Effects of testosterone suppression, hind limb immobilization and recovery on [^{3}H]ouabain binding site content and Na^+, K^+-ATPase isoforms in rat soleus muscle

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Running Head: Testosterone loss, disuse, recovery and muscle Na^+,K^+-pumps

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ABSTRACT

We investigated the effects of testosterone suppression, hindlimb immobilization and recovery on skeletal muscle Na^+,K^+-ATPase (NKA), measured via [^{3}H]ouabain binding site content (OB) and (NKA) isoform abundances (α_{1-3}, β_{1-2}). Male rats underwent Castration or Sham surgery plus 7 d rest, 10 d unilateral immobilization (cast) and 14 d recovery, with soleus muscles obtained at each time from cast and non-cast legs. Testosterone reduction did not modify OB or NKA isoforms in non-immobilized control muscles. With Sham, OB was lower after immobilization in the cast than both the non-cast leg (-26%, p=0.023) and the non-immobilized control (-34%, p=0.001), but subsequently recovered. With Castration, OB was lower after immobilization in the cast leg than in the non-immobilized control (-34% p=0.001), and remained depressed at recovery (-34%, p=0.001). NKA isoforms did not differ after immobilization or recovery in Sham. After Castration, α_2 in the cast leg was ~60% lower than the non-cast leg (p=0.004) and non-immobilized control (p=0.004) and after recovery remained lower than the non-immobilized control (-42%, p=0.039). After immobilization, β_1 was lower in the cast than the non-cast leg (-26%, p=0.018), with β_2 lower in the cast, than the non-cast leg (-71%, p=0.004) and non-immobilized control (-65%, p=0.012). No differences existed for α_1 or α_3. Thus, both OB and α_2 decreased after immobilization and recovery in Castration, with α_2, β_1 and β_2 isoform abundances decreased with immobilization compared to Sham. Therefore, testosterone suppression in rats impaired restoration of immobilization-induced lowered number of functional NKA and α_2 isoforms in soleus muscle.

New and newsworthy:

The Na^+,K^+-ATPase (NKA) is vital in muscle excitability and function.

In rats, immobilization depressed soleus muscle NKA, with declines in [^{3}H]ouabain binding, which was restored after 14 days recovery. After testosterone suppression by castration, immobilization depressed [^{3}H]ouabain binding, α_2, β_1 and β_2 isoforms, and abolished subsequent recovery in [^{3}H]ouabain binding and α_2 isoforms.

This may have implications for functional recovery for inactive men with lowered testosterone levels, such as in prostate cancer or aging.

INTRODUCTION

In skeletal muscle, the Na^+,K^+-ATPase (NKA) plays a critical role in regulating [Na^+] and [K^+] gradients across sarcolemmal and t-tubular membranes and therefore affecting membrane excitability, muscle function and fatigue (6, 8) In muscle, the NKA is expressed as three α (α_1 - α_3), and three β (β_1 - β_3) isoforms (47) The α_1 isoform exerts a cellular "housekeeping role" in regulating trans-membrane [Na^+] and [K^+] gradients, affects muscle contractile force (44) and also mass in oxidative muscle (36). The α_2 plays a significant role in preserving muscle contractions and resisting fatigue (56), whereas the exact role of the α_3 isoform in skeletal muscle is unclear. The β subunit is responsible for regulating NKA activity, transporting and stabilising movement of the α subunit from the endoplasmic reticulum to the plasma membrane 19. In skeletal muscle, NKA has traditionally been measured by the [^{3}H]ouabain binding site content (OB), with [^{3}H]ouabain (usually 10^{-6}M) binding to the α subunits, which can then be quantified (8). In rodent muscles OB detects the α_2 isoform, due to differing ouabain affinities of the three α isoforms (8). Hence OB measurements in rat muscles using this standard assay do not detect α_1 and α_3 isoforms and therefore this measure should not be referred to as total content.

Testosterone stimulates protein synthesis and inhibits protein degradation, with ensuing muscle hypertrophy and strength gains (25, 37, 62, 67). Decreased testosterone concentration is therefore associated with declines in muscle mass and strength (4, 40) and can occur with aging (15) and clinical conditions including trauma (57), obesity (63), prostate cancer (58, 27) and type 2 diabetes (64, 65). Patients with prostate cancer receiving androgen deprivation therapy demonstrated reduced lean tissue mass (17), strength and physical performance (18), and orchiectomy in rats lowered muscle strength (5). Despite the importance of NKA for muscle function and of the NKA α_1 isoform for muscle strength and mass, no studies have investigated the impacts of testosterone reduction on skeletal muscle OB or NKA isoforms. Possible testosterone effects on NKA in muscle are however, suggested by findings in other tissues in rats (20, 61). Testosterone reduction via castration led to 47 - 73% decreases in NKA activity in erythrocyte membranes over 1 - 9 months (61). Four weeks following castration, NKA activity was decreased in the mediobasal hypothalamus; subsequent injection of 50 μg testosterone elevated NKA activity 4-fold in the preoptic-suprachiasmatic region of the brain (20). This likely reflects activity associated with the α_3 isoform, given its high abundance in

the brain (3). We therefore investigated whether testosterone suppression via castration would reduce OB and NKA isoform abundances in skeletal muscle.

Immobilization causes substantial muscle atrophy, with increased proteolysis, decreased protein synthesis and a marked decline in muscle strength (27, 35, 55). The effects of immobilization on factors affecting muscle excitability, especially the NKA protein family, are therefore also of interest. Disuse induced by hindlimb immobilization decreased muscle OB, by 20 - 25% in soleus muscle after one week immobilization in rats (29, 69), by 23 - 25% in gastrocnemius and 18-19% in plantaris muscle after 2 - 3 weeks immobilization in guinea pigs (41) and by 39% in vastus lateralis muscle after 9 weeks immobilization in sheep (28). With subsequent recovery, the muscle OB had returned to baseline levels during a 3-21 d period (41, 69). None of these studies investigated the effects of hindlimb immobilization or recovery on specific NKA isoforms, making it unclear whether multiple α, or β isoforms were downregulated. Recently, two studies investigated the effects of short-term hindlimb immobilization, for only 6-12 hours and 1-3 days, on selected NKA isoforms in soleus muscle in rats (31,32). No changes were found in α_1, but the α_2 isoform protein abundance actually increased after brief hindlimb immobilization, which was paradoxically associated with a 72% - 89% decline in electrogenic activity of the α_2 isoform (31,32). Neither study investigated the impacts on OB, nor on the other NKA isoforms expressed in muscle. Furthermore, no studies have investigated the effects of immobilization on muscle NKA using the combined measurements of OB and isoform abundances; this is important for understanding of the effects of hindlimb immobilization and recovery, since OB measures the number of functional NKA. Whether testosterone reduction and hindlimb immobilization might have combined effects on depressing skeletal muscle NKA content and NKA isoform abundances is unknown and given the importance of NKA for muscle function, was also explored here.

This study therefore investigated the effects of testosterone suppression (via castration), hindlimb immobilization and recovery on OB and NKA isoform protein abundances, in soleus muscle in rats. It was hypothesised that both castration and immobilization would reduce skeletal muscle OB as well as the NKA α_2 and β_1 isoform abundances.

METHODS

Animals and Study Design

Male Fischer (F344) rats (n = 47, body mass = 187.5 ± 4.3 g) were purchased at ~8 weeks of age (Animal Resource Centre, Canning Vale, WA, Australia). The study design is indicated in Figure 1. The rats underwent castration or sham surgery; each then recovered for 7 d whilst consuming a standard diet and housed as described elsewhere (43). Rats were randomly allocated into Castration or Sham groups, which were then each divided into two sub-groups, that underwent unilateral hindlimb immobilization to induce substantial muscle atrophy, or served as non-immobilization controls. The unilateral hindlimb immobilization group had the right hindlimb casted for 10 d and the left served as a non-cast control. The sham group comprised non-immobilization controls (n = 8), unilateral immobilization (n = 8), and after 14 d of recovery (n = 8). The castration group comprised non-immobilization controls (n = 7), unilateral immobilization (n = 8), and after 14 d of recovery (n = 8). The design enabled two different control comparisons. The non-immobilized control group underwent either sham or castration surgery but did not undergo immobilization/recovery; thus this served as a control group for comparison of these intervention effects. The benefit of the non-immobilized control group was that this enabled comparison of the limb casting intervention within each of the sham and castration arms. The non-casted leg provided a within-intervention control for comparison against the casted leg. The benefit of the casted leg within either the sham or castrated groups was that this enabled direct comparison between casted and non-casted legs within that intervention arm. One disadvantage of the latter was that the non-casted leg may undergo increased weight bearing activity to compensate for the immobilized limb. This might amplify any differences between legs than if the control was from an animal where neither leg was immobilized. Thus comparisons were made against both non-immobilized control group and the non-casted leg within each of the sham- and castration surgery arms of the study. The non-immobilized 7 d post-surgery refers to control group in both sham and castration groups and labelled as CON in each figure. All experiments and procedures were approved by the Animal Ethics Committee at Victoria University and in accordance with the Australian code of practice for the care and use of animals in scientific research.

142

143 **Experimental Procedures**

144 ***Castration and Sham Surgery Procedures.*** Testosterone concentration was reduced via
145 orchiectomy surgery, which induced a reduction in testosterone of more than 90% as we previously
146 reported (43). The animals were anesthetised by 4% isoflurane anaesthesia in an induction chamber
147 before being transfered to a face mask until the animals were unresponsive to tactile stimuli.
148 Buprenorphine hydrochloride (0.5 mg/kg Meloxicam, Therapon, Burwood, VIC, Australia) was injected
149 at least 30 min before induction, and then the flank was shaved on both sides, area sterilised and
150 incision made. A ligature was placed around the vas deferens and the blood vessels and each testis
151 removed before the incision was closed. After surgery animal were allowed to recover with pain
152 management (0.5 $mg.kg^{-1}$Meloxicam). In sham surgery conducted in control rats, the procedures were
153 identical except that the testes were exposed but not removed.
154 ***Immobilization Procedure.*** A unilateral hindlimb immobilization was performed in all rats except for
155 the non-casted control animals. Following light anaesthesia with 2 - 4% isoflurance, the right limb of
156 each animal was immobilised with casting material in a neutral foot position, such that neither the
157 extensor digitorum longus (EDL) nor soleus muscles were fully lengthened or shortened, respectively.
158 The hindlimb was wrapped with several layers of casting material before being immobilised by a
159 thermoplastic splint. Additional tape was placed to secure the casting material in place. The contra-
160 lateral leg remained loose to allow for ambulation and was utilised as an active control for the
161 immobilised leg of each animal. The casts were inspected and repaired daily as necessary. At the end
162 of 10 d immobilization, the same anaesthetic procedures were used to remove the casting material to
163 begin the regrowth period. As it has been shown previously that non-casted limbs of unilaterally
164 immobilized rats does not differ from non-casted animal limbs (1, 30, 33), the contralateral limb was
165 used as a control.
166 ***Animal sacrifice, muscle sampling, mass and force measures.*** Animals were deeply
167 anaesthetised with sodium pentobarbital (60 $mg.kg^{-1}$; Therapon), and soleus muscles were obtained,
168 before the immobilization period from non-immobilised controls only, following 10 days immobilization
169 and after 14 days of recovery from the cast and non-cast leg (Fig.1). Phenotypic measures were
170 undertaken as part of a larger study (Hanson et al., unpublished). In brief, muscle force and mass
171 (wet weight) were determined at each time point. For force measures, muscles were excised tendon

172 to tendon and were attached to force transducers in a custom-made organ bath (30 °C, pH of 7.4)
173 containing Krebs-Henseleit Ringer's solution bubbled with carbogen. After establishing optimal length,
174 maximal isometric force was determined using supramaximal stimulation (train duration 500 msec, 12
175 volts) of increasing frequency with three minutes of rest between contractions. Following the
176 contractile experiments, muscles were blotted dry and weighed on an analytical balance. The muscle
177 samples were then snap-frozen in liquid nitrogen before being stored at -80 °C for later analyses.
178 Immediately after muscle excision, the animals were culled via an overdose of anaesthetic (sodium
179 pentobarbital 375 mg/kg; Therapon).

180 **Muscle [^{3}H]ouabain binding site content**

181 Approximately 20 mg of muscle was analysed in quadruplicate using the vanadate-facilitated
182 [^{3}H]ouabain binding content method as previously described (50, 54). Each sample was washed for 2
183 x 10 min at 37° C in vanadate buffer (250 mM sucrose, 10 mM Tris·HCl, 3 mM $MgSO_4$, 1 mM $NaVO_4$;
184 pH 7.3). Muscle samples were then incubated for 2 h at 37°C in vanadate buffer with the addition of
185 [^{3}H]ouabain (2.0 Ci^{-1}ml and 10^{-6} M, PerkinElmer, Boston, MA). The muscle was then placed in ice-
186 cold vanadate solution for 4 x 30 min to remove any unbound [^{3}H]ouabain. Muscle samples were
187 blotted on filter paper and weighed before being soaked overnight in 500 µl of 5% trichloroacetic acid
188 and 0.1 mM ouabain. Following this, 2.5 ml of scintillation cocktail (Opti-Fluor, Packard, PerkinElmer,
189 Boston, MA) was added before liquid scintillation counting of [^{3}H]ouabain. The [^{3}H]ouabain binding
190 site content was then calculated on the basis of the sample wet weight and specific activity of the
191 incubation buffer and samples (50, 54). The final [^{3}H]ouabain binding site content (OB) was calculated
192 using a correction factor of 1.33 as previously described for rat muscle (9) to allow for impurity of
193 [^{3}H]ouabain, loss of specifically bound [^{3}H]ouabain during washout and incomplete saturation during
194 the equilibration of muscle with [^{3}H]ouabain, with OB expressed as pmol.g ww^{-1}.

195 **Western Blotting**

196 To determine skeletal muscle Na^+,K^+-ATPase α and β isoform relative protein abundances, ~20 mg of
197 frozen muscle was analysed using western blotting (52) . Muscle proteins were lysed in ice-cold buffer
198 containing 20 mM Tris pH 7.8 (Bio-Rad Laboratories, Hercules, CA), 137 mM NaCl, 2.7 mM KCl
199 (Merck, Kilsyth, Australia), 1 mM $MgCl_2$, 5 mM $Na_4O_7P_2$, 10 mM NaF, 1% Triton X-100, 10% Glycerol
200 (Ajax Finechem, Australia), Protein Inhibitor Cocktail (P8340). All reagents were analytical grade
201 (Sigma-Aldrich, St Louis, MI). Samples were homogenised (1:40 dilution) using a tissueLyser 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 (22). Therefore, muscle Na^+,K^+-ATPase isoform analyses did not include any membrane isolation steps, to maximise recovery of Na^+,K^+-ATPase enzymes (47). Aliquots of the muscle homogenate were mixed with Laemmli sample buffer and proteins were separated on a 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 Na^+,K^+-ATPase isoforms (α_1, α_2, α_3, β_1, and β_2), 10 µg of total protein per sample were loaded in each gel. To ensure that blot density was within the linear range of detection (48), a four to five-point (2.5 - 12.5 µg) calibration curve of whole-muscle crude homogenate was loaded onto every gel. The homogenate was prepared from an equal amount of 5 µg 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_3 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 x5 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 (48).

The following antibodies were used for NKA isoform α_1 (monoclonal α6F, developed by D. Fambrough, obtained from the Developmental Studies Hybridoma Bank, maintained by the University of Iowa, USA), α_2 (polyclonal anti-HERED, Millipore, # 07- 674), α_3 (monoclonal, Thermo Scientific,

Rockford, IL, # MA3-915), β_1 (Millipore, # 05- 382), β_2 (Proteintech # 22338-1-AP) and β_3 (BD Bioscience, # 610993). Validation of antibodies was performed with positive and negative controls using mixed human skeletal muscle homogenate, rat EDL, rat soleus, rat heart and rat kidney as shown in Figure 2. Representative blots for NKA α_1, α_2, α_3, β_1 and β_2 isoforms are shown in Figure 3. The NKA β_3 isoform could not be detected, despite attempts at several total protein amounts and using two different antibodies.

Statistical Analysis

Data were assessed for normality using the Shapiro-Wilk test. A log transformation was used if required, to ensure a normal distribution of data before subsequent analysis. A one-way ANOVA was used to assess the effects of immobilization, castration and recovery on soleus muscle [^{3}H]ouabain binding site content and NKA isoform abundances, within the respective sham group and castration groups. To also determine the effect of castration only on muscle OB and NKA isoforms, the non-immobilised control in the sham group was compared to the castration group non-immobilised control and the non-cast legs, using a one-way ANOVA. Thus, the two main comparisons were firstly to determine the effects of immobilization and recovery on muscle NKA (within sham-only arm) and secondly, to determine any additional or different effects due to testosterone reduction, by examining the effects of immobilization, recovery plus testosterone reduction (within castration arm). A third comparison was utilized to determine whether testosterone reduction per se affected muscle NKA in muscle, by comparing the muscle NKA in the non-immobilization groups across the two arms. Pairwise comparisons utilised the least significant difference post-hoc test. Statistical analyses were conducted using SPSS version 24. Effect size was calculated using Cohen's *d*, where <0.2, 0.2-0.5 and 0.5-0.8 and >0.8 are considered trivial, small, moderate and large, respectively (10). Data are presented as mean ± standard deviation (SD). Statistical significance was accepted at $P < 0.05$.

RESULTS

Testosterone Concentration

The testosterone concentration at day 0 post-immobilization and 14 d recovery for sham rats was 3.11 ±0.63 and 3.61 ±1.68 ng.ml^{-1} and were substantially lower for castrated rats at 0.29 ±0.09 and 0.22 ±0.07 ng.ml^{-1}, respectively (P<0.001).

Soleus Muscle Mass

Within-Sham group comparisons.

Post immobilization, muscle mass in the cast leg was 37% lower than in the non-cast leg (66 ± 6 vs 105 ± 7 mg, respectively, p = 0.001, d = 5.98), and 31% lower than the non-immobilized control group (66 ± 6 vs 96 ± 5 mg, p= 0.001, 5.43). At 14 d recovery, soleus muscle mass in the cast leg was 16% lower than in non-cast leg (92 ± 16 vs 110 ± 13 mg, respectively, p = 0.033, d = 1.23), but did not differ from the non-immobilized control group (92 ± 16 vs 96 ± 5 mg, p= 0.404 d = 0.337).

Muscle mass in the non-cast leg was greater than in the non-immobilized control group at post immobilization (105 ± 7 vs 96 ± 5 mg, respectively, p = 0.031, d = 1.74) and at 14 d recovery (110 ± 13 vs 96 ± 5 mg, respectively, p = 0.019, d = 1.52). To determine whether changes were related to normal body growth or muscle hypertrophy, soleus mass was also expressed relative to body mass. Normalised mass did not differ between the non-cast leg and the non-immobilized control group either post immobilization (0.45 ± 0.023 vs 0.46 ± 0.039 mg.g^{-1}, p= 0.63), or at 14 d recovery (0.39 ± 0.041 vs 0.46 ± 0.039 mg.g^{-1}, p = 0.15).

Within-Castration group comparisons.

Post immobilization, muscle mass in the cast leg was 31% lower than in non-cast leg (70 ± 9 vs 102 ± 8 mg, respectively, p = 0.001, d = 3.75), and 30% lower than non-immobilized control group (70 ± 9 vs 100 ± 7 mg, p = 0.001, d = 3.53). At 14 d recovery, soleus muscle mass in the cast leg was 20% lower than in non-cast leg (79 ±14 vs 99 ± 4 mg, respectively, p = 0.013, d = 1.16), and 21% lower than the non-immobilized control group (79 ±14 vs 100 ± 7 mg, p = 0.036, d =.1.18).

Muscle mass in the non-cast leg did not differ between the non-immobilized control group either at post immobilization (102 ± 8 vs 100 ± 7 mg, respectively, p = 0.64, d = 0.27) or at 14 d recovery (99 ± 4 vs 100 ± 7 mg, respectively, p = 0.83, d = 0.175). Normalised mass at post immobilization was greater in the non-cast leg than the non-immobilized control group (0.46 ± 0.027 vs 0.43 ± 0.020 $mg.g^{-1}$, p= 0.030), but did not differ at 14 d recovery (0.42 ± 0.023 vs 0.43 ± 0.020 $mg.g^{-1}$, p = 0.93).

Soleus Muscle Force

Within-Sham group comparisons.

Post immobilization, muscle force in the cast leg was 62% lower than in the non-cast leg (61 ± 32 vs 159 ± 93 g, respectively, p = 0.001, d = 1.41), and 58% lower than the non-immobilized control group (61 ± 32 vs 144 ± 42 g, p = 0.001, d= 2.92). At 14 d recovery, muscle force in the cast leg was 19% lower than in non-cast leg (149 ± 56 vs 184 ± 59 g, respectively, p = 0.026, d = 0.63), but did not differ from the non-immobilized control group (149 ± 56 vs 143 ± 42 g, p = 0.835, d = 0.121).

Muscle force in the non-cast leg did not differ from the non-immobilized control group either at post immobilization (159 ± 93 vs 144 ± 42 g, respectively, p = 0.81, d = 0.207) or at 14 d recovery (184 ± 59 vs 144 ± 42 g, respectively, p = 0.29, d = 0.781).

Within-Castration group comparisons.

Post immobilization, muscle force in the cast leg was 44% lower than in non-cast leg (76 ± 54 vs 135 ± 52 g, respectively, p = 0.025, d = 1.123), and 38% lower than non-immobilized control group (76 ± 54 vs 123 ± 12 g, p = 0.034, d = 1.20). At 14 d recovery, muscle force in the cast leg did not differ from the non-cast leg (79 ± 42 vs 109 ± 41 g, respectively, p = 0.208, d = 0.69), but was 35% lower than the non-immobilized control group (79 ± 42 vs 122 ± 12 g, p = 0.036, d = 1.39).

Muscle force in the non-cast leg did not differ between the non-immobilized control group either at post immobilization (135 ± 5 vs 123 ± 12 g, respectively, p = 0.65, d = 1.30) or at 14 d recovery (109 ± 41 vs 123 ± 12 g, respectively, p = 0.53, d = 0.463).

Effects of immobilization and testosterone reduction on [^{3}H]ouabain binding site content

Within-Sham group comparisons.

Post-immobilization, the muscle OB in the cast leg was 26% lower than in the non-cast leg ($p = 0.023$; $d = 1.34$) and 34% lower ($p = 0.001$; $d = 1.69$) than in the non-immobilized control group (Fig. 4A). At 14 d recovery, the muscle OB had recovered, such that there were no differences in the cast leg compared to either the non-cast leg ($p = 0.391$; $d = 0.540$) or non-immobilized control group ($p = 0.833$; $d = 0.093$, Fig. 4A).

Within-Castration group comparisons.

Post immobilization, the muscle OB in the cast leg did not differ significantly from the non-cast leg ($p = 0.163$; $d = 0.996$), but was 34% lower than in the non-immobilized control group ($p= 0.001$; $d = 3.03$, Fig.4B). At 14 d recovery, the OB in the cast leg remained depressed, being 34% lower than in the non-immobilized control group ($p = 0.001$; $d = 2.02$, Fig 4B), but was not different from the non-cast leg ($p = 0.456$; $d = 0.30$).

Effects of immobilization and testosterone reduction on NKAα and β isoform abundances

Within-Sham group comparisons.

NKA α_1, α_2 and α_3 isoforms. There were no significant differences between groups within-sham comparisons for NKA α_1 ($p = 0.876$, Fig. 5A), α_2 although considerable variability was noted ($p = 0.835$, Fig. 6A), or for α_3 ($p = 0.990$, Fig 7A).

NKA β_1 and β_2 isoforms. There were no significant differences between groups within-sham group comparisons for β_1 ($p = 0.661$, Fig. 8A) or β_2 ($p = 0.656$, Fig. 9A).

Within-Castration group comparisons.

NKA α_1 and α_3 isoforms. There were no significant differences between groups within-castration comparisons for NKA α_1 ($p = 0.754$, Fig. 5B) or for α_3 ($p = 0.641$, Fig. 7B).

NKA α_2 isoform. Post-immobilization, the NKA α_2 isoform abundance in the cast leg was ~60% lower than in both the non-cast leg ($p = 0.004$; $d = 1.38$) and non-immobilized control group ($p = 0.004$; $d = 1.37$, Fig. 6B). At 14 d recovery, the NKA α_2 isoform abundance remained depressed in the cast leg being 42% lower than the non-immobilized control group ($p = 0.039$; $d = 0.980$), but was not different from the non-cast leg ($p = 0.812$, $d = 0.340$, Fig 6B).

NKA β_1 isoform. Post-immobilization, the NKA β_1 isoform abundance in the cast leg was 26 % lower than in the non-cast leg ($p = 0.018$, $d = 1.09$), but did not differ from the non-immobilized control group ($p = 0.321$, $d = 0.43$, Fig. 8B). At 14 d recovery, there was no significant differences in the NKA β_1

isoform abundance in cast leg compared to either the non-cast leg ($p = 0.427$, $d = 0.65$) or non-immobilized control group ($p = 0.251$, $d = 0.51$, Fig. 8B).

NKA β_2 isoform. Post-immobilization, the NKA β_2 isoform abundance was 71% lower than the non-cast leg ($p = 0.004$, $d = 1.60$) and 65% lower than the non-immobilized control group ($p = 0.012$; $d = 0.76$, Fig. 9B). At 14 d recovery, the NKA β_2 isoform abundance in the cast leg was not different from the non-cast leg ($p = 0.674$; $d=0.293$) or non-immobilized control group ($p = 0.267$; $d=0.35$, Fig. 9B).

Effects of testosterone reduction on [^{3}H]ouabain binding site content and NKA isoform abundance in non-immobilized muscle

The soleus muscle OB and NKA isoform abundances in the sham non-immobilized control group were compared to castration non-immobilized control group (7 d post-surgery) and to those of the non-immobilized legs in the castration groups (17 d and 31d post-surgery), to determine the effects of testosterone suppression via castration alone, i.e. independent of hindlimb immobilization.

[^{3}H]ouabain binding site content. The sham control OB did not differ from the castration control (i.e., at 7 d post-surgery, $p=0.884$, $d=0.73$), but was higher than in the other castration non-cast legs, at post-immobilization (i.e., 17 d post-surgery, $p=0.016$, $d=1.363$) and recovery (i.e., 31 d post-surgery, $p=0.0123$, $d=1.425$), indicating a depressive effect of testosterone suppression on muscle OB.

NKA α_2 isoform. The NKA α_2 isoform abundance in the sham non-immobilized control did not differ from the castration non-immobilized control (7 d post-surgery, $p=0.695$, $d=-0.193$) or post-immobilization groups (17 d post-surgery, NS, $p=0.51$, $d=0.021$), but was greater than castration non-immobilized recovery (31 d post-surgery, $p=0.020$, $d=1.639$).

Other NKA isoforms. There were no differences between the sham control and the castration control non-immobilized legs (7 d post-surgery) for the NKA α_1 ($p = 0.228$; $d = 0.050$), α_3 ($p = 0.255$; $d = -0.374$), β_1 ($p = 0.859$; $d = 0.075$) or β_2 isoforms ($p = 0.358$; $d = -0.098$). There were also no significant differences with these isoforms at 17 d or 31 d post-surgery, although a tendency was noted to a lower α_3 in the castration recovery non-cast legs ($p=0.055$, $d = 1.124$).

DISCUSSION

This study investigated the effects of testosterone suppression via castration, as well as hindlimb immobilization for 10 d and subsequent 14 d recovery, on NKA downregulation and restoration, measured via both [^{3}H]ouabain binding site content (OB) and isoform protein abundances in soleus muscle in rats. There were three novel findings. First, when animals underwent disuse via hindlimb immobilization, reductions in muscle OB occurred in both sham and castrated groups, but whilst muscle OB had returned to control levels in sham animals after 14 d recovery, this remained depressed in the castration group. Despite the reduction with immobilization in muscle OB in the sham group, there were no changes in any of the α_1, α_2, α_3, β_1 or β_2 isoforms. Second, when castration was combined with hindlimb immobilization, depressive effects on NKA isoforms were found in each of the NKA α_2, β_1 and β_2 isoform abundances following the hindlimb immobilization, and with NKA α_2 remaining suppressed at 14 d in the castration group. Third, whilst testosterone suppression per se did not initially adversely affect the muscle OB or NKA isoform abundances in the non-immobilized muscle at 7 d post-surgery, a depressive effect on OB was manifest at both 17 d and 31 d post-surgery; furthermore the α_2 isoform abundance was also lower at 31 days post-surgery. This suggests that reduced testosterone also lowered skeletal muscle NKA. Hence, reduced testosterone lowered muscle OB and impaired recovery of NKA post-immobilization, with declines also evident independent of immobilization. This may have important implications for rehabilitation of patients that suffer inactivity, reduced muscle mass and lowered testosterone, such as those undergoing androgen deprivation therapy.

Testosterone reduction downregulated muscle NKA and prevented restoration of muscle OB after hindlimb immobilization

Castration per se, which resulted in a 90% reduction in testosterone (43), did not initially reduce NKA in rat soleus muscle, with no changes found in muscle OB, or in any NKA isoforms at 7 days post-surgery. This was despite substantial 31% and 58% reductions in muscle mass and force after immobilization, compared with non-immobilized control animals respectively, and 37% and 62% reductions compared with the non-cast leg. Importantly, however, when the control sham non-immobilized leg was compared to the castration non-immobilized legs, muscle OB was depressed

after the 10 d period associated with immobilization and also the further 14 d recovery, with α_2 also depressed in the longer recovery period. This implies that androgen depletion treatments which markedly suppress testosterone levels, as applied to prostate cancer patients, might also be expected to reduce muscle NKA, although this remains to be determined in human muscle.

Hindlimb immobilization for 10 d reduced muscle OB by 34% in rat soleus muscle, in animals that underwent sham surgery. This is consistent with previously reported reductions in rat soleus muscle (29, 66), guinea pig gastrocnemius muscles (41) and sheep vastus lateralis muscle (28). This finding is also consistent with the ~23-34% decreases in muscle OB in humans characterised by greatly reduced muscle activity, including patients with inactive muscle shoulder impingement syndrome (42), complete spinal cord injury 12 and knee-ligament injury (51). Furthermore, the muscle OB had returned to the baseline at 14 days post-immobilization, also consistent with two earlier reports (41, 66). Thus the expected immobilization-induced suppression and subsequent recovery of muscle OB was observed in sham animals.

The major finding is that testosterone suppression prevented the recovery of muscle OB after hindlimb immobilization, with OB depressed by 34% both after immobilization and at 14 days recovery. This clearly contrasts the full recovery in OB found in the sham group and indicates a reduction in the number of functional NKA. This reduction was evident in non-immobilized muscle indicating a systemic effect of testosterone suppression. This may therefore be important during muscle contractions, potentially impairing cellular Na^+ efflux, K^+ influx, membrane potential and excitability, with implications for early muscular fatigue (8, 45, 60).

The α_2 is the most abundant of the α subunits in rat soleus muscles, comprising ~ 80 - 85% of α subunits, with α_1 representing most of the remaining α subunits 24. The α_3 abundance in muscle is unknown, but thought to be low (22, 24). In rat muscle, the standard ouabain binding site method employed here detects the α_2 isoform, due to its high affinity for ouabain (8). Thus depressed OB with immobilization and testosterone suppression is expected to represent primarily downregulation of the NKA α_2 isoform. Consistent with this, a clear finding in the castrated animals was the 60% downregulation in the α_2 isoform following hindlimb immobilization, and which remained 42% depressed after 14 days of recovery, compared to non-immobilized control muscles. These reductions coincided with the acute and sustained reductions in muscle OB. A further interesting finding was that

422 the decrease in NKA α_2 isoform protein abundance after immobilization (60%) was twice the decline in
423 OB (32%). However, the NKA α_2 isoform was not reduced in the sham group following hindlimb
424 immobilization, despite the substantial reduction of [^{3}H]ouabain binding in the same group; although a
425 moderate effect size was found after immobilization even after 14 days of recovery. This lack of
426 decrease in NKA α_2 was surprising, and may be due to the semi-quantitative, non-molar and variable
427 nature of western blot analysis, making it difficult to detect the changes. This finding appears to
428 contrast with recent studies where short-term hindlimb suspension in the rat for 6-12 hours and for 1-3
429 days, initially increased the NKA α_2 protein, but depressed the associated electrogenic activity (29,
430 30, 31, 32). Whether this discrepancy simply reflects a time-course effect of an initial increase
431 followed by reductions is unclear.
432 The qualitative disparity between the reductions in muscle OB and the NKA α_2 isoform after
433 immobilization and recovery in the castrated animals, as well as the lack of change in NKA α_2 isoform
434 after immobilization in sham animals, may reflect biological and methodological factors. Firstly, the
435 [^{3}H]ouabain binding site content method detects binding in functional pumps (i.e. αβ complexes),
436 whereas the western blot measures all isoforms present in the tissue. It is therefore possible that a
437 considerable fraction of the α_2 isoforms are not present as functional pump complexes, which might
438 change with the immobilization intervention. However, a closer correspondence between findings
439 using the different methods was found in recovery. We recently observed a similar discrepancy after
440 injury and chronic inactivity in humans, where the muscle α_2 isoform abundance was 63% lower in the
441 knee-injured leg than the non-injured leg, associated with ~20% lower muscle OB (51). In contrast,
442 after short-term inactivity alone, induced by unilateral lower limb suspension, both the α_2 isoform
443 abundance and muscle OB were unchanged (53). The [^{3}H]ouabain binding technique is based on
444 intact portions of muscle tissue and is a fully quantitative method, whereas the western blot analysis is
445 based on a standardised total protein concentration from a muscle homogenate. Discrepancies in α_2
446 isoform findings between the groups may in part also reflect the variable nature of western blot
447 analysis of NKA proteins (7).
448 Regardless, this sustained reduction in the muscle OB and α_2 isoform with castration and
449 immobilization may contribute to impaired muscle excitability and contractility. The NKA α_2 isoform
450 plays a major role in Na^+/K^+ transport during muscle contractions (11), partial global knockout of NKA
451 α_2 caused impaired contractile force in mouse EDL muscle (44), whilst muscle-specific NKA α_2

knockout in mice reduced muscle strength, increased muscle fatigability and reduced exercise performance (56). A clear finding from the current study was that the NKA α_1 isoform was not affected by testosterone suppression, or hindlimb immobilization. This is the first time this has been shown for the more extended immobilization periods over which OB has typically been measured. This is important given that α_1 represents ~15 - 25% of the NKA α isoforms in rat soleus muscle (21). Thus, observed reductions in muscle OB with immobilization were not underestimated due to the method failing to detect additional reductions in α_1 isoforms. The unchanged α_1 isoform protein abundance with immobilization is also consistent with unchanged α_1 isoform electrogenic activity after short-term hindlimb immobilization for 6 hours and 1-3 days in rat soleus muscle (31, 32).

This is the first study showing that the NKA α_3 isoform abundance was not affected by hindlimb immobilization or testosterone reduction. A tendency to lowered α_3 with testosterone suppression per se was however evident in non-immobilized muscles compared to the sham controls, suggesting a possible small effect 31 days after surgery. In human vastus lateralis muscle, the NKA α_3 isoform abundance measured in muscle homogenates was not reduced after either chronic knee-injury or 23 d of unilateral lower limb suspension (51, 53). However, in type I single muscle fibres, the NKA α_3 isoform abundance decreased after unilateral lower limb suspension (53). In skeletal muscle, the function and abundance of the NKA α_3 remain unknown, but the lack of change in α_3 indicates these isoforms are unlikely to play major adverse roles in either immobilization, or castration.

This study also demonstrated NKA β_1 and β_2 isoform downregulation with immobilization in the castration, but not in the sham group. There were large variations in the measurements of β_1 and β_2 isoforms, consistent with recent reports (7), which should be considered together with the small sample size when interpreting the data. In the cast leg, the β_1 isoform decreased in the castration group by 26% only compared to non-cast leg, whereas the β_2 isoform was 71% and 65% lower than in the non-cast leg and control group, respectively. This suggests that a reduction in both $\alpha_2\beta_1$ and $\alpha_2\beta_2$ complexes account for the reduction in muscle OB with immobilization. In the rat, the β_1 isoform is the most abundant in muscles rich in slow-twitch fibres such as in soleus muscle, whilst the β_2 is more abundant in muscles rich in fast twitch fibres such as in EDL muscle (16, 70). However, the relative function of these NKA β isoforms remains incompletely understood, in skeletal muscle. The β isoform is essential for regulating NKA activity (39), and transporting and stabilising the movement of the α isoform from the endoplasmic (sarcoplasmic) reticulum to the plasma membrane (39, 59). Therefore,

482 downregulation in NKA β_1 and β_2 isoform abundances with castration and immobilization are likely to
483 be functionally important, and may reflect reduced skeletal muscle NKA activity thereby potentially
484 impacting adversely on muscle NKA activity and fatigue (39). We are unclear as to why reductions in
485 β_1 and β_2 isoform did not occur with immobilization in the sham group.
486 Reductions in testosterone have been reported to lead to increased total fat mass (37). An increased
487 fat infiltration into muscle, might then artefactually lower the [^{3}H]ouabain binding site content, since
488 this is expressed per gram of muscle. It seems unlikely that this effect could however, account for the
489 large reductions evident in OB after immobilization in the castrated rats (-34%) and since similar
490 reductions also occurred in the sham group (-26%, -34%) when testosterone was normal. Thus this
491 reduction is unlikely to be due to a fat infiltration artefact. In addition, future studies should measure
492 fat free mass to correct for any such effect of the measured [^{3}H]ouabain binding site content. This
493 effect would probably be less on Western Blot measurements of isoform abundances since these are
494 conducted on samples of known protein content, but a possible small effect cannot be excluded.
495 ***Mechanisms of NKA downregulation in muscle with castration and immobilization***
496 The mechanisms underlying the decrease in muscle OB and the α_2 isoform in skeletal muscle with
497 castration and immobilization cannot be ascertained from the results of this study. One possibility is
498 that the contractile-induced increases in muscle intracellular [Na^+] (13, 49) are greatly lessened with
499 immobilization simply due to reduced muscle contractions. However, increased intracellular Na^+
500 content was not associated with elevated NKA mRNA (46). Although no initial effects of testosterone
501 were observed here on muscle OB or any α or β isoforms in the non-immobilization control muscle,
502 depression of OB was evident in subsequent measures in non-immobilized muscles and a lowered α_2
503 was also evident in recovery; this also means that recovery of already depressed muscle OB and α_2
504 isoform was absent in castrated rats. The possible mechanism of testosterone suppression effects on
505 recovery of already lowered abundance of NKA might also occur via muscle Na^+ ion regulation, as
506 testosterone regulates intracellular Na^+ in other tissues (14, 38). Reduced muscle protein synthesis
507 was found with androgen deprivation therapy (23). This suggests one possible mechanism
508 responsible for the lack of recovery of OB after castration, but no evidence exists to support or refute
509 this. Further research is warranted on testosterone effects on intracellular ions, fluid dynamics and
510 NKA regulation in skeletal muscle, including signalling mechanisms such as ERK1/2. A possible
511 mechanism for decreased [^{3}H]ouabain binding site content is via impairment or lesser activity of

signalling pathways via the non-ion transducing role of NKA. This may occur as result of direct-protein interactions between NKA and its neighbouring proteins, which triggers a signalling cascade culminating in decreased NKA gene transcription (2, 67, 68). Since this effect has been found to be specific to the α_1 isoform, this mechanism seems unlikely given no changes in α_1 were observed with immobilization and castration. However, the recently reported role for α_1 in muscle mass regulation in oxidative muscles (36) indicates this pathway requires further research with disuse. Finally, the impacts of these changes on muscle NKA activity were not studied here but may be of considerable importance. This seems likely given the large reductions in electrogenic activity of the NKA α_2 with brief immobilization in rat soleus muscle (31, 32) and would be worthy of further exploration.

Conclusions

Testosterone suppression via castration did not initially modify muscle NKA in control muscles, or alter the reduction with immobilization, but subsequent depressive effects were found for NKA content and this also clearly prevented its recovery. This was linked with reduction in the α_2 isoform, whereas neither hindlimb immobilization nor testosterone suppression affected the muscle NKA α_1 or α_3 isoform abundances. Both the muscle NKA β_1 and β_2 isoforms were also decreased with immobilization in the castration group, indicating likely reductions in both NKA $\alpha_2\beta_1$ and $\alpha_2\beta_1$ complexes. These findings open a new promising area of research into the effects of testosterone on skeletal muscle NKA, and also point to a need for further study in humans. These results may have important implications in determining mechanisms to facilitate recovery in men with lower testosterone levels, after significant period of disuse, such as in prostate cancer patients undergoing androgen deprivation therapy, type 2 diabetes and in the elderly.

535 **Figure 1.** Experimental design overview showing A) time line of surgery, immobilization and recovery,
536 and B) Castration and Sham groups, subgroups and sample sizes.
537 Animals were sacrificed and soleus muscles were obtained from F344 inbred male rats at each of the
538 following time points: 7 d after surgery and just prior to immobilization period (-10 d) only from non-
539 immobilised controls; immediately following the 10 d immobilization period (+0); and after 14 d of
540 recovery (+14) from both sham and castration groups.
541
542 **Figure 2.** Validation of antibodies used to quantify NKA isoforms.
543 Total of 10 µg protein of mixed human skeletal muscle homogenate (Hu), rat extensor digitorum
544 longus muscle (EDL), rat soleus muscle (SOL), rat heart (Hrt) and rat kidney (Kid) were loaded onto
545 each gel. Values at right indicate molecular weight of bands and at left indicate the NKA isoform.
546 The NKA α_1 antibody (#a6F) detected a band at the predicted molecular weight ~105 kDa in human
547 skeletal muscle, rat EDL muscle, rat SOL muscle, rat heart and rat kidney. The NKA α_2 polyclonal
548 antibody (Millipore #07-674) detected a clear band at the predicted molecular weight of ~105 kDa in
549 human skeletal muscle, rat EDL muscle, rat SOL muscle and rat heart, but was absent in rat kidney.
550 The NKA α_3 antibody (Thermo Scientific #MA3-915) detected band at the predicted molecular weight
551 of ~105 kDa in rat SOL muscle but was absent in human skeletal muscle, rat EDL muscle, rat heart
552 and rat kidney. The NKA β_1 antibody (Thermo Scientific #MA3-930) specifically designed to detect β_1
553 protein only in human muscle detected a clear band at the predicted molecular weight ~50kDa in
554 human skeletal muscle and was absent in rat EDL muscle, rat SOL muscle, rat heart and rat kidney.
555 The second NKA β_1 antibody (Millipore, # 05- 382) detected a band at the expected molecular weight
556 of ~50kDa in human skeletal muscle, rat EDL muscle, rat SOL muscle and rat heart, but was absent
557 in rat kidney. The NKA β_2 antibody (Proteintech #22338-1-AP) detected a band at the predicted
558 molecular weight in human skeletal muscle, rat EDL muscle (albeit weak), rat SOL muscle, rat heart
559 and rat kidney. The NKA β_3 could not be detected by two different antibodies used (BD Bioscience, #
560 610993) and BD Bioscience, # 610992).
561
562

563 **Figure 3.** Representative immunoblots of NKA α_1, α_2, α_3, β_1 and β_2 isoforms in homogenates of rat
564 soleus muscle.

565 Values at left indicate molecular weight of bands. Protein bands from left to right are (SC) sham non-
566 immobilised control group, (CC) castration non-immobilised control group, (SNCL) sham non-cast leg,
567 (SCL) sham cast leg, (CNCL) castration non-cast leg, (CCL) castration cast leg. 1, 2, 3 and 4 are
568 calibration curve loaded with 2.5.- 12.5µg whole-muscle crude homogenate. The homogenate was
569 prepared from an equal amount from each sample.

570

571 **Figure 4.** Effects of 10 d hindlimb immobilization and castration in rats on soleus muscle [^{3}H]ouabain
572 binding site content from (A) sham and (B) castration groups.
573 CON, non-immobilization control group; Post immob, 0 d following immobilization; Recovery, 14 d of
574 recovery after immobilization. Values are mean ± SD, n = 7 - 8 per group. Samples were compared by
575 one-way ANOVA, *cast leg less than control group, $p < 0.001$. & cast leg less than non-cast leg, $p <$
576 0.05

577

578 **Figure 5.** Effects of 10 d hindlimb immobilization and castration in rats on soleus muscle NKA α_1
579 isoform protein abundance from (A) sham group and (B) castration group.
580 CON, non-immobilization control group; Post immob, 0 d following immobilization; Recovery, 14 d of
581 recovery after immobilization. Values are mean ± SD in arbitrary units (a.u.), n = 7 - 8 per group.
582 Samples were compared by one-way ANOVA,

583

584 **Figure 6.** Effects of 10 d hindlimb immobilization and castration in rats on soleus muscle NKA α_2
585 isoform protein abundance from (A) sham group and B) castration group.
586 CON, non-immobilization control group; Post immob, 0 d following immobilization; Recovery, 14 d of
587 recovery after immobilization. Values are mean ± SD in arbitrary units (a.u.), n = 7 - 8 per group.
588 Samples were compared by one-way ANOVA, *cast leg less than control group $p < 0.001$, & cast leg
589 less than non-cast leg, $p < 0.05$.

590

591 **Figure 7.** Effects of 10 d hindlimb immobilization and castration in rats on soleus muscle NKA α_3
592 isoform protein abundances from (A) sham group and (B) castration group.
593 CON, non-immobilization control group; Post immob, 0 d following immobilization; Recovery, 14 d of
594 recovery after immobilization. Values are mean ± SD in arbitrary units (a.u.), n = 7 - 8 per group.
595 Samples were compared by one-way ANOVA,

596 **Figure 8.** Effects of 10 hindlimb immobilization and castration in rats on soleus muscle NKA β_1
597 isoform protein abundance from (A) sham group and (B) castration group.
598 CON, non-immobilization control group; Post immob, 0 d following immobilization; Recovery, 14 d of
599 recovery after immobilization. Values are mean ± SD in arbitrary units (a.u.), n = 7 - 8 per group.
600 Samples were compared by one-way ANOVA, & cast leg less than non-cast leg, $p < 0.05$.
601

602 **Figure 9.** Effects of 10 d hindlimb immobilization and castration in rats on soleus muscle NKA β_2
603 isoform protein abundances from (A) sham group and (B) castration group.
604 CON, non-immobilization control group; Post immob, 0 d following immobilization; Recovery, 14 d of
605 recovery after immobilization.Values are mean ± SD in arbitrary units (a.u.), n = 7 - 8 per group.
606 Samples were compared by one-way ANOVA, *cast leg less than non-immoblisation control group, p
607 < 0.05, & cast leg less than non-cast leg, $p < 0.05$.
608
609

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