·Original Paper·

Effect of downhill exercise on sarcoplasmic reticulum function in rat skeletal muscle

CHEN Wan[1] Ruell PA[2] Thompson MW[2]

Abstract **Objective:** To investigate the effect of downhill (eccentric) exercise (ECE) on sarcoplasmic reticulum (SR) Ca^{2+}–ATPase activity, Ca^{2+} uptake and release in rat skeletal muscle, in terms of both magnitude and time course. In addition, ionophore stimulation was determined to assess vesicle integrity by measuring the ratio of calcium–dependent ATPase activities in the presence and absence of A23187. **Method:** Adult male SD rats were randomly assigned to control and ECE groups. The ECE rats were sacrificed at the 0th, 4th, 24th, 48th, 72nd and 144th h following ECE (n=7). The ECE protocol consisted of 90min continuous downhill exercise (–16 deg; 15m·min^{-1}). Red vastus muscles were sampled separately for each group and muscle homogenates were prepared. The rates of SR Ca^{2+}–ATPase activity, Ca^{2+} uptake and release were measured in vitro. **Result:** SR Ca^{2+} uptake was significantly lower (P<0.05) compared with control values [19.25±1.38 nmol·min^{-1}·(mg protein)$^{-1}$], by 29% and 36% immediately and 4h after ECE, respectively, and remained depressed (P<0.05) 24h following ECE. SR Ca^{2+} release was also significantly lower (P<0.05) compared with control values [31.06±2.36nmol·min^{-1}·(mg protein)$^{-1}$], by 37% and 39% immediately and 4h after ECE, respectively, and remained depressed (P<0.05) 24h following ECE. SR Ca^{2+}–ATPase activity measured with ionophore was 31% lower (P<0.05) 4h after ECE, and remained lower (P<0.05) 24h following ECE. The ratio of Ca^{2+}–ATPase activities in the presence and absence of A23187 was not significantly changed following ECE, indicating that membrane integrity was not altered by the exercise. **Conclusion:** The present results suggest that a bout of low–intensity, prolonged downhill exercise results in a long–lasting depression of SR function that is not fully restored after two days of recovery, which may underlie some muscle functional impairments induced by ECE. These changes could be the results of stress from sarcomere length inhomogeneities during eccentric contractions.

Author's address Department of Fundamental Theories, Shandong Sport University, Jinan, 250063

Key words eccentric exercise; sarcoplasmic reticulum; Ca^{2+}–ATPase activity; Ca^{2+} uptake; Ca^{2+} release

下坡运动对大鼠骨骼肌肌浆网功能的影响/陈万，Ruell PA，Thompson MW//中国康复医学杂志，2007，22(12)：1070—1074

摘要 **目的**:观测研究下坡(离心)运动对大鼠骨骼肌肌浆网 Ca^{2+}–ATP 酶活性,Ca^{2+}摄取与释放在量与时程上的影响。此外,测定离子载体的刺激作用,即测定在含与不含(Ca^{2+}离子载体)A23187 时Ca^{2+}–ATP 酶活性的比值,用以评定囊泡的完整性。**方法**:成年雄性 SD 大鼠随机分为对照与离心运动组, 离心运动的大鼠分别于运动后即刻, 4, 24, 48, 72 和 144h 后取样 (n=7). 离心运动方式采用 90min 持续跑台下坡运动(–16°;15m/min)。取大鼠红股肌制备组织匀浆, 测定肌浆网Ca^{2+}–ATP 酶活性,Ca^{2+}摄取与释放。**结果**:与对照组[19.25±1.38 nmol ·min^{-1}·(mg protein)$^{-1}$]相比, 肌浆网Ca^{2+}摄取分别于运动后即刻和 4h 下降了 29% and 36% (P<0.05), 24h 依然降低(P<0.05). 肌浆网 Ca^{2+}释放与对照组[31.06±2.36 nmol·min^{-1}·(mg protein)$^{-1}$] 相比,也分别于运动后即刻和 4h 下降了 37% and 39% (P<0.05), 24h 持续降低(P<0.05). 用含离子载体测定的肌浆网 Ca^{2+}–ATP 酶活性运动后 4h 降低了 31%(P<0.05), 并于运动后 24h 仍然降低 (P<0.05)。运动后, 含与不含 A23187 时测定的 Ca^{2+}–ATP 酶活性的比值未见显著性改变, 表明该运动没有明显改变肌浆网膜的完整性。**结论**:一次性低强度,长时间下坡运动导致肌浆网功能长时间降低, 运动后恢复期两天尚未完全恢复, 亦可构成离心运动诱导的骨骼肌某些功能降低的基础。提示这些变化可能产生于离心收缩时肌节长度不匀一性所造成的张力应激。

关键词 离心运动; 肌浆网; Ca^{2+}–ATP 酶活性; Ca^{2+}摄取; Ca^{2+}释放

中图分类号: R493 **文献标识码**:A **文章编号**:1001–1242(2007)–12–1070–05

The studies of sarcoplasmic reticulum (SR) function in skeletal muscle damaged by eccentric exercise (ECE) can yield information on the mechanism of excitation–contraction coupling failure. However, such studies are few and yield

1 Department of Fundamental Theories, Shandong Sport University, Jinan, 250063, China

2 School of Exercise & Sport Science, Faculty of Health Sciences, The University of Sydney, Sydney1825NSW, Australia

conflicting results. Yasuda et al.[1] have demonstrated that SR membrane integrity is altered in rat skeletal muscles following eccentric contractions induced by percutaneous electrical stimulation with no changes in muscle SR Ca^{2+}–ATPase pump function. Whereas, in a human study that examined changes in SR Ca^{2+} uptake and Ca^{2+}–ATPase activity in exercised vastus lateralis with 60 min of eccentric cycling exercise [2], SR Ca^{2+} uptake was depressed during the recovery period but not immediately following the exercise, the SR Ca^{2+}–ATPase activity showed a biphasic response. In contrast, no change in SR function was found during the first 24h following ECE in young men even though there were significant changes in muscle contractility [3]. There are no studies that have investigated both the magnitude and time course of changes in SR function including Ca^{2+} uptake, Ca^{2+} release and Ca^{2+}–ATPase over a longer time course. Therefore, there is a clear need for further research in this area.

1 MATERIAL AND METHODS

1.1 Animal care and exercise protocol

Adult male Sprague–Dawley rats were used in this experiment. The rats were placed in standard rat cages (2—3/cage) in a light (light/dark cycle was altered opposite to day/night) and temperature(21—22°C) controlled environment. A 7–day acclimatization period was allowed during which ad libitum standard rat chow and water were offered. At time of sacrifice for the experiments the animals weighed 395±5.9g (mean±SEM). Animal holding facilities and all experimental protocols were approved by the University of Sydney Animal Ethics Committee. All rats were randomly assigned to control group and ECE group. The ECE rats were sacrificed at the 0, 4th, 24th, 48th, 72nd and 144th h following ECE (n=7). The ECE protocol consisted of 90min continuous downhill exercise (–16deg; 15 $m \cdot min^{-1}$). Rat rectal temperature was measured by inserting a thermometer probe into rectum to a depth of approximately 5cm on sedentary control rats and those of immediate postexercise after injection of anesthetic.

1.2 Sample preparation

The rats were anaesthetized by intraperitoneal injection of ketamine/xylazine at a dose of 104.3/15.6mg per kg body weight, and then humanely killed by excision of heart. Red parts of m. vastus were immediately removed from both hind limbs and immediately placed on a petri dish with ice–cold physiological saline. After dissection, the muscle sample was freed from fat and connective tissue.A mass of 70—80 mg muscle were weighed, diluted 1:10 (wt/vol) in cold homogenizing buffer (40 mM Tris, 0.3M sucrose, pH 7.9) and then homogenized on ice at 18,000 rpm for 3×15 s with 15s rests (Omni 2000, USA). This process results in a suspension of fragmented SR membranes which resealed into numerous small vesicles, allowing direct measurements of the rates of Ca^{2+}–uptake and Ca^{2+}–release, and Ca^{2+}–ATPase activity[4]. The homogenizing procedure for each sample was completed within 5 minutes and the suspension was kept cool by packing the sample in ice. The homogenate was then rapidly frozen in liquid nitrogen for later analyses. On any given day, seven samples from each group were analyzed together. All measurements were completed within 50min after thawing of the sample. Muscle homogenate protein content was determined in triplicate according to the methods of Markwell et al.[5].

1.3 Determination of the Ca^{2+}–ATPase activity

The assays were performed at 37℃ using a spectrophotometer (UV–1601PC; Shimadzu, Japan) at 340 nm as previously described [4]. The assay buffer consisted of 18 mM HEPES buffer, pH 7.5, 180 mM KCl, 13mM $MgCl_2$, 1 mM EGTA, 9mM NaN_3, 0.3mM NADH, 9mM phosphoenolpyruvate, 22 U/ml lactate dehydrogenase, 16 U/ml pyruvate kinase and 4 mM ATP. When the intactness of SR vesicles was assessed, the assay mixture additionally contained 2.6μM Ca^{2+} ionophore A23187 (Boehringer Mannheim, Germany). Ionophore stimulation was determined to evaluate vesicle integrity by calculating the ratio of Ca^{2+}–ATPase activities in the presence and absence of A23187. Muscle homogenate (10 μl) was added to 1ml assay buffer and the reaction was initiated with the addition of 1.26 mM $CaCl_2$. This concentration was determined from a pilot experiment, in which the calcium concentration producing the highest total activity was determined. The basal ATPase activity was then measured after the addition of 15 μl of 2 M $CaCl_2$, giving a final concentration of 26.8 mM $CaCl_2$, which completely inhibited the Ca^{2+}–ATPase activity. The SR Ca^{2+}–ATPase activity was determined from the total minus basal activities.

1.4 Determination of the Ca^{2+} uptake and Ca^{2+} release

The rates of oxalate–supported Ca^{2+} uptake and silver nitrate ($AgNO_3$) induced Ca^{2+} release were analyzed at 37℃ using the Ca^{2+}–fluorescent dye Indo–1 on a dual–emission luminescence spectrofluorometer (Series 2, Aminco Bowman, SLM Instruments, USA) in a reaction medium (2.2 ml) that consisted of 20 mM Hepes, 150 mM KCl, 10 mM NaN_3, 6.8 mM oxalate, 4.5 mM MgATP, 5 μM TPEN, and 1 μM Indo–1, pH 7.0[4]. Addition of extra $CaCl_2$ was not necessary as the Ca^{2+} in the assay buffers gave a starting $[Ca^{2+}]_f$ of approximately 1 μM. The excitation wavelength was 349 nm and the emission wavelength alternated between 410 nm and 485 nm (for Ca^{2+}–bound and Ca^{2+}–free Indo–1, respectively). Excitation and emission band–pass widths were set at 1 and 8 nm, respectively. Once the homogenate was added,

ratiometric data was collected every 1 s for the next 100 s. The decrease in $[Ca^{2+}]_f$ due to uptaking by SR was determined from the ratio of emission signals at 410 and 485 nm according to the equation of reference [6]. A Ca^{2+}–Indo–1 dissociation constant of 170 nM was used [4]. The maximal rate of Ca^{2+} release was determined by the addition of $AgNO_3$ (141 μM) once $[Ca^{2+}]_f$ had declined to a plateau, approximately 100 s after homogenate addition (Figure 1), as described previously [4]. Minimum and maximum ratios were determined at the completion of assay by the addition of EGTA (3.5 mM) and $CaCl_2$ (3.8 mM), respectively. Maximal rates of Ca^{2+} uptake and release were calculated as described previously[4]. Ca^{2+}–ATPase activity, Ca^{2+} uptake and release were corrected for protein content and expressed as nmoles per minute per milligram of muscle protein.

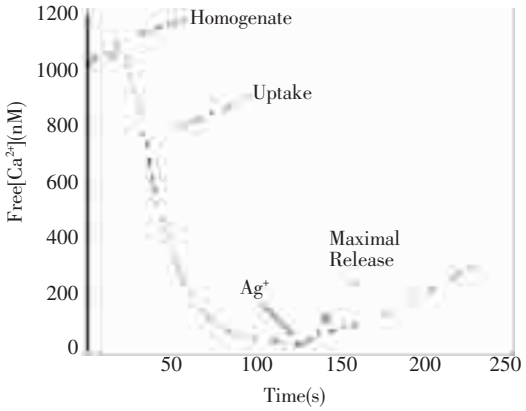

Figure 1 A typical graph showing Ca^{2+} fluxes over time from which the maximal rates of Ca^{2+} uptake and release from SR were measured with Indo–1.

1.5 Statistical analysis

A one–way analysis of variance and a Duncan′s post–hoc analysis were used to calculate the difference between individual groups. Significance of the difference of rat rectal temperature between control group and immediately post–exercise group was assessed through the Independent–Samples *t*–test. For all comparisons, the level of significance was set at $P<0.05$. All statistics were calculated using the SPSS(11.0) Data are expressed as means±SEM(Standard error of the mean)unless otherwise indicated.

2 Results

The seven groups of rats used in this study were control rats and rats from six groups sacrificed at immediately, the 4, 24th, 48th, 72nd and 144th h following the downhill exercise. There was no significant difference in body weight between any of the groups as shown in Table 1. In addition, no difference was observed in protein content of muscle samples of red vastus muscles before and after ECE (Table 1). The rectal temperature of rats immediately after ECE bout increased by 1.77 ℃ compared with that of control rats (39.39±0.12 ℃ vs. 37.62±0.08 ℃, n=7, $P<0.01$).

2.1 SR Ca^{2+} uptake and release

In rat red vastus muscle, the rate of SR Ca^{2+} uptake for whole muscle homogenate from control animals was 19.25±1.38 nmol min^{-1} (mg $protein^{-1}$). The Ca^{2+} uptake rate was significantly($P<0.05$) depressed by 29% immediately after eccentric exercise, further reduced ($P<0.05$) by 36% at the 4h post exercise, and remained depressed ($P<0.05$) by 20% at 24th h post–exercise (Table 2). The rate of Ca^{2+} release from SR for whole muscle homogenate from control animals was 31.06±2.36 nmol min^{-1} (mg $protein^{-1}$). The Ca^{2+} release rate followed a similar profile in exercise and recovery to Ca^{2+} uptake rate. Similar to Ca^{2+} uptake rate, Ca^{2+} release rate was decreased ($P<0.05$) by 37 % immediately after exercise, further reduced ($P<0.05$) by 39% at the 4th h post exercise, and remained depressed ($P<0.05$) by 26% at the 24th h post–exercise (Table 2).

2.2 SR Ca^{2+}–ATPase activity

Ca^{2+}–ATPase activities, measured in homogenates with the ionophore A23187, were found to be decreased by 31% and 24% at the 4th h and 24th h post –exercise, respectively ($P<0.05$) (Table 3). When measured in the absence of ionophore, Ca^{2+}–ATPase activity in red vastus decreased at the 4th h post –exercise only. In these experiments, there was no change detected in basal activity over the recovery period after exercise (Table 3). To determine whether SR membrane integrity was altered by eccentric exercise in this study, Ca^{2+}–ATPase activity was assessed with and without the Ca^{2+} ionophore A23187. The ratio of Ca^{2+}–ATPase activity with the ionophore to that

Table 1 Rat body weight and muscle protein content in red vastus muscles from rats following downhill exercise ($\bar{x}\pm SEM$,n=7)

	Control	0h	4h	24h	48h	72h	144h
Body Weight (g)	400.9±16.1	413.7±20.6	399.9±11.8	374.1±20.4	399.7±6.7	381.6±10.3	396.1±19.1
Muscle Protein	214.1±4.9	226.2±8.0	214.6±8.5	212.6±9.4	207.8±9.0	212.8±4.6	200.3±6.7

There was no difference($P>0.05$) between groups. 0h=immediately following exercise.Muscle protein contents are given in mg protein($g\cdot muscle^{-1}$)

Table 2 SR Ca^{2+} uptake and release for muscle homogenates in red vastus muscles from rats following downhill exercise ($\bar{x}\pm SEM$,n=7)

	Control	0h	4h	24h	48h	72h	144h
Ca^{2+} uptake	19.25±1.38	13.59±1.23①	12.23±1.3①	15.55±0.69①	16.74±1.23	18.94±1.03	20.35±1.3
Ca^{2+} release	31.06±2.36	19.49±1.88①	18.72±2.87①	23.24±1.98①	27.08±2.49	33.49±3.35	35.4±2.69

Values are in nmol min^{-1} (mg $protein^{-1}$).0h = immediately following exercise.①$P<0.05$(exercise vs. control).

Table 3 SR Ca^{2+}–ATPase, basal and total ATPase activities in presence and absence of Ca^{2+} ionophore A23187 for muscle homogenates in red vastus muscles from rats following downhill exercise ($\bar{x}\pm SEM$,n=7)

Group	(+ Ionophore)			(– Ionophore)			Ratio (+/–)
	Ca^{2+}–ATPase	Basal–ATPase	Total–ATPase	Ca^{2+}–ATPase	Basal–ATPase	Total–ATPase	
Control	206.5±24.6	13.6±3.6	220.1±25.5	92.4±5.6	16.1±2.9	108.5±7.5	2.2±0.2
0h	174.1±17.4	14.9±4.7	189.0±16.2	91.7±7.2	15.0±1.7	106.6±7.5	1.9±0.2
4h	141.2±15.9①	13.7±3.0	154.9±15.3①	73.0±6.4①	15.5±3.8	88.5±5.6	1.9±0.2
24h	156.1±3.0①	15.4±3.5	171.5±4.0	78.0±4.0	13.2±2.9	91.2±3.6	2.0±0.1
48h	162.5±14.2	16.9±3.0	179.5±14.8	79.4±6.3	14.6±2.6	94.0±6.6	2.0±0.1
72h	166.4±11.4	13.9±2.3	180.2±10.9	80.8±3.5	15.5±2.6	96.3±4.1	2.1±0.1
144h	165.6±12.8	15.5±4.1	181.1±14.4	83.1±5.2	20.1±2.7	103.3±7.3	2.0±0.1

Values are in nmol min^{-1} (mg $protein^{-1}$). 0h = immediately following exercise. h = hours postexercise, ①$P<0.05$ (exercise vs. control). A ratio of Ca^{2+}–ATPase activity in presence of ionophore to that in absence of ionophore (+/–) was determined.

without ionophore (+/–) was calculated to provide an index of membrane alterations. A 2.2– fold increasing in Ca^{2+}–ATPase activity with ionophore was observed in homogenate from control group (Table 3). From the results there was no change in the ratio, hence membrane integrity was not altered by exercise in all experimental groups.

3 Discussion

This study observed significant depressions in SR functional characteristics of Ca^{2+}–ATPase activity, Ca^{2+} uptake and release in red vastus muscle from rats after downhill exercise. In addition, there was no evident change in SR membrane integrity since the ratio of Ca^{2+}–ATPase activity in presence of ionophore to the activity in absence of ionophore did not decrease significantly. Similar downhill exercise protocols were previously shown to result in marked injury to fibres in the deep slow extensor muscle of rats[7]. Armstrong and colleagues [7] demonstrated that a prolonged bout of decline running of untrained rats produced histological and biochemical changes in muscles, particularly the vastus intermedius, the deep red layer of knee extensors, indicative of cell damage. They suggested that these postural muscles underwent eccentric contractions while lowering the animal down the decline, acting as 'brakes', but underwent concentric contractions during incline running. Comparisons between experiments with extremely different exercise protocols should be made with caution since high–intensity contractions would change the recruitment order of motor units, when the fast–twitch–glycolytic fibres presumably were recruited [8]. This was evident with evoking maximal eccentric contractions via electrical stimulation, where the damage was confined to the type 2 muscle fibres in general[9].

In an investigation of rats subjected to downhill running, the triceps brachii muscles showed a number of ultrastructural abnormalities including more longitudinal T–tubule segments, changes in the direction and disposition of triads, caveolar clusters and apposition of multiple T–tubule segments with terminal cisternae elements[10]. Development of T–tubular vacuoles in eccentrically damaged mouse muscle fibres has been observed in a recent study [11]. These investigators suggested that T–tubules are susceptible to rupture during eccentric contraction probably due to the relative movement associated with inhomogeneity of sarcomere lengths. Shearing damage to T–tubules by eccentric contraction was proposed to result in an increase in intracellular $[Na^+]$ and $[Ca^{2+}]$, which would cause localized swelling of T–system and activation of proteases and phospholipases[11—12]. It suggested that sarcomere length inhomogeneities during eccentric contractions could damage the SR and impair its connection with the T tubules [13]. Damage to SR could also produce an elevation in calcium concentration within the fibres, activating proteolytic enzymes[14].

This investigation is the first to report the effect of ECE on Ca^{2+} uptake, Ag^+–induced Ca^{2+} release, and Ca^{2+}–ATPase activity in rat skeletal muscle, both in terms of magnitude and time course. Both silver nitrate ($AgNO_3$) and 4–chloro–m–cresol (4–CMC)were used to induce calcium release, and it showed that these agents exert their release effects differently[15]. Nevertheless the rate of release reduced to a similar extent in a fatigue study comparing Ag^+ and 4–CMC induced Ca^{2+} release [16], An alternative method used in vivo nerve stimulation to induce eccentric muscle damage[17] resulted in depressions in SR Ca^{2+} uptake and release with $AgNO_3$ used to stimulate Ca^{2+} release. With the more physiologically relevant animal model of voluntary exercise employed, which consisted of 90min of downhill exercise, we demonstrated that depressions in SR Ca^{2+} uptake and Ca^{2+} release in rat red vastus muscles as measured in vitro occur immediately after exercise. In addition, interesting findings were the further reduction in both variables at the 4th h post–exercise with both remaining depressed at the 24th h of recovery period. Ca^{2+}–ATPase activity with ionophore in muscle homogenate showed significant depression during recovery period at the 4th h and 24th h post–exercise. However, the ratio of Ca^{2+}–ATPase activity in presence and absence of ionophore A23187 in exercised muscles was not significantly different from that of control rats (1.9 vs. 2.2, immediately post–exercise vs. control), which indicated that membrane integrity was not changed by downhill exercise. It

was showed previously that SR membrane damage was induced in an animal model, in which maximal eccentric contractions were elicited via electrical stimulation [1]. This disparity may be due to several factors, for example different protocol and experimental duration. It appears that the current exercise intensity and/or volume were insufficient to change the SR membrane integrity.

SR Ca^{2+} uptake is higher in muscles that are predominantly fast twitch, with a correlation between % type II myosin heavy chain and SR Ca^{2+} uptake[3]. There are no previous study comparing SR function in slow and fast twitch muscle following eccentric exercise, however differences were noted following concentric exercise. SR Ca^{2+} uptake depressed to a greater extent in soleus muscle compared with plantaris muscle following an acute bout of concentric exercise to exhaustion, and this was thought to be due to recruitment order of the muscles [18]. In general, the major protein responsible for Ca^{2+} transport is the 105 KDa ATPase which translocates 2mol of Ca^{2+} across the SR bilayer membrane upon the hydrolysis of 1 mol of ATP[19]. A depression in Ca^{2+} uptake may not only affect relaxation but reduce Ca^{2+} loading into the SR, resulting in less Ca^{2+} available for release during subsequent action potentials [19]. Moreover, inefficiency of Ca^{2+} transport or slowed Ca^{2+} uptake during repeated muscle contractions would expose the fibers to increase $[Ca^{2+}]_f$ for prolonged periods of time, consequently activating Ca^{2+}-sensitive proteolytic and phospholipolytic degradative pathways (e.g. calpain, phospholipase) involved in myofibrillar degradation[14,20].

The rat rectal temperature increased from 37.62℃ to 39.39℃ after ECE, although muscle temperature was not recorded in this animal study due to technical limitations. A higher temperature in muscle compared with rectal temperature of rat would occur because of higher heat production within the lengthening muscle. Intramuscular temperatures up to 42℃ was recorded previously in human skeletal muscles with eccentric exercise [21]. A recent study[22] showed that exposure of homogenates to a temperature (41℃) typically experienced in exercise resulted in reduction in the coupling ratio that was reflected in large reductions of SR Ca^{2+} uptake and only modest reductions in maximal Ca^{2+}-ATPase activity, due to an increasing in membrane permeability to Ca^{2+}. Temperature elevating could result in protein unfolding, exposing hydrophobic domains and leading to oligomerization which was defined as aggregation from more active, low-molecular-weight aggregates to less active, high-molecular-weight aggregates [22]. In this experiment, Ca^{2+} uptake depressed immediately after exercise with no reduction in Ca^{2+}-ATPase activity, however this cannot be attributed to alterations in membrane permeability resulting from elevated muscle temperature, as the effect of Ca^{2+} ionophore did not change after the ECE.

Our results suggest that a bout of low-intensity, prolonged downhill exercise results in long-lasting depression in SR function in rat red vastus muscles that is not fully restored after two days of recovery, which may underlie some muscle functional impairments induced by eccentric exercise. These changes could be the results of stress from sarcomere length inhomogeneities during eccentric contractions. However, change in SR membrane integrity was not evident, which may be due to the relatively low intensity of the downhill exercise in the present experiment.

Acknowledgements: This study was partly supported by an International Postgraduate Research Scholarship Scheme funded by the Australian Department of Education, Science and Training. We wish to acknowledge the input of Professor Green for his participation in discussions when planning the study.

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