α-lipoic acid are both potent antioxidants and their effectiveness is increased when they are combined due to the ability of α-lipoic acid to recycle vitamin E through interactions with ascorbate and glutathione [8,9,28]. In the current study, we found that administration of both α-lipoic acid and vitamin E in vitro elicited a greater Bcl-2 response than either antioxidant alone. Haramaki et al. [29,30] also demonstrated a synergistic action between these two antioxidants and reported that the combination of α-lipoic acid and vitamin E was more effective in increasing cardioprotection than either antioxidant alone. Interestingly, in vitro data in the current study indicates that vitamin E elevates Bcl-2 to a greater extent than α-lipoic acid and, therefore, it is possible that vitamin E was primarily responsible for the increase in endothelial cell Bcl-2 seen in vivo. We have reported previously that decreases in skeletal muscle properties seen in vivo following supplementation with both vitamin E and α-lipoic acid were primarily caused by vitamin E [31].

To our knowledge, this is the first study to report an endothelial cell-specific response to Bcl-2 with antioxidant supplementation. We have shown that endothelial Bcl-2 increases in response to dietary supplementation of vitamin E and α-lipoic acid with no change in myocardial Bcl-2. As discussed, our in vitro data suggest that in this study the vitamin E was a major cause of Bcl-2 elevation in endothelial cells and while antioxidant supplementation significantly increased vitamin E levels in endothelial cells by 26%, there was no change in myocardial vitamin E. The lack of vitamin E uptake in the myocardium was surprising, but would explain the lack of Bcl-2 response in this tissue. The level of vitamin E in the antioxidant-supplemented diet was based on the findings of Machlin and Gabriel [32] who showed that consumption of 1000 IU dl-α-tocopherol/kg diet by rats yielded plasma concentrations of vitamin E equivalent to those found in humans following the commonly recommended daily supplementation of 400 mg/d α-tocopherol.

The finding that endurance exercise training caused a significant increase in resting levels of MDA in endothelial cells is another novel finding of the present study. Acute exercise of moderate to high intensity is known to augment endothelial oxidative stress and this is thought to be due to increases in shear stress associated with an elevated cardiac output during the exercise [33]. However, exercise-trained animals in this study were sacrificed 24–48 h after their final training session to avoid any acute effect of exercise, therefore, any changes observed were training-induced. Our findings are different to Rush et al. [34] who reported decreased resting levels of MDA in aortic endothelial cells following exercise training in pigs. These conflicting results may be due to the differences in training intensities between the two studies as Rush et al. [34] utilised a training intensity of 40–50% VO₂max whereas rats in our study were exercised at 75% VO₂max. Thus, training at a higher intensity may elicit a higher resting level of oxidative stress in the endothelium.

In summary, antioxidant supplementation with vitamin E and α-lipoic acid, but not exercise training, increases endothelial cell levels of the anti-apoptotic protein Bcl-2. In vitro experiments confirmed the ability of these antioxidants to increase Bcl-2. Thus, as epidemiological studies suggest that antioxidant supplementation is ineffective and exercise is effective in the prevention of cardiovascular disease, our findings imply that exercise-induced endothelial cell protection operates by a mechanism other than the Bcl-2 pathway. In addition, there appears to be a delineation between the responses of the endothelium and myocardium to vitamin E and α-lipoic acid supplementation that warrants further investigation.

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Table 2
Effects of antioxidant supplementation and/or endurance exercise training on vitamin E and MDA in LVECs

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Antioxidant</th>
<th>Exercise trained</th>
<th>Antioxidant</th>
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</thead>
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<tr>
<td>Endothelial cells</td>
<td>(n = 23)</td>
<td>(n = 14)</td>
<td>(n = 15)</td>
<td>(n = 14)</td>
</tr>
<tr>
<td>Vitamin E (µmol/g protein)</td>
<td>5.02 ± 0.34</td>
<td>6.35 ± 0.34*</td>
<td>4.96 ± 0.40</td>
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<tr>
<td>MDA (mmol/g protein)</td>
<td>42.2 ± 1.9</td>
<td>54.8 ± 4.6*</td>
<td>54.8 ± 4.0*</td>
<td>44.2 ± 3.6</td>
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<tr>
<td>Myocardial tissue</td>
<td>(n = 7)</td>
<td>(n = 8)</td>
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<td></td>
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<tr>
<td>Vitamin E (µmol/g protein)</td>
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<td>3.65 ± 0.41</td>
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<td>–</td>
</tr>
<tr>
<td>MDA (mmol/g protein)</td>
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Values are mean ± S.E.M. * P < 0.05 compared to sedentary, control diet.
ments used in this study were a generous gift from Herron Pharmaceuticals. This study was funded by The University of Queensland.

References


