

**Research Bank**

Journal article

**Resistance training upregulates skeletal muscle Na<sup>+</sup>, K<sup>+</sup>-ATPase content, with elevations in both  $\alpha$ 1 and  $\alpha$ 2, but not  $\beta$  isoforms**

**Altarawneh, Muath M., Petersen, Aaron C., Farr, Trevor, Garnham, Andrew, Broatch, James R., Halson, Shona, Bishop, David J. and McKenna, Michael J.**

This version of the article has been accepted for publication, after peer review (when applicable) and is subject to Springer Nature's AM terms of use, but is not the Version of Record and does not reflect post-acceptance improvements, or any corrections. The Version of Record is available online at: <https://doi.org/10.1007/s00421-020-04408-3>

**Resistance training upregulates skeletal muscle Na<sup>+</sup>,K<sup>+</sup>-ATPase content, with elevations in both  $\alpha_1$  and  $\alpha_2$ , but not  $\beta$  isoforms**

Muath M. Altarawneh<sup>1</sup>, Aaron C. Petersen<sup>1</sup>, Trevor Farr<sup>1</sup>, Andrew Garnham<sup>1</sup>, James R. Broatch<sup>1,2</sup>, Shona Halson<sup>2,3</sup>, David J. Bishop<sup>1</sup> and Michael J. McKenna<sup>1</sup>

<sup>1</sup> Institute for Health and Sport (iHeS), Victoria University, Melbourne, Australia

<sup>2</sup> Department of Physiology, Australian Institute of Sport (AIS), Canberra, Australia

<sup>3</sup> School of Behavioural and Health Sciences, Australian Catholic University, Brisbane, Australia

**Author for Correspondence:**

Professor Michael J. McKenna

Institute for Health and Sport (iHeS),

Victoria University, PO Box 14428, Melbourne, Victoria, 8001, Australia

Email: michael.mckenna@vu.edu.au

Phone: (+61 3) 9919 4499

<b>Author</b>	<b>ORCID</b>
Muath Altarawneh	0000-0003-2354-4951
Aaron Petersen	0000-0003-1508-748X
Trevor Farr	
Andrew Garnham	
James Broatch	0000-0002-0082-3168
Shona Halson	0000-0002-1047-3878
David Bishop	0000-0002-6956-9188
Michael J. McKenna	0000-0001-9998-0093

**List of abbreviations**

1RM	One repetition maximum
$\alpha$	Alpha subunit
$\beta$	Beta subunit
CWI	Cold water immersion
$K^+$	potassium ion
$[K^+]$	potassium ion concentration
$Na^+$	sodium ion
$[Na^+]$	sodium ion concentration
NKA	$Na^+,K^+$ -ATPase
RT	Resistance Training
$[^3H]$ ouabain binding	tritiated ouabain binding

**Abstract****Purpose**

The Na<sup>+</sup>,K<sup>+</sup>-ATPase (NKA) is important in regulating trans-membrane ion gradients, cellular excitability and muscle function. We investigated the effects of resistance training in healthy young adults on the adaptability of NKA content and of the specific  $\alpha$  and  $\beta$  isoforms in human skeletal muscle.

**Methods**

Twenty-one healthy young males (22.9  $\pm$ 4.6 y; 1.80  $\pm$ 0.70 m, 85.1  $\pm$ 17.8 kg, mean $\pm$ SD) underwent 7 weeks of resistance training, training three times per week (RT, n=16) or control (CON, n=5). The training program was effective with a 39% gain in leg press muscle strength (p=0.001). A resting vastus lateralis muscle biopsy was taken before and following RT or CON and assayed for NKA content ([<sup>3</sup>H]ouabain binding site content) and NKA isoform ( $\alpha_1$ ,  $\alpha_2$ ,  $\beta_1$ ,  $\beta_2$ ) abundances.

**Results**

After RT, each of NKA content (12%, 311  $\pm$ 76 vs 349  $\pm$ 76 pmol.g wet weight<sup>-1</sup>, p=0.01), NKA  $\alpha_1$  (32%, p=0.01) and  $\alpha_2$  (10%, p<0.01) isoforms were increased, whereas  $\beta_1$  (p=0.18) and  $\beta_2$  (p=0.22) isoforms were unchanged. NKA content and isoform abundances were unchanged during CON.

**Conclusions**

Resistance training increased muscle NKA content through upregulation of both  $\alpha_1$  and  $\alpha_2$  isoforms, which were independent of  $\beta$  isoform changes. In animal models, modulations in  $\alpha_1$  and  $\alpha_2$  isoform abundances in skeletal muscle may affect fatigue resistance during exercise, muscle hypertrophy and strength. Whether similar in-vivo functional benefits of these NKA isoform adaptations occurs in human muscle with resistance training remains to be determined.

**Keywords:** Resistance training, [<sup>3</sup>H]ouabain binding site content, Na<sup>+</sup>,K<sup>+</sup>-ATPase, skeletal muscle.

## Introduction

The Na<sup>+</sup>,K<sup>+</sup>-ATPase (NKA) is vital in maintaining steep transmembrane [Na<sup>+</sup>] and [K<sup>+</sup>] gradients, and in skeletal muscle has an essential role in maintaining membrane excitability and contractility (Clausen 2003). The NKA is expressed in human skeletal muscle as three catalytic  $\alpha$  subunit isoforms ( $\alpha_1$  -  $\alpha_3$ ) and three regulatory  $\beta$  subunit isoforms ( $\beta_1$  -  $\beta_3$ ) (Murphy et al. 2004; Wyckelsma et al. 2015). The  $\alpha_1$  is important in skeletal muscle for basal Na<sup>+</sup>/K<sup>+</sup> exchange, and affects muscle strength and mass (Radzyukevich et al. 2013; Lingrel et al. 2003; Kutz et al. 2018). In contrast, the  $\alpha_2$  has an important role in Na<sup>+</sup>/K<sup>+</sup> transport in contracting muscle, resisting fatigue and enhancing exercise performance (DiFranco et al. 2015; Radzyukevich et al. 2013). Thus, tight regulation of NKA content and isoforms is vital for preservation of muscle function (Clausen 2013; Sejersted and Sjogaard 2000).

The effects of resistance training on increasing muscle size and strength are well known (Candow and Burke 2007; Fyfe et al. 2019) but the effects on muscle NKA are less clear. Different types of exercise training, including sprint and endurance training, induce similar upregulation of muscle NKA content (measuring all functional  $\alpha\beta$  complexes) in human skeletal muscle, as measured by ~15% increases in the [<sup>3</sup>H]ouabain binding site content (Wyckelsma et al. 2019). However, the effects of resistance training on muscle NKA content and NKA isoform abundances in healthy young adults have not been comprehensively determined (Wyckelsma et al. 2019). An early, cross-sectional comparison indicated that older participants (mean age 68 years) who had undertaken strength training for 12-17 years had 40% greater NKA content than untrained age-matched controls (Klitgaard and Clausen 1989). It is unclear whether this substantial upregulation reflected the long period of training and/or other differences between the groups. Two subsequent studies utilising a longitudinal resistance training design reported increased muscle NKA content (Green et al. 1999b; Medbo et al. 2001). In healthy, untrained young adults, muscle NKA content was increased by 16% after seven weeks of resistance training (Green et al. 1999b). In trained male athletes, who were already strength trained, three months of resistance training increased muscle NKA content by 15%, when all data were pooled (Medbo et al. 2001). One limitation of the latter finding was that the analysis with pooled data was based on participants who undertook different amounts of training.

Only a single study has examined the effects of resistance training on NKA isoforms in skeletal muscle (Dela et al. 2004). In healthy, untrained, older participants (mean age 61 years), 6 weeks of resistance training increased each of the NKA  $\alpha_1$ ,  $\alpha_2$  and  $\beta_1$  isoforms, by 37%, 21% and 33%, respectively, in the

trained compared to the untrained leg (Dela et al. 2004). These were measured in fractionated membrane samples, which typically have a low total protein yield and thus may not necessarily be representative of the total pool of NKA in muscle (Hansen and Clausen 1988). It remains unclear whether similar NKA isoform adaptability occurs in young adults with resistance training as found in older individuals (Dela et al. 2004). Whilst there is evidence of NKA isoform upregulation with other types of exercise training in young adults, there appears little consistency in adaptability of specific isoforms and in their magnitude of change (Wyckelsma et al 2019). When undertaken concurrently with repeated 30-s sprint running, resistance training over 8 weeks failed to upregulate NKA  $\alpha_1$ ,  $\alpha_2$  or  $\beta_1$  isoforms in moderately trained endurance runners (Skovgaard et al. 2014). Combined strength and speed endurance training on separate days over 4 weeks similarly did not change NKA  $\alpha_1$ , or  $\alpha_2$  isoforms, but  $\beta_1$  was increased by 15% (Vorup et al. 2016). However, no increases were found in NKA content after unilateral lower limb suspension followed by 4 weeks of resistance training, or in isoforms measured in whole-muscle homogenates, although fibre-type specific changes were found (Perry et al. 2016). Thus, the effects of resistance training per se on NKA isoforms in young adults are unclear. Furthermore, whether elevations in both  $\alpha_1$  and  $\alpha_2$  isoforms enable an increased NKA content with resistance training cannot be confirmed, since no study has combined measures of the quantitative [ $^3\text{H}$ ]ouabain binding site content method along with determination of NKA isoform protein abundances, in the same age group and without confounding concurrent training or earlier inactivity. The possible effects of resistance training on the  $\alpha_3$ ,  $\beta_2$  and  $\beta_3$  NKA isoforms also expressed in human skeletal muscle remain unknown.

Therefore, this study investigated the adaptability of NKA isoforms in skeletal muscle after resistance exercise training in healthy, young, untrained adults, to explore which isoforms underpin the anticipated increase in NKA content. It was hypothesised that muscle NKA content and protein abundances of each of the  $\alpha_1$ ,  $\alpha_2$ , and  $\beta_1$  isoforms would be increased following resistance exercise training.

## **Methods**

### **Participants and overview**

Twenty-one healthy young adult males that were recreationally-active but not well-trained gave written informed consent prior to participating in this study, which was approved by the Victoria University Human Research Ethics Committee. The physical characteristics of the participants were: age:  $22.9 \pm$

4.6 y; height:  $1.80 \pm 0.70$  m; body mass:  $85.1 \pm 17.8$  kg (mean $\pm$ SD). Participants undertook 7 weeks of resistance training (RT, n = 16) or control (CON, n = 5). Each participant underwent a muscle biopsy prior to and following RT or CON. This study was part of a larger study that included investigation of the effects of cold water immersion (CWI) recovery after resistance training on muscle strength, hypertrophy and associated signalling pathways (Fyfe et al 2019). One-half of the participants in the RT group underwent CWI during recovery, which was initiated within 5 min of completing each workout, whilst the others undertook passive recovery. CWI participants were immersed to their umbilicus for 15 minutes in 10°C water in a recovery pool (iCool Sport, Australia), whilst non-CWI participants sat for 15 minutes in ambient room air ( $22 \pm 0.8^\circ\text{C}$ ).

### **Resistance Training Program**

The RT protocol comprised three training sessions per week, for 7 weeks. Participants in the training group were familiarised with the RT exercises to ensure correct techniques, which were monitored throughout the training program. A standardised 5-min warm up at  $1\text{W}\cdot\text{kg}^{-1}$  body mass on a cycle ergometer was conducted prior to every training session. Training loads were determined during a familiarisation session performed prior to commencing the training program. Participants performed three sets of 10 - 12 repetitions of each exercise, interspersed with 60 - 90 s of recovery between sets. To induce progressive overload, the training weight was incremented by ~5% when a participant completed 12 repetitions of the three total sets. The training program involved upper- and lower-body exercises and comprised: back squat, leg raise, leg press, bench press, incline bench press, lateral pulldown, lunge, dumbbell shoulder press, dumbbell bicep curl, lying triceps extensions, bent-over row, upright row, barbell biceps curl, dips and sit-ups. The training was conducted in the laboratory under supervision. Participants in CON were asked to continue their regular daily activities during the control period; these comprised only recreational activities and they were specifically requested not to undertake any high-intensity exercise. Compliance with the RT program was  $92 \pm 5\%$ . RT was effective; one repetition maximum (1RM) was increased after RT for bench press (9%, pre-train,  $77.6 \pm 16.2$  vs post-train  $84.9 \pm 17.3$  kg,  $P < 0.001$ ) and for leg press (38%, pre-train,  $342.3 \pm 65.7$  vs post-train  $472.4 \pm 106.3$  kg,  $P < 0.001$ ). Increases were also found after RT for each of whole-body lean mass (4.4%, pre-train,  $58.65 \pm 7.34$  vs post-train,  $61.22 \pm 8.50$  kg,  $P < 0.001$ ), lower body lean mass (4.5%, pre-train,  $21.02 \pm 2.07$  vs post-train,  $21.96 \pm 2.85$  kg,  $P < 0.01$ ) and upper body lean mass (5.6%, pre-train,  $7.81 \pm 1.05$  vs post-train,  $8.25 \pm 1.14$  kg,  $P < 0.01$ ) (data from Fyfe et al. 2019). Control participants did not

undergo strength or lean mass measurements, but only provide a muscle biopsy sample before and after the control period. Leg press 1RM did not differ between CWI and non-CWI RT participants at pre- (CWI  $346 \pm 55$  kg, non-CWI  $338 \pm 78$  kg) or post-training (CWI  $480 \pm 118$  kg, non-CWI  $464 \pm 111$  kg). Bench press 1RM also did not differ between CWI and non-CWI RT participants at pre- (CWI  $75.6 \pm 16$  kg, non-CWI  $79.5 \pm 17.2$  kg) or post-training (CWI  $83.4 \pm 14.5$  kg, non-CWI  $86.4 \pm 20.6$  kg).

### **Muscle sampling.**

Participants in the RT and CON groups underwent a resting *vastus lateralis* muscle biopsy prior to and after the 7 weeks training period, with the latter biopsy in RT taken 72 - 96 h after the final training session. Participants were asked to refrain from exercise and alcohol in the 24 h preceding the muscle biopsies and reported to the lab in a fasted state after ingesting a standardized dinner (containing 53.1 g carbohydrate, 41 g protein and 10.9 g fat) the night before. After injection of local anaesthetic into the subcutaneous skin and fascia (1% Xylocaine, Astra Zeneca), an incision was placed in skin overlying the *vastus lateralis* muscle. A muscle sample (~100 - 120mg) was extracted from the mid-thigh using a Bergström biopsy needle with suction. Muscle was rapidly frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for later analyses of NKA content and isoform protein abundances.

### **[ $^3\text{H}$ ]-ouabain binding site content**

Analysis of skeletal muscle NKA content was determined by the [ $^3\text{H}$ ]-ouabain binding site content assay. Approximately 20 mg of muscle was analysed in quadruplicate using the vanadate-facilitated [ $^3\text{H}$ ]ouabain binding content method as previously described (Nørgaard et al., 1984, Petersen et al., 2005). Each sample was washed for 2 x 10 min at  $37^{\circ}\text{C}$  in vanadate buffer (250 mM sucrose, 10 mM Tris-HCl, 3 mM  $\text{MgSO}_4$ , 1 mM  $\text{NaVO}_4$ ; pH 7.3). Muscle samples were then incubated for 2 h at  $37^{\circ}\text{C}$  in vanadate buffer with the addition of [ $^3\text{H}$ ]ouabain (2.0 Ci-1ml and  $10^{-6}$  M, PerkinElmer, Boston, MA). The muscle was then placed in ice-cold vanadate solution for 4 x 30 min to remove any unbound [ $^3\text{H}$ ]ouabain. Muscle samples were blotted on filter paper and weighed before being soaked overnight in 500  $\mu\text{l}$  of 5% trichloroacetic acid and 0.1 mM ouabain. Following this, 2.5 ml of scintillation cocktail (Opti-Fluor, Packard, PerkinElmer, Boston, MA) was added before liquid scintillation counting of [ $^3\text{H}$ ]ouabain. The [ $^3\text{H}$ ]ouabain binding site content was then calculated on the basis of the sample wet weight and specific activity of the incubation buffer and samples (Nørgaard et al. 1984; Petersen et al. 2005). The final [ $^3\text{H}$ ]ouabain binding site content was calculated using a correction factor of 1.13 as previously described (Petersen et al. 2005) to allow for impurity of [ $^3\text{H}$ ]ouabain, loss of specifically bound



[<sup>3</sup>H]ouabain during washout and incomplete saturation during the equilibration of muscle with [<sup>3</sup>H]ouabain, with binding site content expressed as pmol.g ww<sup>-1</sup>.

### **Western blotting**

To determine skeletal muscle NKA  $\alpha$  and  $\beta$  isoform relative protein abundances, ~20 mg of frozen muscle was analysed using western blotting (Perry et al. 2013). Muscle proteins were lysed in ice-cold buffer containing 20 mM Tris pH 7.8 (Bio-Rad Laboratories, Hercules, CA), 137 mM NaCl, 2.7 mM KCl (Merck, Kilsyth, Australia), 1 mM MgCl<sub>2</sub>, 5 mM Na<sub>4</sub>O<sub>7</sub>P<sub>2</sub>, 10 mM NaF, 1% Triton X-100, 10% Glycerol (Ajax Finechem, Australia), Protein Inhibitor Cocktail (P8340). All reagents were analytical grade (Sigma-Aldrich, St Louis, MI). Samples were homogenised (1:40 dilution) using a tissue Lyser II (QIAGEN, Hilden, Germany) followed by gentle rocking for 60 min at 4°C. Protein concentration of the homogenate was determined using a commercially available kit (DC Protein Assay, Bio Rad Laboratories, USA). Repeated steps of centrifugation of muscle and membrane separation have resulted in very low recovery of NKA, yielding a final sample that may be unrepresentative of the whole muscle NKA (Hansen and Clausen 1988). Therefore, muscle NKA isoform analyses did not include any membrane isolation steps, to maximise recovery of NKA enzymes (Murphy et al. 2004). Aliquots of the muscle homogenate were mixed with Laemmli sample buffer and proteins were separated on 26 well Criterion Stain Free precast gels (8 - 16%, Criterion TGX, Bio-Rad Laboratories, USA) for 45 min at 200 V and 400 mA.

For the analysis of protein abundance of the NKA isoforms ( $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_3$ ,  $\beta_1$ , and  $\beta_2$ ), 10  $\mu$ g of total protein per sample were loaded in each gel. To ensure that blot density was within the linear range of detection (Murphy and Lamb 2013), a four to five-point (2.5 - 12.5  $\mu$ g) calibration curve of whole-muscle crude homogenate was loaded onto every gel. The homogenate was prepared from an equal amount of 5  $\mu$ g from each sample. Following electrophoresis, proteins were transferred to polyvinylidene fluoride membranes (TurboTransfer pack, Bio-Rad Laboratories, USA) for 7 min at 320 mA using the semi-dry Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, USA). Membranes were blocked in PBST buffer (10 mM Tris, 100 mM NaCl, 0.02% Tween-20) containing 5% non-fat milk, for 1 h at room temperature. After being washed (4 x 8 min in TBST), membranes were incubated with the appropriate primary antibody overnight at 4°C. Primary antibodies were diluted in PBS buffer containing 0.1% NaN<sub>3</sub> and 0.1% albumin bovine serum. Following incubation with the primary antibodies, membranes were washed in PBST buffer (4 x 5 min) and incubated with the appropriate anti-rabbit (PerkinElmer #

NEF812001EA) or anti-mouse (PerkinElmer # NEF822001EA) horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. After washing the membranes in PBST (4 x 5 min), membranes were incubated for 5 min with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo, Waltham, MA, USA), then stain free images were taken using a ChemiDoc Imaging system (Bio-Rad Laboratories, USA). The densities of samples were expressed relative to the total protein on the gel and then normalised to the calibration curve (Murphy and Lamb 2013).

The following antibodies were used for NKA isoform  $\alpha_1$  (monoclonal  $\alpha_6F$ , developed by D. Fambrough, obtained from the Developmental Studies Hybridoma Bank, maintained by the University of Iowa, USA),  $\alpha_2$  (polyclonal anti-HERED, Millipore, # 07- 674),  $\alpha_3$  (monoclonal, Thermo Scientific, Rockford, IL, # MA3-915),  $\beta_1$  (Millipore, # 05- 382),  $\beta_2$  (Proteintech # 22338-1-AP) and  $\beta_3$  (BD Bioscience, # 610993). However, neither the NKA  $\alpha_3$  or  $\beta_3$  isoforms could be detected, despite attempts at loading several total protein amounts and using additional antibodies.

### **Statistics**

Data were assessed for normality using the Shapiro-Wilk test, and all data were normally distributed. All NKA data were analysed using a one-tailed paired t-test (pre vs post) with RT due to the hypothesised increase in NKA, with RT and CON groups analysed individually. Data are presented as mean  $\pm$  standard deviation (SD) and statistical significance was set at  $P < 0.05$ . Statistical analyses were conducted using SPSS version 24.

### **Results**

#### **Comparisons of NKA variables between subgroups**

There were no differences in any NKA variables prior to (data not shown) or following RT between the CWI and non-CWI groups, for any of NKA content ( $351 \pm 88$  vs.  $347 \pm 72$  pmol.g wet weight<sup>-1</sup>,  $p = 0.36$ ); NKA  $\alpha_1$  ( $0.87 \pm 0.12$  vs  $0.91 \pm 0.10$  a.u.,  $p = 0.25$ );  $\alpha_2$  ( $0.93 \pm 0.13$  vs  $0.89 \pm 0.08$  a.u.,  $p = 0.31$ ); NKA  $\beta_1$  ( $0.83 \pm 0.07$  vs  $0.87 \pm 0.11$  a.u.,  $p = 0.41$ ); and  $\beta_2$  isoform ( $0.82 \pm 0.09$  vs  $0.88 \pm 0.05$  a.u.,  $p = 0.36$ ). Given the lack of significant differences or tendencies between the CWI and non-CWI groups, the results from both groups were pooled into a single RT group to investigate the effects of RT on NKA content, as well as NKA  $\alpha_1$ ,  $\alpha_2$ ,  $\beta_1$  and  $\beta_2$  isoform abundances.

#### **Resistance Training Effects on Muscle NKA content**

##### ***NKA content***

The muscle NKA content increased by 12% after RT ( $p = 0.01$ ), with no significant changes in CON ( $p = 0.27$ , Fig 1).

### **NKA isoform abundances**

Representative blots for NKA  $\alpha_1$ ,  $\alpha_2$ ,  $\beta_1$  and  $\beta_2$  are shown in Fig 2.

**$\alpha$  isoforms.** Following 7 weeks of RT, the muscle NKA  $\alpha_1$  isoform abundance increased by 32% ( $p = 0.01$ ) and the  $\alpha_2$  isoform by 10% ( $p = 0.001$ ), with no changes found in CON ( $p = 0.17$  and  $p = 0.34$ , respectively, Fig 3).

**$\beta$  isoforms.** The muscle NKA  $\beta_1$  ( $p = 0.18$ ) and  $\beta_2$  ( $p = 0.22$ ) isoforms were not significantly changed following RT, with no changes also in CON ( $p = 0.25$  and  $p = 0.29$ , respectively, Fig 4).

## Discussion

This study demonstrates that resistance training in healthy young adults induced an increase in the NKA content in skeletal muscle that was due to increased abundances of both of the major NKA  $\alpha$  isoforms,  $\alpha_1$  and  $\alpha_2$ . This suggests both adaptations may be important in the phenotypical changes with RT. We further show that RT was not accompanied by any increases in the NKA  $\beta_1$  or  $\beta_2$  isoform abundances in these healthy young adults. This indicates that  $\beta$  isoform upregulation is not an obligatory adaptation to resistance training and suggests that existing  $\beta$  subunits must be in sufficient abundance to form these new functional NKA complexes.

### **Increased skeletal muscle NKA content, $\alpha_1$ and $\alpha_2$ isoforms with resistance training**

The 12% increase in muscle NKA content after 7 weeks of RT is consistent with an earlier 16% increase reported following 7 weeks of resistance training, also in healthy untrained subjects (Green et al. 1999a), and also with the 15% increase after 3 months of resistance training in highly-trained athletes (Medbø et al. 2001). This increase in muscle NKA content with RT is also in broad agreement with other studies investigating different training models that typically induced increases in NKA content of 8 - 16% following sprint training (Harmer et al. 2006; McKenna et al. 1993) and ~16% following endurance training (Evertsen et al. 1997; Madsen et al. 1994). Since most training studies investigated either [<sup>3</sup>H]ouabain binding site content or isoform abundances with training, an important feature of this study utilising both measures is that our findings allow us to determine which  $\alpha$  isoforms form the basis of this upregulation in NKA content with training.

A major finding was that RT in healthy young adults induced increases of ~32% and ~10% in NKA  $\alpha_1$  and  $\alpha_2$  isoform abundances, respectively, indicating that the increased NKA content was derived from increases in both  $\alpha_1\beta$  and  $\alpha_2\beta$  complexes. These increases in NKA  $\alpha_1$  and  $\alpha_2$  are consistent with the only other study to have investigated effects on NKA isoforms of RT, without any other concurrent training modes, which reported 37% and 21% increases in muscle NKA  $\alpha_1$  and  $\alpha_2$  isoform abundances, respectively (Dela et al. 2004). Important differences were that this earlier finding was in older individuals, with no measures of NKA content undertaken (Dela et al. 2004). In human skeletal muscle, the standard [<sup>3</sup>H]ouabain binding assay can detect each of the  $\alpha_1$ ,  $\alpha_2$  and  $\alpha_3$  isoforms since each have a high ouabain affinity (Wang et al. 2001). Hence, whilst the upregulation of muscle NKA content after RT is probably primarily due to increase in the abundance of both of the  $\alpha_1$  and  $\alpha_2$

isoforms, it is possible that this might also occur for the  $\alpha_3$  isoform. Unfortunately, this cannot be confirmed here since the  $\alpha_3$  isoform could not be detected.

In skeletal muscle, the  $\alpha_1$  isoform comprises ~15-20% and the  $\alpha_2$  ~80 - 85% of the NKA (Hansen 2001; Hansen and Clausen 1988; He et al. 2001). The increased  $\alpha_1$  and  $\alpha_2$  isoform abundances would appear to fully account for the increase in [ $^3\text{H}$ ] ouabain binding, from pre-RT of ~311 to 349 pmol.g wet weight<sup>-1</sup>. If  $\alpha_1$  was assumed ~20% and  $\alpha_2$  ~80% of NKA also for human muscle, then the isoform contents would respectively be ~62 and ~249 pmol.g wet weight<sup>-1</sup>. With gains after RT in  $\alpha_1$  of 32% and in  $\alpha_2$  by 10%, the corresponding post-training isoform contents would be 82 and 274 pmol.g wet weight<sup>-1</sup>, respectively. Together the assumed post-RT NKA content would be ~356 pmol.g wet weight<sup>-1</sup>, an 11.4% increase, very similar to the observed 12% increase in NKA content with RT. Together this data demonstrates for the first time that increases in both of the key  $\alpha_1$  and  $\alpha_2$  isoform abundances underpin the muscle NKA content upregulation with RT.

Combining the data from the two groups that underwent cold water immersion and passive recovery into a single pooled RT group is unlikely to have influenced these findings. Firstly, no differences were found between the groups in any NKA variable. This lack of effect on NKA proteins is also consistent with a recent finding that cold water immersion recovery during 6 weeks of sprint training did not alter the NKA  $\alpha_1$ ,  $\alpha_2$ ,  $\beta_1$  or  $\beta_2$  isoform responses to (Christiansen et al. 2018). Utilising an unbalanced design with fewer individuals in the control than in the RT group is a limitation. However, this seems unlikely to be a major limitation, nor likely to introduce undue bias, for two reasons. First, increases in muscle NKA content after training were robust in the RT group, being elevated in 15 of the 16 participants after RT ( $P < 0.01$ ), but in only 2 of the 5 participants in the control group (trivial effect size,  $d = 0.157$ , Cohen 1988). Second, pre- versus post-training analyses were conducted separately within each group rather than between groups to alleviate possible bias due to uneven numbers. Similar robust upregulatory responses to training were also found for NKA  $\alpha_1$  and  $\alpha_2$ , being increased after RT in 14, and in all of the 16 participants, respectively.

A high NKA capacity and activity is needed to effectively regulate rates of  $\text{Na}^+$  influx into cells and  $\text{K}^+$  efflux from contracting muscle cells, in order to sustain muscle excitability and contractility (Clausen 2010; Sejersted and Sjøgaard 2000). Thus, the upregulation of NKA content with training is consistent with beneficial outcomes of delayed or minimised muscle fatigue and improved exercise performance (Harmer et al. 2006; McKenna et al. 1993). The upregulation of, and quantitatively different adaptive

responses between the  $\alpha_1$  and  $\alpha_2$  isoforms suggests that each may confer different specific benefits to enhanced muscle function with training (Wyckelsma et al. 2019). This is consistent with evidence that the  $\alpha_1$  and  $\alpha_2$  isoforms play different physiological roles in skeletal muscle function, based on findings in gene-targeted mice that express only one or no copies of the  $\alpha_1$  or  $\alpha_2$  isoform genes. The NKA  $\alpha_1$  isoform is believed to be vital in  $\text{Na}^+/\text{K}^+$  exchange during basal conditions, also affecting muscle strength, at least in oxidative muscle fibres and also muscle mass (He et al. 2001; Lingrel et al. 2003; Radzyukevich et al. 2013; Kutz et al. 2018). The  $\alpha_2$  isoform plays a key role in regulating transmembrane  $\text{Na}^+/\text{K}^+$  exchange during muscle contractions, thus enhancing fatigue resistance and enabling improved exercise performance (Radzyukevich et al. 2013). Therefore, we speculate that the upregulation in both  $\alpha_1$  and  $\alpha_2$  isoform proteins with RT might differentially benefit muscle performance, with elevated  $\alpha_1$  supporting more rapid recovery between resistance exercise bouts, and possibly promoting developments of mass and strength (Kutz et al. 2018), whilst the increased  $\alpha_2$  isoform may enhance maintenance of contractility during resistance exercise bouts. Further work investigating intracellular signalling pathways responsible for NKA  $\alpha$  isoform upregulation, as well as determining specific benefits are required to determine the importance of these potential impacts in muscle. We acknowledge that our data do not provide specific evidence that the functional benefits of specific isoform upregulation evidenced in murine models, would also be conferred in human muscle after RT.

#### **Lack of change in muscle NKA $\beta$ isoforms with resistance training.**

A surprising finding was that RT had no effect on the NKA  $\beta_1$  isoform abundance. This contrasts with a previous report of increased NKA  $\beta_1$  after RT in older men (Dela et al. 2004). The reason for the lack of change in the  $\beta_1$  isoform with RT here is unknown. However, it is interesting that this lack of effect is consistent with several previous findings of unchanged  $\beta_1$  isoform after intense intermittent exercise training (Nielsen et al. 2004), endurance training (Green et al. 2004) and acute high-intensity interval cycling training (Aughey et al. 2007). There was also no change in NKA  $\beta_2$  isoform abundance after RT. This is consistent with similar findings reported following high-intensity interval cycling training in already well-trained cyclists (Aughey et al. 2007) and endurance training in untrained participants (Benziane et al. 2011), although others have reported increased  $\beta_2$  with endurance training (Green et al. 2008). The reason for discrepancies between these studies remains unclear, with further research needed to clarify the effects of training on the NKA  $\beta_1$  and  $\beta_2$  isoform abundances (Wyckelsma et al.

2019). The lack of change in  $\beta_1$  and  $\beta_2$  with RT found here may not indicate a lack of adaptability of the isoforms, but point instead to the possibility that this simply reflects an overall excess of  $\beta$  over  $\alpha$  subunits in skeletal muscle, which was suggested to be vital for NKA activity (Lavoie et al. 1997). Thus, it is possible that an existing relative overabundance of  $\beta$  isoforms may enable myocytes to efficiently form an increased number of functional NKA complexes, without upregulation of further  $\beta$  subunits, at least with short-term resistance training lasting only a few months. However, an overabundance of  $\beta$  over  $\alpha$  subunits is yet to be substantiated in human skeletal muscle.

This study sampled *vastus lateralis* muscle, which is of mixed fibre type composition (Staron et al. 2000). Recent studies have examined the responses of isoforms in specific fibre types to training, revealing some differences in Type I or II fibres (Perry et al. 2016; Wyckelsma et al. 2017; Wyckelsma et al. 2015; Christiansen et al. 2018) and also that changes in homogenates, which are composed of mixed muscle fibres, did not necessarily mirror changes at the single fibre level. It is possible that fibre-specific adaptations underpin these current findings. Further research is required to determine whether fibre-specific adaptations in NKA isoforms also occurred with RT.

Finally, the increased NKA content and  $\alpha$  isoform abundances in *vastus lateralis* muscle after RT, together with the increased upper and lower body lean mass (Fyfe et al 2019), indicates that the whole-body muscle NKA abundance and thus  $\text{Na}^+/\text{K}^+$  transport capacity would also have increased with RT. This suggests the potential for enhanced systemic  $\text{K}^+$  clearance during and following acute exercise after RT, although this remains to be determined.

## **Conclusions**

This study demonstrated that resistance training induced an increase in muscle NKA content that could be accounted for by increases in abundance of both the  $\alpha_1$  and  $\alpha_2$  NKA isoforms, but without increased net synthesis of  $\beta$  subunits. An increased number of functional NKA complexes in muscle may confer benefits on muscle performance that are evident both during training bouts and recovery, through preservation of muscle excitability. Further work is required to explore the possibility that intracellular signalling pathways associated with NKA  $\alpha_1$  upregulation may also play a role in muscle hypertrophy and strength development with resistance training.

**Figure 1. Skeletal muscle NKA [<sup>3</sup>H]ouabain binding site content before and following 7 wk of resistance training in healthy young adults.**

Data are presented as a box and whisker plot, where the top and bottom lines of the box represents the third and the first quartiles, with the median also indicated, whilst the whiskers represent the maximum and minimum values. \*post-training greater than pre-training,  $p < 0.05$ ,  $n = 16$  resistance training,  $n = 5$  control).

**Figure 2. Representative immunoblots of NKA  $\alpha_1$ ,  $\alpha_2$ ,  $\beta_1$ , and  $\beta_2$  isoforms in homogenates of the human vastus lateralis muscle.**

Values at left indicate molecular weight of bands. Protein bands from left to right are (T1) pre-training, (T2) post-training, (C1) pre-control, (C2) post control. 1, 2, 3, 4 and 5 are calibration curve loaded with 2.5 - 12.5 $\mu$ g whole-muscle crude homogenate. The homogenate was prepared from an equal amount from each sample.

**Figure 3. Skeletal muscle NKA (A)  $\alpha_1$  and (B)  $\alpha_2$  isoform abundances before and following 7 wk of resistance training in healthy young adults.**

Data are presented as a box and whisker plot, where the top and bottom lines of the box represents the third and the first quartiles, with the median also indicated, whilst the whiskers represent the maximum and minimum values. Data are expressed in arbitrary units (a.u.).

\*post-training greater than pre-training,  $p < 0.05$ ,  $n = 16$  resistance training,  $n = 5$  control.

**Figure 4. Skeletal muscle NKA (A)  $\beta_1$  and (B)  $\beta_2$  isoform abundances before and following 7 wk of resistance training in healthy young adults.**

Data are presented as a box and whisker plot, where the top and bottom lines of the box represents the third and the first quartiles, with the median also indicated, whilst the whiskers represent the maximum and minimum values. Data are expressed in arbitrary units (a.u.)  $n = 16$  resistance training,  $n = 5$  control.



## References

- Aughey RJ, Murphy KT, Clark SA, Garnham AP, Snow RJ, Cameron-Smith D, Hawley JA, McKenna MJ (2007) Muscle Na<sup>+</sup>-K<sup>+</sup>-ATPase activity and isoform adaptations to intense interval exercise and training in well-trained athletes. *Journal of Applied Physiology* 103 (1):39-47
- Bangsbo J, Gunnarsson TP, Wendell J, Nybo L, Thomassen M (2009) Reduced volume and increased training intensity elevate muscle Na<sup>+</sup>-K<sup>+</sup> pump  $\alpha$ 2-subunit expression as well as short- and long-term work capacity in humans. *J Appl Physiol* 107 (6):1771-1780. doi:10.1152/jappphysiol.00358.2009
- Benziane B, Widegren U, Pirkmajer S, Henriksson J, Stepto NK, Chibalin AV (2011) Effect of exercise and training on phospholemman phosphorylation in human skeletal muscle. *American Journal of Physiology-Endocrinology and Metabolism* 301 (3):E456-E466
- Candow DG, Burke DG (2007) Effect of short-term equal-volume resistance training with different workout frequency on muscle mass and strength in untrained men and women. *Journal of strength and conditioning research* 21 (1):204
- Christiansen D, Bishop DJ, Broatch JR, Bangsbo J, McKenna MJ, Murphy RM (2018) Cold-water immersion after training sessions: Effects on fiber type-specific adaptations in muscle K<sup>+</sup> transport proteins to sprint-interval training in men. *Journal of Applied Physiology* 125 (2): 429-444
- Clausen T (2003) Na<sup>+</sup>-K<sup>+</sup> pump regulation and skeletal muscle contractility. *Physiological reviews* 83 (4):1269-1324
- Clausen T (2010) Hormonal and pharmacological modification of plasma potassium homeostasis. *Fundamental & clinical pharmacology* 24 (5):595-605
- Clausen T (2013) Quantification of Na<sup>+</sup>, K<sup>+</sup> pumps and their transport rate in skeletal muscle: Functional significance. *J Gen Physiol* 142 (4):327-345. doi:10.1085/jgp.201310980
- Dela F, Holten M, Juel C (2004) Effect of resistance training on Na, K pump and Na<sup>+</sup>/H<sup>+</sup> exchange protein densities in muscle from control and patients with type 2 diabetes. *Pflügers Archiv* 447 (6):928-933
- DiFranco M, Hakimjavadi H, Lingrel JB, Heiny JA (2015) Na, K-ATPase  $\alpha$ 2 activity in mammalian skeletal muscle T-tubules is acutely stimulated by extracellular K<sup>+</sup>. *The Journal of General Physiology:jgp*. 201511407
- Evertsen F, Medbo J, Jebens E, Nicolaysen K (1997) Hard training for 5 mo increases Na (+)-K<sup>+</sup> pump concentration in skeletal muscle of cross-country skiers. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 272 (5):R1417-R1424
- Fyfe JJ, Broatch JR, Trewin AJ, Hanson, ED, Argus CK, Garnham AP, Petersen AC. (2019). Cold water immersion attenuates anabolic signaling and skeletal muscle fiber hypertrophy, but not strength gain, following whole-body resistance training. *Journal of Applied Physiology*, 127(5), 1403-1418
- Green H, Barr D, Fowles J, Sandiford S, Ouyang J (2004) Malleability of human skeletal muscle Na<sup>+</sup>-K<sup>+</sup>-ATPase pump with short-term training. *Journal of Applied Physiology* 97 (1):143-148
- Green H, Dahly A, Shoemaker K, Goreham C, Bombardier E, Ball-Burnett M (1999a) Serial effects of high-resistance and prolonged endurance training on Na<sup>+</sup>-K<sup>+</sup> pump concentration and enzymatic activities in human vastus lateralis. *Acta physiologica Scandinavica* 165 (2):177-184
- Green H, Duhamel T, Stewart R, Tupling AR, Ouyang J (2008) Dissociation between changes in muscle Na<sup>+</sup>-K<sup>+</sup>-ATPase isoform abundance and activity with consecutive days of exercise and recovery. *American Journal of Physiology-Endocrinology and Metabolism* 294 (4):E761-E767
- Green H, Goreham C, Ouyang J, Ball-Burnett M, Ranney D (1999b) Regulation of fiber size, oxidative potential, and capillarization in human muscle by resistance exercise. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 276 (2):R591-R596
- Hansen O (2001) The  $\alpha$ 1 isoform of Na<sup>+</sup>, K<sup>+</sup>-ATPase in rat soleus and extensor digitorum longus. *Acta physiologica scandinavica* 173 (3):335-341

- Hansen O, Clausen T (1988) Quantitative determination of Na<sup>+</sup>-K<sup>+</sup>-ATPase and other sarcolemmal components in muscle cells. *American Journal of Physiology-Cell Physiology* 254 (1):C1-C7
- Harmer AR, Ruell PA, McKenna MJ, Chisholm DJ, Hunter SK, Thom JM, Morris NR, Flack JR (2006) Effects of sprint training on extrarenal potassium regulation with intense exercise in Type 1 diabetes. *Journal of applied physiology* 100 (1):26-34
- He S, Shelly DA, Moseley AE, James PF, James JH, Paul RJ, Lingrel JB (2001) The  $\alpha$ 1- and  $\alpha$ 2-isoforms of Na-K-ATPase play different roles in skeletal muscle contractility. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 281 (3):R917-R925
- Klitgaard H, Clausen T (1989) Increased total concentration of Na<sup>+</sup>,K<sup>+</sup>-pumps in vastus lateralis muscle of old trained human subjects. *J Appl Physiol* 67 (6):2491-2494
- Kutz LC, Mukherji ST, Wang X, Bryant A, Larre I, Heiny JA, Lingrel JB, Pierre SV, Xie Z (2018) Isoform-specific role of Na/K-ATPase  $\alpha$ 1 in skeletal muscle. *American Journal of Physiology-Endocrinology and Metabolism* 314 (6):E620-E629. doi:10.1152/ajpendo.00275.2017
- Lavoie L, Levenson R, Martin-Vasallo P, Klip A (1997) The Molar Ratios of  $\alpha$  and  $\beta$  Subunits of the Na<sup>+</sup>-K<sup>+</sup>-ATPase Differ in Distinct Subcellular Membranes from Rat Skeletal Muscle. *Biochemistry* 36 (25):7726-7732
- Lingrel J, Moseley A, Dostanic I, Cougnon M, He S, James P, Woo A, O'CONNOR K, Neumann J (2003) Functional Roles of the  $\alpha$  Isoforms of the Na, K-ATPase. *Annals of the New York Academy of Sciences* 986 (1):354-359
- Madsen K, Franch J, Clausen T (1994) Effects of intensified endurance training on the concentration of Na, K-ATPase and Ca-ATPase in human skeletal muscle. *Acta physiologica Scandinavica* 150 (3):251-258
- McKenna M, Schmidt T, Hargreaves M, Cameron L, Skinner S, Kjeldsen K (1993) Sprint training increases human skeletal muscle Na<sup>+</sup>-K<sup>+</sup>-ATPase concentration and improves K<sup>+</sup> regulation. *Journal of Applied Physiology* 75 (1):173-180
- Medbo JJ, Jebens E, Vikne H, Refsnes PE, Gramvik P (2001) Effect of strenuous strength training on the Na<sup>+</sup>,K<sup>+</sup>-pump concentration in skeletal muscle of well-trained men. *Eur J Appl Physiol* 84 (1-2):148-154
- Murphy KT, Snow RJ, Petersen AC, Murphy RM, Mollica J, Lee J-S, Garnham AP, Aughey RJ, Leppik JA, Medved I (2004) Intense exercise up-regulates Na<sup>+</sup>, K<sup>+</sup>-ATPase isoform mRNA, but not protein expression in human skeletal muscle. *The Journal of physiology* 556 (2):507-519
- Murphy RM, Lamb GD (2013) Important considerations for protein analyses using antibody based techniques: down-sizing Western blotting up-sizes outcomes. *The Journal of physiology* 591 (23):5823-5831
- Nielsen JJ, Mohr M, Klarskov C, Kristensen M, Krstrup P, Juel C, Bangsbo J (2004) Effects of high-intensity intermittent training on potassium kinetics and performance in human skeletal muscle. *The Journal of Physiology* 554 (3):857-870. doi:10.1113/jphysiol.2003.050658
- Nørgaard A, Kjeldsen K, Clausen T (1984) A method for the determination of the total number of 3H-ouabain binding sites in biopsies of human skeletal muscle. *Scandinavian Journal of Clinical & Laboratory Investigation* 44 (6):509-518
- Petersen AC, Murphy KT, Snow RJ, Leppik JA, Aughey RJ, Garnham AP, Cameron-Smith D, McKenna MJ (2005). Depressed Na<sup>+</sup>-K<sup>+</sup>-ATPase activity in skeletal muscle at fatigue is correlated with increased Na<sup>+</sup>-K<sup>+</sup>-ATPase mRNA expression following intense exercise. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 289(1):266-274.
- Perry BD, Levinger P, Serpiello FR, Caldow MK, Cameron-Smith D, Bartlett JR, Feller JA, Bergman NR, Levinger I, McKenna MJ (2013) The effects of osteoarthritis and age on skeletal muscle strength, Na<sup>+</sup>-K<sup>+</sup>-ATPase content, gene and isoform expression. *Journal of Applied Physiology* 115 (10):1443-1449
- Perry BD, Wyckelsma VL, Murphy RM, Steward CH, Anderson M, Levinger I, Petersen AC, McKenna MJ (2016) Dissociation between short-term unloading and resistance training effects on

- skeletal muscle Na<sup>+</sup>,K<sup>+</sup>-ATPase, muscle function, and fatigue in humans. *J Appl Physiol* 121 (5):1074-1086. doi:10.1152/jappphysiol.00558.2016
- Radzyukevich TL, Neumann JC, Rindler TN, Oshiro N, Goldhamer DJ, Lingrel JB, Heiny JA (2013) Tissue-specific role of the Na, K-ATPase  $\alpha$ 2 isozyme in skeletal muscle. *Journal of Biological Chemistry* 288 (2):1226-1237
- Sejersted OM, Sjogaard G (2000) Dynamics and consequences of potassium shifts in skeletal muscle and heart during exercise. *Physiol Rev* 80 (4):1411-1481
- Skovgaard C., Christensen P M., Larsen S., Andersen TR, Thomassen M., Bangsbo J. (2014). Concurrent speed endurance and resistance training improves performance, running economy, and muscle NHE1 in moderately trained runners. *Journal of Applied Physiology*, 117(10): 1097-1109
- Staron RS, Hagerman FC, Hikida RS, Murray TF, Hostler DP, Crill MT, Ragg KE, Toma K (2000) Fiber type composition of the vastus lateralis muscle of young men and women. *Journal of histochemistry & cytochemistry* 48 (5):623-629
- Wang J, Velotta JB, McDonough AA, Farley RA (2001) All human Na<sup>+</sup>-K<sup>+</sup>-ATPase  $\alpha$ -subunit isoforms have a similar affinity for cardiac glycosides. *American Journal of Physiology-Cell Physiology* 281 (4):C1336-C1343
- Vorup J, Tybirk J, Gunnarsson TP, Ravnholt T, Dalsgaard S, Bangsbo J (2016) Effect of speed endurance and strength training on performance, running economy and muscular adaptations in endurance-trained runners. *European Journal of Applied Physiology* 116 (7):1331-1341. doi:10.1007/s00421-016-3356-4
- Wyckelsma VL, Levinger I, Murphy RM, Petersen AC, Perry BD, Hedges CP, Anderson MJ, McKenna MJ (2017) Intense interval training in healthy older adults increases skeletal muscle [3H]ouabain-binding site content and elevates Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha$ 2 isoform abundance in Type II fibers. *Physiol Rep* 5 (7). doi:10.14814/phy2.13219
- Wyckelsma VL, McKenna MJ, Serpiello FR, Lambole CR, Aughey RJ, Stepto NK, Bishop DJ, Murphy RM (2015) Single-fiber expression and fiber-specific adaptability to short-term intense exercise training of Na<sup>+</sup>-K<sup>+</sup>-ATPase  $\alpha$ - and  $\beta$ -isoforms in human skeletal muscle. *Journal of Applied Physiology* 118 (6):699-706
- Wyckelsma, VL., Perry BD., Bangsbo J., McKenna MJ. (2019). Inactivity and exercise training differentially regulate abundance of Na<sup>+</sup>-K<sup>+</sup>-ATPase in human skeletal muscle. *Journal of Applied Physiology* 127(4): 905-920.







