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Des-aspartate-angiotensin I attenuates ICAM-1 formation in hydrogen peroxide-treated L6 skeletal muscle cells and soleus muscle of mice subjected to eccentric exercise

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ABSTRACT

L6 skeletal muscle cells overexpressed ICAM-1 when treated with H_2O_2. Maximum effect was observed at 200 μM H_2O_2. Des-aspartate-angiotensin I (DAA-I) concentration-dependently attenuated the overexpression. Maximum attenuation occurred at 10^{-10} M DAA-I. H_2O_2 activated NFκB and its translocation into the nucleus of L6 muscle cells suggesting that NFκB mediates the H_2O_2-induced overexpression of ICAM-1. DAA-I inhibited the activation and translocation of NFκB. H_2O_2 is a major oxidant formed during skeletal muscle contraction and is implicated in oxidative stress and skeletal muscle damage in excessive unaccustomed exercise. The data show that DAA-I has antioxidant action, and its action was further investigated in the soleus muscle of mice subjected to 240 min of eccentric exercise on a rodent treadmill. The eccentric exercise induced superoxide formation and overexpression of ICAM-1 in the soleus muscle of the mice at 3 days post exercise. DAA-I (0.2 nmole/kg/day) administered orally on day 1 (pre-exercise) and 2 days post-exercise attenuated both the ROS formation and ICAM-1 overexpression. Earlier studies show that DAA-I acts as an agonist on the angiotensin AT_1 receptor and elicits responses opposing those of angiotensin II. The present and earlier findings support the recent suggestion that angiotensin II is involved in skeletal muscle damage, and curtailment of its actions via ACE inhibitors and losartan protects and improves skeletal muscle damage. These findings open up new avenues for treatment and management of skeletal muscle damage via the interventions of the renin angiotensin system.

1. Introduction

Unaccustomed and excessive strenuous exercise causes skeletal muscle damage. Both in vivo and in vitro studies indicate that reactive oxygen species (ROS) play a critical role in the damage [1–5]. Oxidative stress, structural muscle damage, and muscle inflammation (resulting from the exercise) generate excess ROS that overwhelm cellular antioxidant defenses and cause tissue damage. Paradoxically, elevated ROS are also involved in endurance exercises that impart resistance to excessive exercise-induced muscle damage. The likely cytoprotective proteins that are induced by the ROS during endurance exercise include heat shock protein 70, Mn-SOD [1], and haem oxygenase-1 [6]. These proteins curtailed the potential damage of greater amount of ROS released in subsequent excessive exercises. Hence, there are uncertainties regarding the prophylactic and acute use of antioxidants in reducing oxidative stress, decreasing muscle damage and improving exercise performance [7]. Recent studies have shown that angiotensin II is involved in skeletal muscle hypertrophy [8], and angiotensin AT_1 receptor blockade or ACE inhibition improves skeletal muscle features in myopathic states [9–12]. These studies show that angiotensin II is involved in the pathology of skeletal muscle damage, and attenuation of its action offers new interventions for skeletal muscle maladies. Des-aspartate-angiotensin I (DAA-I), a nine-aminoacid angiotensin peptide, has been shown to attenuate the early inflammatory processes in animal models of diseases in which angiotensin II has been implicated [13–17]. DAA-I has been shown to act as an agonist on the angiotensin AT_1 receptor and its actions are blocked by indomethacin indicating that prostaglandins mediate its actions [13,15–18]. The present study investigated the possible antioxidant actions of DAA-I on L6 muscle cells subjected to oxidative stress, and on soleus muscle of mice subjected to excessive eccentric exercise. As ROS are known to induce intercellular adhesion molecule-1 (ICAM-1) formation in endothelial and epithelial cells, [19,20], ICAM-1 was investigated as a mediator of inflammation in the present study. Ischemia-induced injury caused vascular endothelial cells to express ICAM-1, which recruited leucocytes [21]. The recruited leucocytes extravasated and caused inflammation including the expression of ICAM-I [21]. The role of ICAM-1 in skeletal muscle damage arising from lengthening contraction is less known.

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2. Methods

2.1. Cell culture

L6 skeletal muscle cells (CRL-1458) were purchased from American Type Culture Collection (ATCC) Maryland, USA. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; ATCC, 30–2002) containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum. The cells were grown to confluence in 25 cm^2 flasks (Nunc, EW-01930-48).

2.2. H_2O_2 incubation and ICAM-1 determination

L6 skeletal muscle cells were incubated in culture medium containing various concentrations of H_2O_2 (50–1600 μM). Cells were then prepared for ICAM-1 determination as described previously [22]. Briefly, cells from each sample were washed, trypsinized, transferred to 2-ml microtubes and treated with anti-ICAM-1 antibody (sc-1151-R, Santa Cruz, CA) for 45 min at ambient temperature. After washing, the cells were further incubated with FITC-conjugated secondary antibody (sc-2090, Santa Cruz, CA). Cells were similarly washed to remove unbound secondary antibody. The cells were counted using a hemocytometer, and diluted with PBS to a concentration of 10^6 cells per ml, and cell fluorescence (at 494 nm excitation and 518 nm emission) quantified by flow cytometry (Beckman CyAn/538R, Beckman Coulter, London, UK). The data were analyzed using the instrument software (SUMMIT 4.3.02). The effects of DAA-1 on the H_2O_2-treated L6 muscle cells were studied by incubating the muscle cells with various concentrations of DAA-I for 16 h prior to and during H_2O_2 incubation.

2.3. Nuclear extraction and Western blot assay

L6 muscle cells were incubated in culture medium containing 200 μM H_2O_2 for various time duration (1, 2, and 3 h). The cells were collected and subjected to nuclear extraction. Briefly, the cells were dissolved in a lysis buffer containing 10 mM HEPES/NaOH (pH 7.9), 1 mM MgCl2, 10 mM KCl, 1 mM dithiothreitol (DTT) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and set on ice for 10 min. The lysate was centrifuged at 10,000 ×*g* for 15 s, and the nucleus-containing pellet was reconstituted in a buffer containing 10 mM HEPES/NaOH (pH 7.9), 25% glycerol, 5 mM NaCl, 1 mM MgCl2, 0.5 mM EDTA, 1 mM DTT and 0.1 mM PMSF. The nucleus fraction was then put on the ice for 15 min, and centrifuged at 10,000 ×*g* for 5 min. The supernatant was collected and was stored at −70 °C. Activated NF-κB that had translocated into the nucleus was determined by Western blot. Each isolated nuclear fraction (40 μg) was resolved by SDS-PAGE and probed with anti-NF-κB antibody (sc-109, Santa Cruz, CA), anti-lamin a/c antibody (sc-20681, Santa Cruz, CA), anti-β-actin antibody (sc-130656, Santa Cruz, CA) and anti-rabbit secondary antibody (sc-2004, Santa Cruz, CA). The specific nuclear protein lamin a/c was assayed to determine the purity of the isolated nuclear fraction, and cytoplasmic protein β-actin to determine the contamination by cytoplasmic protein.

2.4. Animals and DAA-I

Ten to 12-week-old male C57BL/6J mice (22–24 g) were purchased from the Laboratory of Animal Care, National University of Singapore. All animal procedures were carried out in accordance with Institutional Protocols and Guidelines approved by the National University of Singapore and the Institutional Animal Use and Care Committee (Reference No. IACUC 010/10).

DAA-I was purchased from Bachem AG (Bubendorf, Switzerland). 1-ml aliquots of stock solution (1 mM) were prepared and kept frozen at −20 °C. Working solutions were prepared from the stock solution and filtered (with a 22-μm filter) on the day of use.

2.5. Eccentric treadmill running

The protocol was adapted from the experiment described by Armand et al. [23]. Briefly, 20 mice were trained to run down an incline of 14° on a motor-driven rodent treadmill at 6 m/min for 1 h on day 1, and 9 m/min for 1 h on day 2. On the third day, 16 of the trained mice were selected based on their ability to run continuously during the training sessions without electric shock prodding. The selected mice were divided into two groups of 8 mice. Mice in one group were orally administered 0.2 nmole/kg DAA-I in 0.1 ml water (treated group). This was done by preparing three solutions containing 0.2 nmole/kg DAA-I in 0.1 ml for mice of 22, 23, and 24 g weight, respectively. Mice in the other group were similarly administered 0.1 ml water (control group). Following the administration of either DAA-I or water, the animals were then made to run down the same incline for a total of 240 min. The speed and duration during the 240 min run were 6 m/min for 40 min, 10 m/min for 30 min, 15 m/min for 110 min, and 12 m/min for 60 min. After the exercise, the mice were returned to their cage and were similarly administered with DAA-I or water on day 1 and day 2 post-exercise. On the third day, the mice were sacrificed by cervical dislocation and the soleus muscles were removed and immediately frozen in liquid nitrogen and stored at −70 °C. Sixteen untrained mice of matched weight were also sacrificed and the soleus muscles were similarly frozen.

With the two days of training, all mice were able to complete the eccentric exercise on the third day, which ensured that the animals had the same amount of injurious exercise. Preliminary experiment with an early batch of 4 mice showed that the two-day training session did not cause any significant increase in superoxide level in the soleus muscle of these mice as compared to untrained mice (data not shown), and was unlikely to cause adaptation. Adaptation had been reported to occur when mice ran at a speed of 13 m/min for 60 min per day for

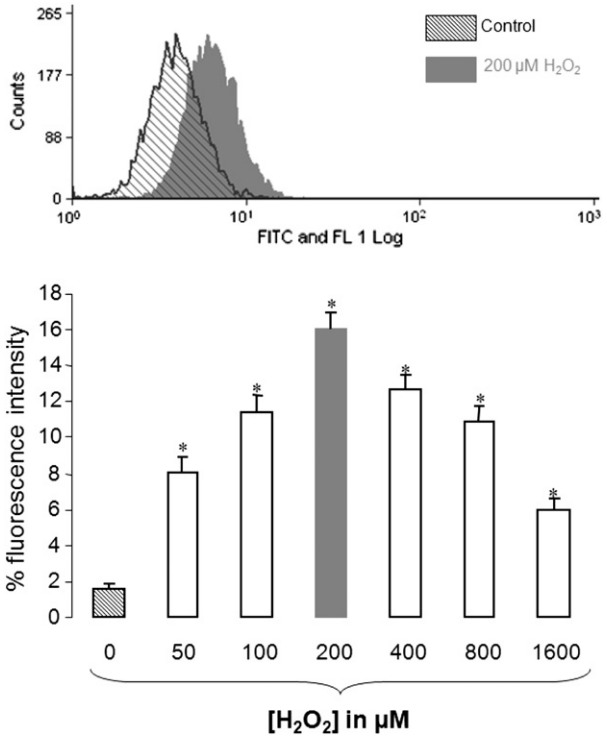

Fig. 1. Effect of H_2O_2 on ICAM-1 expression in L6 skeletal muscle cells. L6 muscle cells were exposed to various concentration of H_2O_2 (50–1600 μM) for 4 h, and data were collected from at least 10,000 gated viable cells by flow cytometry. Upper panel: Tracing of flow cytometric fluorescence of L6 muscle cells in the absence (Control, left tracing) and presence (right tracing) of 200 μM H_2O_2. Lower panel: Histograms of ICAM-1 fluorescence cells. Each value is the mean ± SEM. of three separate experiments. * Significantly different from the control value ($p < 0.05$, one-way ANOVA, post hoc Tukey's test).

4 weeks [24] and at a speed of 12–17 cm/s for 75–135 min per day for 9 days [25].

2.6. In situ detection of superoxide

The study was carried out as described earlier [16]. Briefly, each frozen soleus muscle was mounted with Tissue Freezing Medium (Leica, Germany) onto a specimen block. The mounted samples were allowed to cool in a cryostat (Leica CM 3050S) for 10 min at −28 °C, before cutting into 20 μm-thick sections and picked up onto gelatin-coated slides. The slides were warmed in room temperature for 30 min and fixed in cold acetone for 2 min. After fixation, the slides were washed with PBS and allowed to air-dry in room temperature for 1 h. Each sample was incubated with 2 μmol/L dihydroethidium solution (Biotium, CA) for 30 min at 37 °C. Dihydroethidium is oxidized in the presence of superoxide to form ethidium, which binds specifically to DNA in the nucleus and emits red fluorescence. In situ detections of superoxide in the muscle tissues of the treated animals were then carried out using fluorescence microscope and the intensity was quantified using Imaging software (Image Processor, New York University).

2.7. Western blot determination of ICAM-1

Each pair of soleus muscle (~0.2 g) was minced on an ice pack and homogenized in 10 volume (w/v) of CellLytic MT mammalian tissue lysis reagent and 1% protease inhibitor cocktail (Sigma Chemical, St. Louis, MO) in a Potter-Elvehjem homogenizer immersed in crushed ice for 1 min. The homogenate was incubated on ice for 50 min, and centrifuged at 10,000 ×*g* for 10 min. The supernatant was stored at −70 °C till used. Protein concentration of the muscle supernatant was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Samples of muscle supernatant (0.2 mg protein) were resolved by electrophoresis in 10% SDS-PAGE polyacrylamide gel, for 120 min at 100 V, before transfer to nitrocellulose polyvinylidene difluoride (PVDF) membranes (Sigma Chemical, St. Louis, MO). Membranes were then blocked with 5% BSA at room temperature for 1 h, before incubation with mouse monoclonal ICAM-1 antibody (Sigma Chemical, St Louis, MO) in a 1:200 dilution with 5% BSA solution overnight, at 4 °C. For negative control, membranes were incubated with PBS instead of monoclonal ICAM-1 antibody. Membranes were then rinsed in three changes of 0.1% Tween in TBS (TBS-T), each step lasting 10 min. Membranes were incubated with horseradish peroxidase conjugated donkey anti-mouse secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) in a 1:10,000 dilution in TBS, for 1 h at room temperature before rinsing in 3 changes of TBS-T, each step lasting 10 min. Signal was developed by direct exposure of the PVDF membrane in 200 μl of Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) for 1 min in a gel imager (Gel Doc Ez, Bio Rad Laboratories).

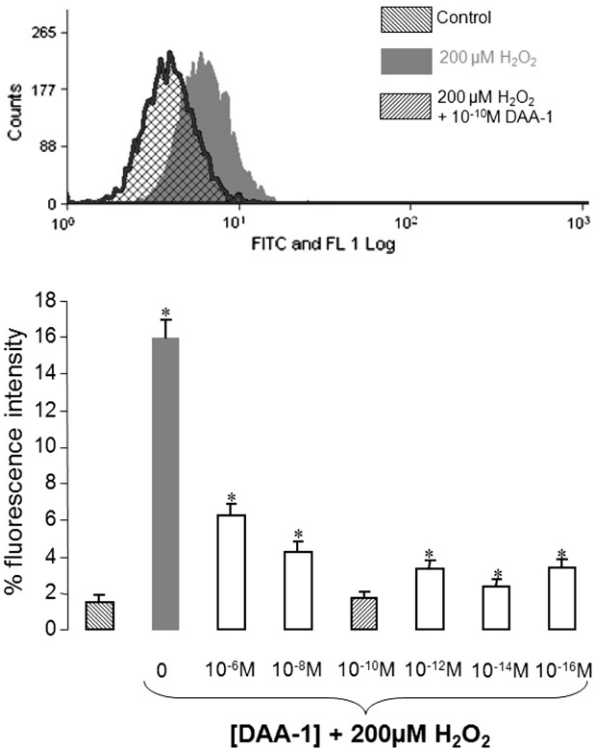

Fig. 2. Effects of DAA-1 on H_2O_2-induced overexpression of ICAM-1 in L6 skeletal muscle cells. Upper panel: Tracing of flow cytometric fluorescence of L6 muscle cells. The tracings for the Control, and cells treated with of 200 μM H_2O_2 were copied from Fig. 1. Left-hatched tracing was from H_2O_2-treated cells in the presence of 10^{-10} M DAA-I. Lower panel: Histograms of ICAM-1 fluorescence cells. Each value is the mean ± SEM. of three separate experiments. * Significantly different from the value of H_2O_2-treated cells ($p < 0.05$, one-way ANOVA, post hoc Tukey's test).

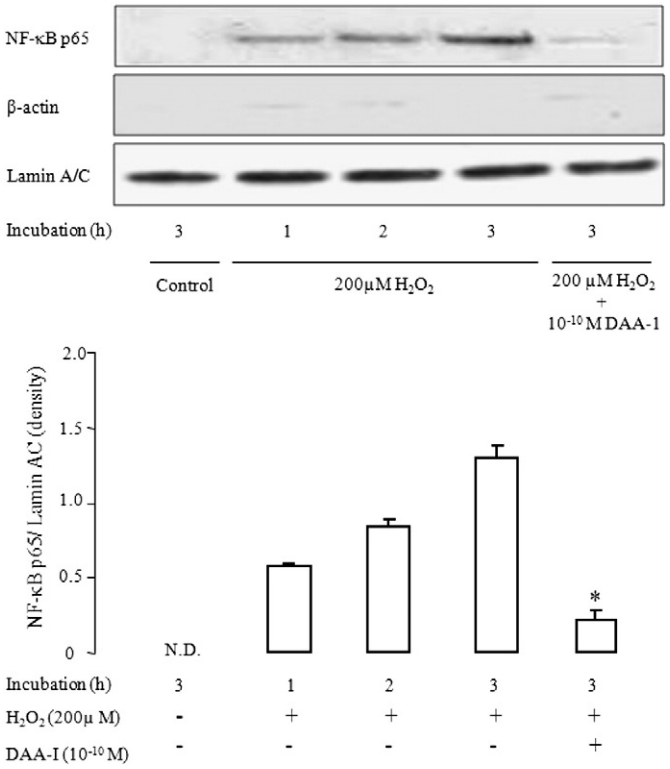

Fig. 3. Effects of DAA-1 on H_2O_2-induced activation and translation of NF-κB to the nucleus in L6 skeletal muscle cells. Upper panel: representative Western blot of activated NF-κB protein, β-actin, and lamin a/c. Lower panel: Ratio of densitometric reading of NF-κB protein/lamin a/c. each value is the mean ± SEM. of three separate experiments. * Significantly different from the corresponding value, cells incubated with H_2O_2 for 3 h ($p < 0.05$. one way ANOVA, post hoc Tukey's test).

2.8. Statistics

Data were expressed as means ± SEM. Vertical error bars in figures denote the SEM. Statistical analysis for significant differences between groups was performed using analysis of variance (ANOVA), with post-hoc Tukey's test. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. DAA-I attenuates ICAM-1 formation in H_2O_2-treated L6 muscle cells

Preliminary experiments showed that, within the concentration range used in Figs. 1 and 2, both H_2O_2 and DAA-I had no effect on the

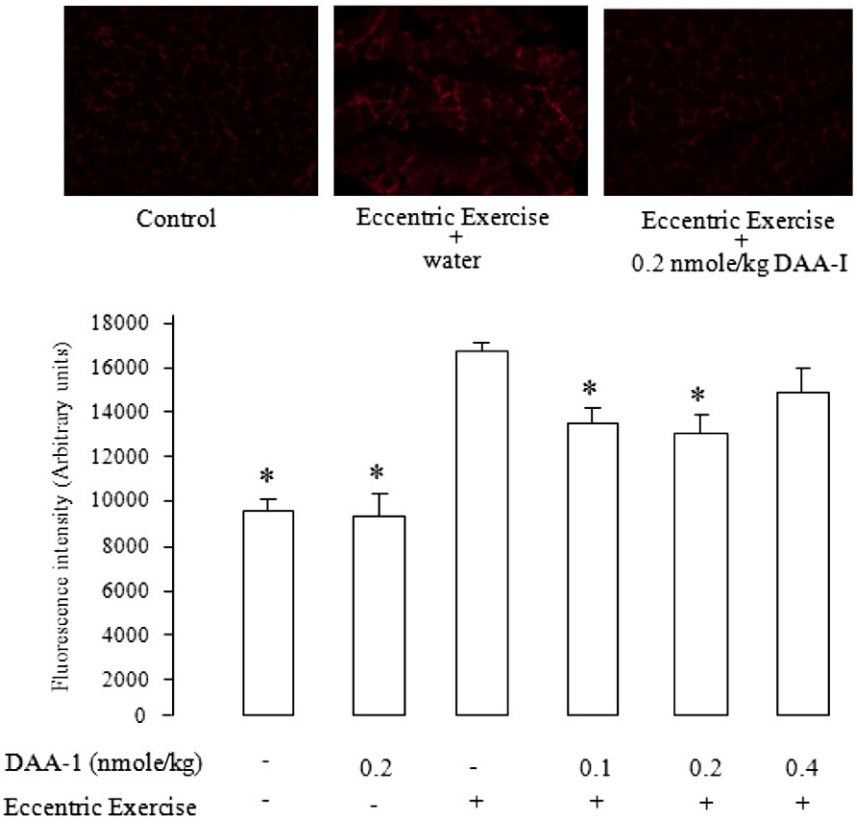

Fig. 4. Effect of DAA-I on ROS production in skeletal muscle of mice subjected to eccentric exercise. Upper panel: Ethidium fluorescence of skeletal muscle cryosections. Lower panel: Fluorescence densitometry of cryosections. Each value represents the mean ± SEM. of 4 individual animals. * Significantly different from the value of eccentric exercised-mice that were treated with vehicle ($p < 0.05$. one way ANOVA, post hoc Tukey's test).

viability of L6 muscle cells (data not shown). H_2O_2 induced overexpression of ICAM-1 in L6 muscle cells. The induction was concentration dependent and maximum effect was observed with a concentration of 200 μM H_2O_2 (Fig. 1). DAA-I attenuated the overexpression of ICAM-1 in L6 muscle cells treated with 200 μM H_2O_2. Maximum attenuation was observed at 10^{-10} M DAA-I, and significant attenuation was seen with concentration as low as 10^{-16} M (Fig. 2). The action of DAA-I at such low concentrations was also seen in earlier in vitro studies [26,27], and could be due to the possibility that circulating DAA-I (present in pmolar range; [28]) was active physiologically. The tissue level of active DAA-I could be much lower than the circulating level.

The procedure used in nuclear extraction yielded a nuclear fraction that had negligible cytoplasmic contamination (Fig. 3). The activation and translocation of NF-κB to the nucleus occurred in L6 skeletal muscle cells treated with 200 μM H_2O_2, and 10^{-10} M DAA-I significantly attenuated both processes (Fig. 3).

3.2. DAA-I attenuates eccentric exercise-induced superoxide and ICAM-1 formation in skeletal muscles

Eccentric exercise of 240-min duration generated superoxide formation in soleus muscle of the mouse. The formation was attenuated by DAA-I, and an oral dose of 0.2 nmole/kg produced the maximum effect (Fig. 4). Soleus muscle also over-expressed ICAM-1 protein following similar eccentric exercise, and an oral dose of 0.2 nmole/kg significantly attenuated the over-expression (Fig. 5). Constitutively expressed ICAM-1 in skeletal muscle was barely detectable by the Western blot employed in this study. Similar observations have also been reported by other investigators using different protocols [29,30].

4. Discussion

In the present study, the treatment of L6 muscle cell with H_2O_2,a major ROS in skeletal muscle [31], caused overexpression of ICAM-1, an early inflammatory protein involved in the recruitment of leucocytes. The concurrent activation and translocation of NF-κB to the nucleus

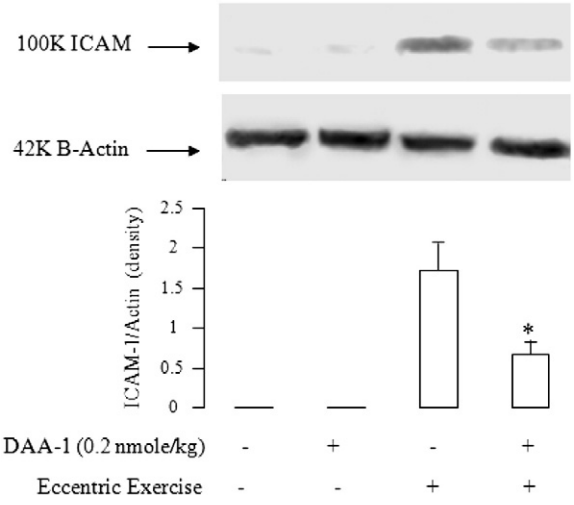

Fig. 5. Effects of DAA-I on the expression of ICAM-1 protein in skeletal muscle of mice subjected to eccentric exercise. Upper panel: Representative Western blots of ICAM-1 protein. Lower panel: Density ratio of ICAM-1/actin. Each value represents the mean ± SEM. of four animals. * Significantly different from the value of eccentric exercised-mice that were treated with vehicle ($p < 0.05$, one way ANOVA, post hoc Tukey's test).

were also observed, and this supports the contention that NF-κB is a major nuclear transcriptional activator that mediates the action of ROS [32], and the overexpression of ICAM-1 observed in the L6 muscles cells. Similar findings on these two actions of H_2O_2 have also been reported [4,5]. DAA-I inhibited both the translocation of NF-κB to the nucleus and overexpression of ICAM-1 indicating that the nonapeptide could be an effective antioxidant in combating oxidative stress in skeletal muscles. This possibility was investigated in mice that had undergone excessive eccentric exercise on a rodent treadmill.

The protocol of the eccentric exercise was designed to induce increase in superoxide (precursor of ROS) production and overexpression of ICAM-1 in the soleus muscle of the mice. Preliminary experiments showed that maximum production of superoxide and ICAM-1 occurred on the third day after eccentric exercise. This duration is in accordant with findings showing that initial mechanical injury in skeletal muscles initiates a cascade of events that lead to more severe late onset injury that peaks in rodents at approximately day 3 post exercise [33–36]. DAA-I attenuated both the superoxide production and overexpression of ICAM-I in soleus muscle at day 3 post exercise. This finding is a first demonstration of an antioxidant and inflammatory action DAA-I in late onset skeletal muscle damage arising from eccentric exercise. Antioxidant like polyethylene glycol-superoxide dismutase has also been shown to protect mouse muscle from late onset injury caused by plyometric contractions [34]. In vitro L6 muscle cell experiments show that DAA-I directly attenuates the action of ROS, and this could also occur in the soleus muscle resulting in the observed reduced formation of ICAM-1. DAA-I has been shown to attenuate the upregulation of gp91 subunit of NADPH oxidase in skeletal muscle of hyperglycemic mice, and lung of mice that were exposed to half sulfur mustard intoxication resulting in the reduction of ROS formation in these two tissues [16,37]. It is also likely that DAA-I could act to curtail the upregulation of NADPH oxidase as membrane-bound enzymes in skeletal muscles have been suggested to generate superoxide in contractile activity-induced oxidative stress [38]. ICAM-1 appears to be an important molecule involved in skeletal muscle injury and disease. Ischemia-induced injury caused vascular endothelial cells in mouse skeletal muscle to express ICAM-1, which recruited leucocytes. The recruited leucocytes transmigrated and caused inflammation including the expression of ICAM-I in skeletal muscle cells [39]. Acute prolonged exercise caused delayed-onset skeletal muscle damage in the rat and this is related to inflammation via phagocyte infiltration caused by ROS [4]. However, there is a paucity of information on the role of ICAM-1 in skeletal muscle damage arising from lengthening contraction; and avenues of therapeutic interventions on this molecule await further investigation.

As shown in Figs. 2 and 4, the action of DAA-I was less effective at concentrations higher than the maximal effective concentration, but this profile was also characteristic of other DAA-I activities [15–17,22,40]. Although the exact mechanism for this biphasic phenomenon is not known, it could be a hormetic response [41] that involves receptor down-regulation by different mechanisms at high doses of DAA-I [42–44]. DAA-I being an endogenous angiotensin could also, at higher doses, bind to the classical angiotensin II inositol triphosphate-generating AT_1 receptor and annul its more specific action mediated by the indomethacin-sensitive receptor. The possibility is supported by earlier findings showing that DAA-I produced differential responses in adjacent sections of the rabbit pulmonary artery. DAA-I produced contraction in the pulmonary end of the artery and relaxation in the cardiac end of the artery. The contraction was inhibited by losartan but not indomethacin, while the relaxation was inhibited by both losartan and indomethacin [18].

Evidence for the involvement of angiotensin II in skeletal muscle damage comes from the study by Bedair et al. [45]. They showed that injured skeletal muscles had more angiotensin AT_1 receptors than normal muscles, and losartan significantly reduced fibrosis and increased the number of regenerating myofibers in acutely injured skeletal muscles of the mouse. Other studies showed that ACE inhibitors improved muscular performance and decreased rate of muscle wasting in hypertensive patients [46], and enalapril was able to blunt angiotensin II-dependent activation of pro-inflammatory and pro-oxidant pathways in exercised dystrophic mdx mice [12]. Both losartan and ACE inhibitors curtail that activity of angiotensin II, albeit by different mechanisms, the former by blocking the angiotensin AT_1 receptor and the latter by inhibiting the formation of angiotensin II from angiotensin I. Earlier studies show that DAA-I acts as an agonist on the angiotensin AT_1 receptor and exerts actions opposing those of angiotensin II [27,47,48]; and ameliorates symptoms in diseases in which angiotensin II is implicated [13–15,17,37]. DAA-I together with losartan (and possibly other angiotensin receptor blockers) and ACE inhibitors could be considered a new class of drug that protects or ameliorates skeletal muscle damage by curtailing the actions of angiotensin II. They offer potential new approaches and open up new avenues of research in sport injuries and muscle deterioration in the elderly.

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