J Appl Physiol 128: 501–513, 2020.
First published December 19, 2019; doi:10.1152/japplphysiol.01077.2018.

RESEARCH ARTICLE

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

Muath M. Altarawneh,[1] Erik D. Hanson,[1,2] Andrew C. Betik,[1,3] Aaron C. Petersen,[1] Alan Hayes,[1,4] and Michael J. McKenna[1]

[1]*Institute for Health and Sport, Victoria University, Melbourne, Australia;* [2]*Department of Exercise and Sport Science, University of North Carolina at Chapel Hill, North Carolina;* [3]*Institute for Physical Activity and Nutrition, School of Exercise and Nutrition Sciences, Deakin University, Geelong, Australia; and* [4]*Australian Institute for Musculoskeletal Science, Victoria University, Melbourne, Australia*

Submitted 7 December 2018; accepted in final form 18 December 2019

Altarawneh MM, Hanson ED, Betik AC, Petersen AC, Hayes A, McKenna MJ. Effects of testosterone suppression, hindlimb immobilization, and recovery on [^{3}H]ouabain binding site content and Na^+, K^+-ATPase isoforms in rat soleus muscle. *J Appl Physiol* 128: 501–513, 2020. First published December 19, 2019; doi:10.1152/japplphysiol.01077.2018.—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 days of rest, 10 days of unilateral immobilization (cast), and 14 days of recovery, with soleus muscles obtained at each time from cast and noncast legs. Testosterone reduction did not modify OB or NKA isoforms in nonimmobilized control muscles. With sham surgery, OB was lower after immobilization in the cast leg than in both the noncast leg (−26%, $P = 0.023$) and the nonimmobilized control (−34%, $P = 0.001$), but OB subsequently recovered. With castration, OB was lower after immobilization in the cast leg than in the nonimmobilized control (−34%, $P = 0.001$), and remained depressed at recovery (−34%, $P = 0.001$). NKA isoforms did not differ after immobilization or recovery in the sham group. After castration, α_2 in the cast leg was ~60% lower than in the noncast leg ($P = 0.004$) and nonimmobilized control ($P = 0.004$) and after recovery remained lower than the nonimmobilized control (−42%, $P = 0.039$). After immobilization, β_1 was lower in the cast than the noncast leg (−26%, $P = 0.018$), with β_2 lower in the cast leg than in the noncast leg (−71%, $P = 0.004$) and nonimmobilized 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 the castration group, with α_2, β_1, and β_2 isoform abundances decreased with immobilization compared with the sham group. Therefore, testosterone suppression in rats impaired restoration of immobilization-induced lowered number of functional NKA and α_2 isoforms in soleus muscle.

NEW & NOTEWORTHY: 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, depressed 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.

disuse; castration; Na^+,K^+-ATPase; ouabain; testosterone

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, in 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 transmembrane [Na^+] and [K^+] gradients, affects muscle contractile force (44), and also affects 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 and transporting and stabilizing 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 because of 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 (27, 58,), and type 2 diabetes (64, 65). Patients with prostate cancer who received 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

Address for correspondence: M. J. McKenna, Institute for Health and Sport, Victoria Univ., Footscray Park Campus, 70–104 Ballarat Road, Footscray, 3011 Victoria, Australia (e-mail: michael.mckenna@vu.edu.au).

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 mo (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 1-wk immobilization in rats (29, 69), by 23%–25% in gastrocnemius muscle and 18%–19% in plantaris muscle after 2–3-wk immobilization in guinea pigs (41), and by 39% in vastus lateralis muscle after 9-wk immobilization in sheep (28). With subsequent recovery, the muscle OB had returned to baseline levels during a 3–21-day 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, 2 studies investigated the effects of short-term hindlimb immobilization (for only 6–12 h 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 or 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 because 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 hypothesized 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 wk of age (Animal Resource Centre, Canning Vale, WA, Australia). The study design is indicated in Fig. 1. The rats underwent castration or sham surgery; each then recovered for 7 days while consuming a standard diet and housed as described elsewhere (43). Rats were randomly allocated into castration or sham groups that were then each divided into two subgroups that underwent unilateral hindlimb immobilization to induce substantial muscle atrophy or served as nonimmobilization controls. The unilateral hindlimb immobilization group had the right hindlimb casted for 10 days, and the left served as a noncast control. The sham group comprised nonimmobilization controls (n = 8), a unilateral immobilization subgroup (n = 8), and a subgroup after 14 days of recovery (n = 8). The castration

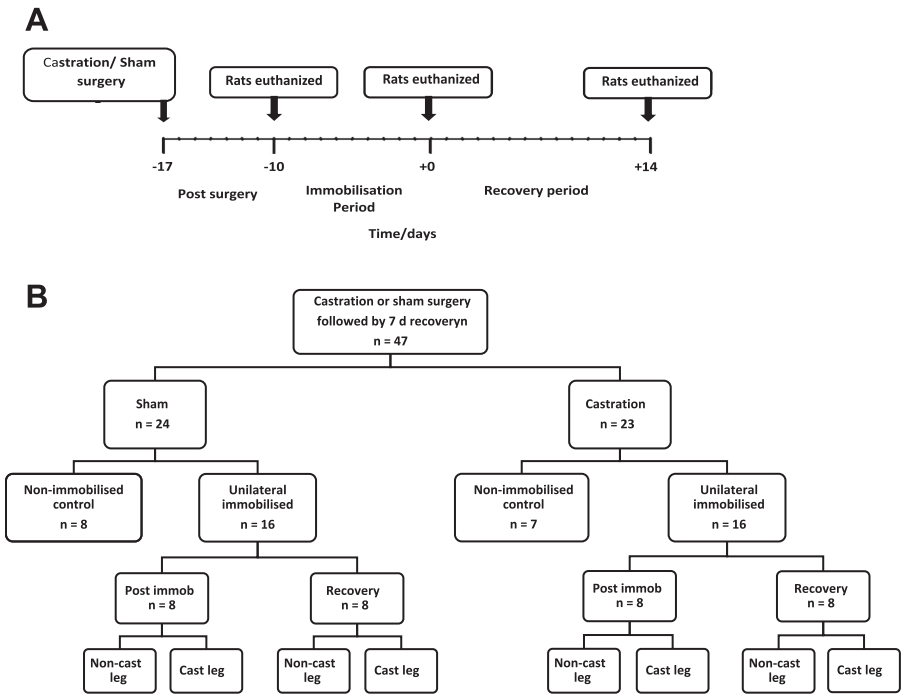

Fig. 1. Experimental design overview. *A*: timeline of surgery, immobilization, and recovery. *B*: castration and sham groups, subgroups, and sample sizes. Animals were euthanized, and soleus muscles were obtained from F344 inbred male rats at each of the following time points: 7 days after surgery (from nonimmobilized controls only), just before immobilization period (−10 days) (from nonimmobilized controls only), immediately following the 10-day immobilization period (+0) (from both sham and castration groups), and after 14 days of recovery (+14) (from both sham and castration groups).

group comprised nonimmobilization controls ($n = 7$), a unilateral immobilization subgroup ($n = 8$), and a subgroup after 14 days of recovery ($n = 8$). The design enabled two different control comparisons. The nonimmobilized 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 nonimmobilized control group was that this enabled comparison of the limb casting intervention within each of the sham and castration arms. The noncasted 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 noncasted legs within that intervention arm. One disadvantage of the latter was that the noncasted leg may have undergone increased weight bearing activity to compensate for the immobilized limb. This might amplify any differences between legs compared with a control from an animal in which neither leg was immobilized. Thus comparisons were made against both nonimmobilized control group and the noncasted leg within each of the sham and castration surgery arms of the study. The nonimmobilized 7 days post surgery refers to control group in both sham and castration groups and is labeled 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.

Experimental Procedures

Castration and sham surgery procedures. Testosterone concentration was reduced via orchiectomy surgery, which induced a reduction in testosterone of more than 90%, as we previously reported (43). The animals were anesthetized by 4% isoflurane anesthesia in an induction chamber before being transferred to a face mask until the animals were unresponsive to tactile stimuli. Buprenorphine hydrochloride (0.5 mg/kg meloxicam, Therapon, Burwood, VIC, Australia) was injected at least 30 min before induction, and then the flank was shaved on both sides, the area was sterilized, and the incision was made. A ligature was placed around the vas deferens and the blood vessels, and each testis was removed before the incision was closed. After surgery, animals were allowed to recover with pain management (0.5 mg/kg meloxicam). In sham surgery conducted in control rats, the procedures were identical except that the testes were exposed but not removed.

Immobilization procedure. A unilateral hindlimb immobilization was performed in all rats except for the noncasted control animals. Following light anesthesia with 2%–4% isoflurane, the right limb of each animal was immobilized with casting material in a neutral foot position, such that neither the extensor digitorum longus (EDL) nor the soleus muscles were fully lengthened or shortened, respectively. The hindlimb was wrapped with several layers of casting material before being immobilized by a thermoplastic splint. Additional tape was placed to secure the casting material in place. The contralateral leg remained loose to allow for ambulation and was utilized as an active control for the immobilized leg of each animal. The casts were inspected and repaired daily as necessary. At the end of 10 days of immobilization, the same anesthetic procedures were used to remove the casting material to begin the regrowth period. As it has been shown previously that noncasted limbs of unilaterally immobilized rats do not differ from noncasted animal limbs (1, 30, 33), the contralateral limb was used as a control.

Animal euthanasia, muscle sampling, mass measures, and force measures. Animals were deeply anesthetized with 60 mg/kg sodium pentobarbital (Therapon), and soleus muscles were obtained before the immobilization period from non-immobilized controls only and were obtained following 10 days of immobilization and after 14 days of recovery from the cast and noncast legs (Fig. 1). Phenotypic measures were undertaken as part of a larger study (Hanson E, Betik A, Timpani CA, Tarle J, Zhang X, Hayes A, unpublished observations). In brief, muscle force and mass (wet weight) were determined at each time point. For force measures, muscles were excised tendon to tendon and were attached to force transducers in a custom-made organ bath (30°C, pH of 7.4) containing Krebs-Henseleit Ringer's solution bubbled with carbogen. After establishing optimal length, maximal isometric force was determined using supramaximal stimulation (train duration 500 ms, 12 V) of increasing frequency with 3 min of rest between contractions. Following the contractile experiments, muscles were blotted dry and weighed on an analytical balance. The muscle samples were then snap-frozen in liquid nitrogen before being stored at −80°C for later analyses. Immediately after muscle excision, the animals were culled via an overdose of anesthetic (375 mg/kg sodium pentobarbital; Therapon).

Muscle [^{3}H]ouabain Binding Site Content

Approximately 20 mg of muscle was analyzed in quadruplicate using the vanadate-facilitated [^{3}H]ouabain binding content method as previously described (50, 54). Each sample was washed twice for 10 min at 37°C in vanadate buffer (250 mM sucrose, 10 mM Tris HCl, 3 mM $MgSO_4$, 1 mM $NaVO_4$; pH 7.3). Muscle samples were then incubated for 2 h at 37°C in vanadate buffer with the addition of [^{3}H]ouabain (2.0 Ci^{-1} mL and 10^{-6} M; PerkinElmer, Boston, MA). The muscle was then placed in ice-cold vanadate solution for 4 times for 30 min to remove any unbound [^{3}H]ouabain. Muscle samples were blotted on filter paper and weighed before being soaked overnight in 500 μL of 5% trichloroacetic acid and 0.1 mM ouabain. Following this, 2.5 mL of scintillation cocktail (Opti-Fluor, PerkinElmer) was added before liquid scintillation counting of [^{3}H]ouabain. The [^{3}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 (50, 54). The final [^{3}H]ouabain binding site content (OB) was calculated using a correction factor of 1.33 as previously described for rat muscle (9) to allow for impurity of [^{3}H]ouabain, loss of specifically bound [^{3}H]ouabain during washout, and incomplete saturation during the equilibration of muscle with [^{3}H]ouabain, with OB expressed as picomoles per gram wet weight.

Western Blotting

To determine skeletal muscle Na^+,K^+-ATPase α and β isoform relative protein abundances, ~20 mg of frozen muscle was analyzed using Western blotting (52). 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 $MgCl_2$, 5 mM $Na_4O_7P_2$, 10 mM NaF, 1% Triton X-100, 10% Glycerol (Ajax Finechem, Australia), and protein inhibitor cocktail (P8340). All reagents were analytical grade (Sigma-Aldrich, St. Louis, MI). Samples were homogenized (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). 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 maximize recovery of Na^+,K^+-ATPase enzymes (47). 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) 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 was loaded in each gel. To ensure that blot density was within the linear range of detection (48), a 4–5-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) for 7 min at 320 mA using the semi-dry Trans-Blot Turbo Transfer System (Bio-Rad Laboratories). Membranes were blocked in phosphate-buffered saline solution with Tween 20 (PBST) buffer (10 mM Tris, 100 mM NaCl, 0.02% Tween 20) containing 5% nonfat milk for 1 h at room temperature. After being washed (4 times for 8 min in PBST), 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 times for 5 min) and incubated with the appropriate anti-rabbit (PerkinElmer, no. NEF812001EA) or anti-mouse (PerkinElmer, no. NEF822001EA) horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. After washing the membranes in PBST (4 times for 5 min), membranes were incubated for 5 min with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo, Waltham, MA), and then stain-free images were taken using a ChemiDoc Imaging system (Bio-Rad Laboratories). The densities of samples were expressed relative to the total protein on the gel and then normalized to the calibration curve (48).

The following antibodies were used for NKA isoforms: α_1 (monoclonal α6F, developed by D. Fambrough, obtained from the Developmental Studies Hybridoma Bank, maintained by the University of Iowa), α_2 (polyclonal anti-HERED, Millipore, no. 07- 674), α_3 (monoclonal, Thermo Scientific, Rockford, IL, no. MA3–915), β_1 (Millipore, no. 05- 382), β_2 (Proteintech, no. 22338–1-AP), and β_3 (BD Biosciences, no. 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 Fig. 2. Representative blots for NKA α_1, α_2, α_3, β_1, and β_2 isoforms are shown in Fig. 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 nonimmobilized control in the sham group was compared with the castration group nonimmobilized control and the noncast legs using a one-way ANOVA. Thus, the two main comparisons were first, to determine the effects of immobilization and

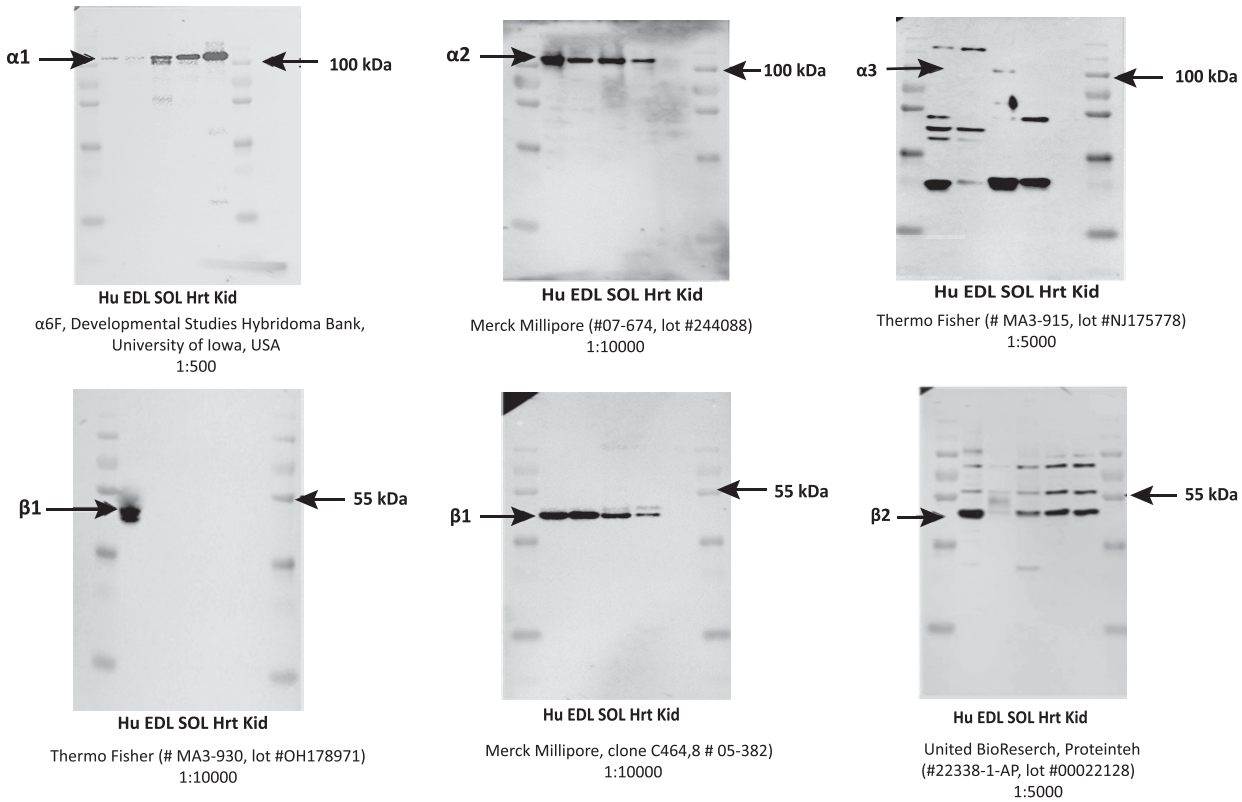

Fig. 2. Validation of antibodies used to quantify Na^+,K^+-ATPase (NKA) isoforms. Total of 10 μg protein of mixed human skeletal muscle homogenate (Hu), rat extensor digitorum longus muscle (EDL), rat soleus muscle (SOL), rat heart (Hrt), and rat kidney (Kid) were loaded onto each gel. Values on the *right* indicate molecular weight of bands and on the *left* indicate the NKA isoform. The NKA α_1 antibody (no. a6F) detected a band at the predicted molecular weight of ~105 kDa in human skeletal muscle, rat EDL muscle, rat SOL muscle, rat heart, and rat kidney. The NKA α_2 polyclonal antibody (Millipore, no. 07–674) detected a clear band at the predicted molecular weight of ~105 kDa in human skeletal muscle, rat EDL muscle, rat SOL muscle, and rat heart, but this was absent in rat kidney. The NKA α_3 antibody (Thermo Scientific no. MA3–915) detected a band at the predicted molecular weight of ~105 kDa in rat SOL muscle, but the band was absent in human skeletal muscle, rat EDL muscle, rat heart, and rat kidney. The NKA β_1 antibody (Thermo Scientific no. MA3–930), which was specifically designed to detect β_1 protein only in human muscle, detected a clear band at the predicted molecular weight of ~50 kDa in human skeletal muscle, and this was absent in rat EDL muscle, rat SOL muscle, rat heart, and rat kidney. The second NKA β_1 antibody (Millipore, no. 05- 382) detected a band at the expected molecular weight of ~50 kDa in human skeletal muscle, rat EDL muscle, rat SOL muscle, and rat heart, but the band was absent in rat kidney. The NKA β_2 antibody (Proteintech, no. 22338–1-AP) detected a band at the predicted molecular weight in human skeletal muscle, rat EDL muscle (albeit weak), rat SOL muscle, rat heart, and rat kidney. The NKA β_3 could not be detected by two different antibodies used (BD Bioscience, no. 610993 and no. 610992).

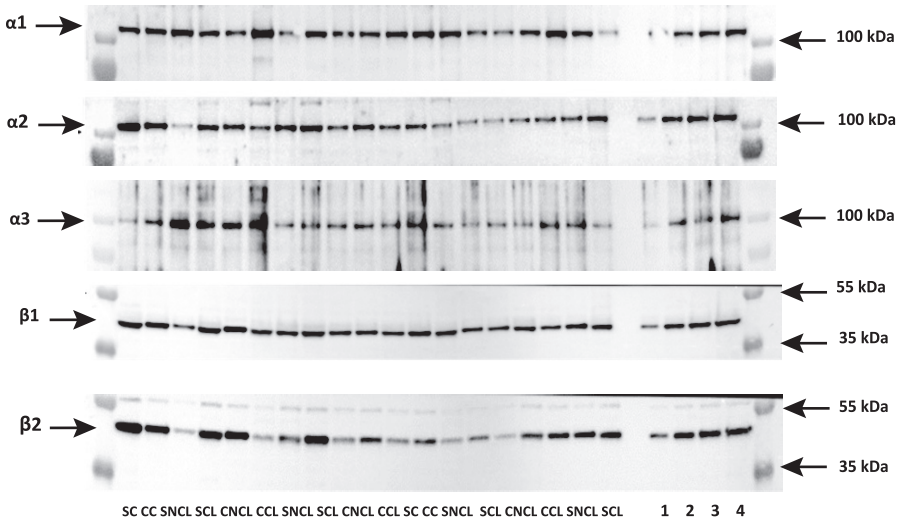

Fig. 3. Representative immunoblots of Na^+,K^+-ATPase α_1, α_2, α_3, β_1, and β_2 isoforms in homogenates of rat soleus muscle. Values on the *left* indicate molecular weight of bands. Protein bands from left to right are sham nonimmobilized control group (SC), castration non-immobilized control group (CC), sham noncast leg (SNCL), sham cast leg (SCL), castration noncast leg (CNCL), and castration cast leg (CCL). 1, 2, 3, and 4 are calibration curve loaded with 2.5–12.5 μg whole muscle crude homogenate. The homogenate was prepared from an equal amount of each sample.

recovery on muscle NKA (within sham-only arm) and second, to determine any additional or different effects because of testosterone reduction by examining the effects of immobilization and 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 nonimmobilization groups across the two arms. Pairwise comparisons utilized the least significant difference post hoc test. Statistical analyses were conducted using SPSS version 24. Effect size was calculated using Cohen's *d*, in which <0.2, 0.2–0.5, 0.5–0.8, and >0.8 are considered trivial, small, moderate, and large, respectively (10). Data are presented as mean ± SD. Statistical significance was accepted at $P < 0.05$.

RESULTS

Testosterone Concentration

The testosterone concentration at *day 0* postimmobilization and *day 14* of recovery for sham rats was 3.11 ± 0.63 ng/mL and 3.61 ± 1.68 ng/m^L and were substantially lower for castrated rats at 0.29 ± 0.09 ng/mL and 0.22 ± 0.07 ng/mL, 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 noncast leg (66 ± 6 mg vs. 105 ± 7 mg, respectively; $P = 0.001$; $d = 5.98$) and 31% lower than the nonimmobilized control group (66 ± 6 mg vs. 96 ± 5 mg; $P = 0.001$; $d = 5.43$). At 14 days of recovery, soleus muscle mass in the cast leg was 16% lower than in noncast leg (92 ± 16 mg vs. 110 ± 13 mg, respectively; $P = 0.033$; $d = 1.23$) but did not differ from the nonimmobilized control group (92 ± 16 mg vs. 96 ± 5 mg; $P = 0.404$; $d = 0.337$).

Muscle mass in the noncast leg was greater than in the nonimmobilized control group post immobilization (105 ± 7 mg vs. 96 ± 5 mg, respectively; $P = 0.031$; $d = 1.74$) and at 14 days of recovery (110 ± 13 mg 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. Normalized mass did not differ between the noncast leg and the nonimmobilized control group either postimmobilization (0.45 ± 0.023 mg/g vs. 0.46 ± 0.039 mg/g; $P = 0.63$), or at 14 days of recovery (0.39 ± 0.041 mg/g vs. 0.46 ± 0.039 mg/g; $P = 0.15$).

Within-castration group comparisons. Post immobilization, muscle mass in the cast leg was 31% lower than in noncast leg (70 ± 9 mg vs. 102 ± 8 mg, respectively; $P = 0.001$; $d = 3.75$) and 30% lower than nonimmobilized control group (70 ± 9 mg vs. 100 ± 7 mg; $P = 0.001$; $d = 3.53$). At 14 days of recovery, soleus muscle mass in the cast leg was 20% lower than in noncast leg (79 ± 14 mg vs. 99 ± 4 mg, respectively; $P = 0.013$; $d = 1.16$) and 21% lower than the nonimmobilized control group (79 ± 14 mg vs. 100 ± 7 mg; $P = 0.036$; $d = 0.1.18$).

Muscle mass in the noncast leg did not differ between the nonimmobilized control group either post immobilization (102 ± 8 mg vs. 100 ± 7 mg, respectively; $P = 0.64$; $d = 0.27$) or at 14 days of recovery (99 ± 4 mg vs. 100 ± 7 mg, respectively; $P = 0.83$; $d = 0.175$). Normalized mass post immobilization was greater in the noncast leg than the nonimmobilized control group (0.46 ± 0.027 mg/g vs. 0.43 ± 0.020 mg/g; $P = 0.030$), but did not differ at 14 days of recovery (0.42 ± 0.023 mg/g vs. 0.43 ± 0.020 mg/g; $P = 0.93$).

Soleus Muscle Force

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

Muscle force in the noncast leg did not differ from the nonimmobilized control group either post immobilization (159 ± 93 g vs. 144 ± 42 g, respectively; $P = 0.81$; $d = 0.207$) or at 14 days of recovery (184 ± 59 g 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 noncast leg (76 ± 54 g vs. 135 ± 52 g, respectively; $P = 0.025$; $d = 1.123$) and 38% lower than nonimmobilized control group (76 ± 54 g vs. 123 ± 12 g; $P = 0.034$; $d = 1.20$). At 14 days of recovery, muscle force in the cast leg did not differ from the noncast leg (79 ± 42 g vs. 109 ± 41 g, respectively; $P = 0.208$; $d = 0.69$) but was 35% lower than the nonimmobilized control group (79 ± 42 g vs. 122 ± 12 g; $P = 0.036$; $d = 1.39$).

Muscle force in the noncast leg did not differ between the nonimmobilized control group either post immobilization (135 ± 5 g vs. 123 ± 12 g, respectively; $P = 0.65$; $d = 1.30$) or at 14 days of recovery (109 ± 41 g 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 noncast leg ($P = 0.023$; $d = 1.34$) and 34% lower ($P = 0.001$; $d = 1.69$) than in the nonimmobilized control group (Fig. 4*A*). At 14 days of recovery, the muscle OB had recovered, such that there were no differences in the cast leg compared with either the noncast leg ($P = 0.391$; $d = 0.540$) or nonimmobilized control group ($P = 0.833$; $d = 0.093$; Fig. 4*A*).

Within-castration group comparisons. Post immobilization, the muscle OB in the cast leg did not differ significantly from the noncast leg ($P = 0.163$; $d = 0.996$) but was 34% lower than in the nonimmobilized control group ($P = 0.001$; $d = 3.03$; Fig. 4*B*). At 14 days of recovery, the OB in the cast leg remained depressed, being 34% lower than in the nonimmobilized control group ($P = 0.001$; $d = 2.02$; Fig. 4*B*), but was not different from the noncast leg ($P = 0.456$; $d = 0.30$).

Effects of Immobilization and Testosterone Reduction on NKA α and β Isoform Abundances

Within-sham group comparisons. NKA α_1, α_2 AND α_3 ISOFORMS. There were no significant differences between groups for within-sham group comparisons for NKA α_1 ($P = 0.876$, Fig. 5*A*), α_2 (although considerable variability was noted; $P = 0.835$; Fig. 6*A*), or for α_3 ($P = 0.990$; Fig. 7*A*).

NKA β_1 AND β_2 ISOFORMS. There were no significant differences between groups for within-sham group comparisons for β_1 ($P = 0.661$; Fig. 8*A*) or β_2 ($P = 0.656$; Fig. 9*A*).

Within-castration group comparisons. NKA α_1 AND α_3 ISOFORMS. There were no significant differences between groups for within-castration comparisons for NKA α_1 ($P = 0.754$; Fig. 5*B*) or for α_3 ($P = 0.641$; Fig. 7*B*).

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

NKA β_1 ISOFORM. Post immobilization, the NKA β_1 isoform abundance in the cast leg was 26% lower than in the noncast leg ($P = 0.018$; $d = 1.09$) but did not differ from the nonimmobilized control group ($P = 0.321$; $d = 0.43$; Fig. 8*B*). At 14 days of recovery, there were no significant differences in the NKA β_1 isoform abundance in cast leg compared with either the noncast leg ($P = 0.427$; $d = 0.65$) or the nonimmobilized control group ($P = 0.251$; $d = 0.51$; Fig. 8*B*).

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

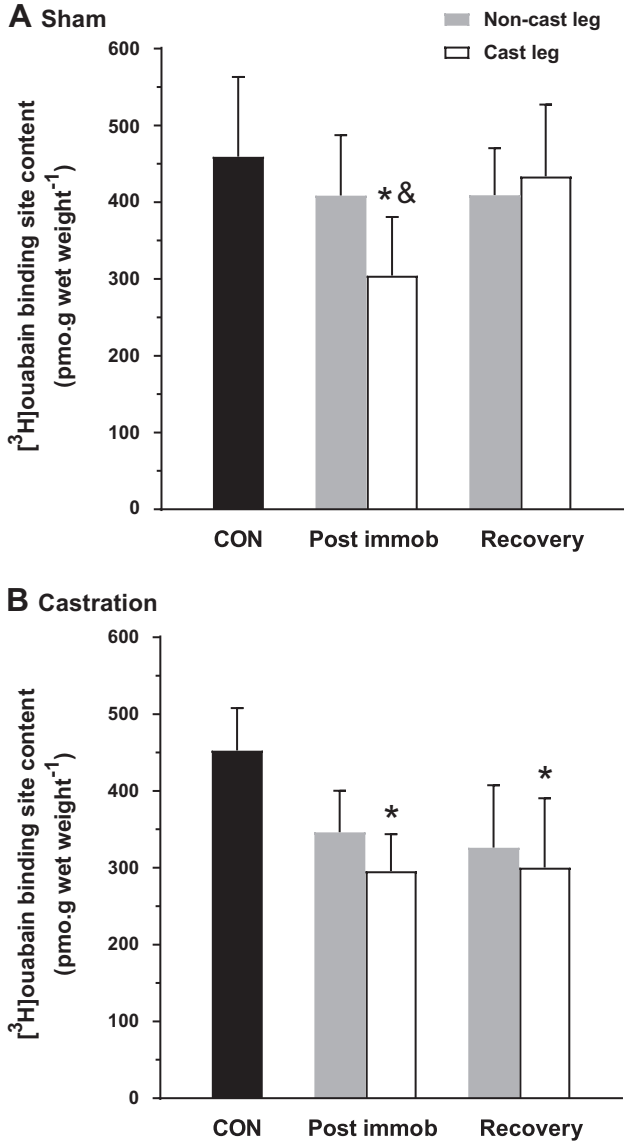

Fig. 4. Effects of 10 days of hindlimb immobilization and castration in rats on soleus muscle [^{3}H]ouabain binding site content from the sham (*A*) and castration (*B*) groups. Samples were compared by one-way ANOVA. Values are means ± SD; $n = 7–8$ per group. *Cast leg content is less than control group content; $P < 0.001$. &Cast leg content is less than noncast leg content; $P < 0.05$. CON, nonimmobilized control group; post immob, 0 days following immobilization; recovery, 14 days of recovery after immobilization.

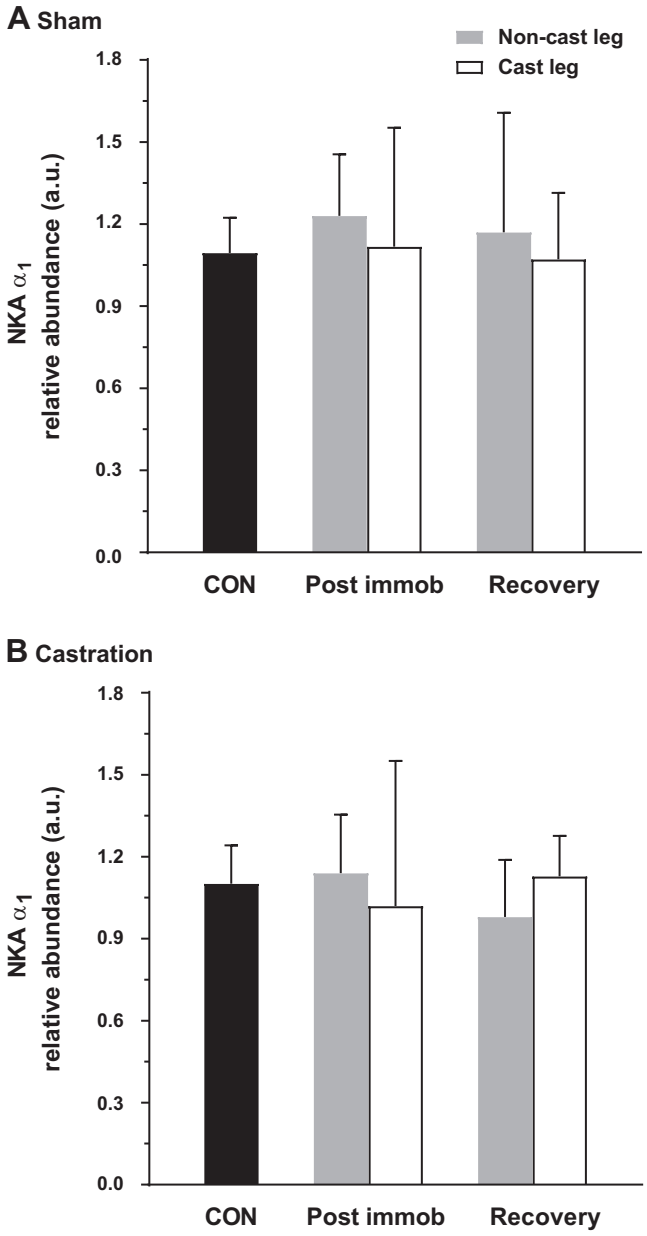

Fig. 5. Effects of 10 days of hindlimb immobilization and castration in rats on soleus muscle Na^+,K^+-ATPase (NKA) α_1 isoform protein abundance from the sham group (*A*) and the castration group (*B*). Values are means ± SD in arbitrary units (a.u.); $n = 7–8$ per group. Samples were compared by one-way ANOVA. CON, nonimmobilization control group; post immob, 0 days following immobilization; recovery, 14 days of recovery after immobilization.

Effects of Testosterone Reduction on [^{3}H]ouabain Binding Site Content and NKA Isoform Abundance in Nonimmobilized Muscle

The soleus muscle OB and NKA isoform abundances in the sham nonimmobilized control group were compared with castration nonimmobilized control group (7 days post surgery) and to those of the nonimmobilized legs in the castration groups (17 d and 31 d 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 days post surgery, $P = 0.884$; $d = 0.73$) but was higher than in the other castration noncast legs post immobilization (i.e., at 17 days post surgery, $P = 0.016$; d = 1.363) and recovery (i.e., at 31 days 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 nonimmobilized control group did not differ from the castration nonimmobilized control (7 days post surgery, $P = 0.695$; $d = -0.193$) or postimmobilization groups (17 days

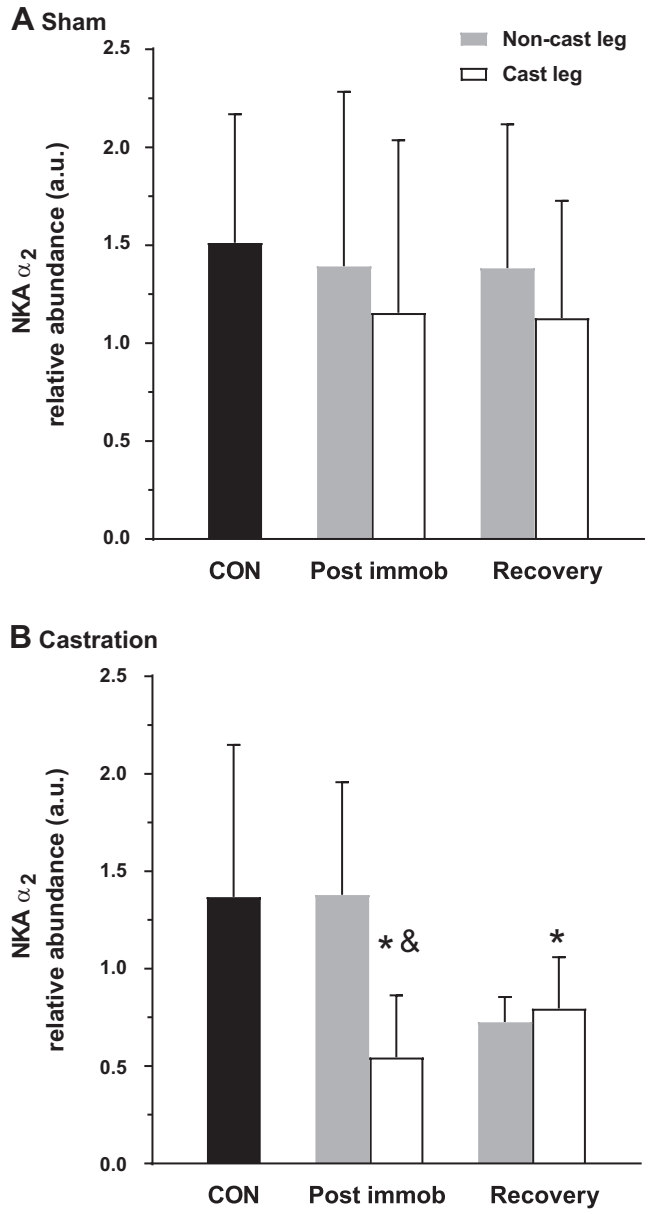

Fig. 6. Effects of 10 days of hindlimb immobilization and castration in rats on soleus muscle Na^+,K^+-ATPase (NKA) α_2 isoform protein abundance from the sham group (*A*) and the castration group (*B*). Values are means ± SD in arbitrary units (a.u.); $n = 7–8$ per group. Samples were compared by one-way ANOVA, *cast leg abundance is less than control group abundance; $P < 0.001$. &Cast leg abundance is less than noncast leg abundance; $P < 0.05$. CON, nonimmobilization control group; post immob, 0 days following immobilization; recovery, 14 days of recovery after immobilization.

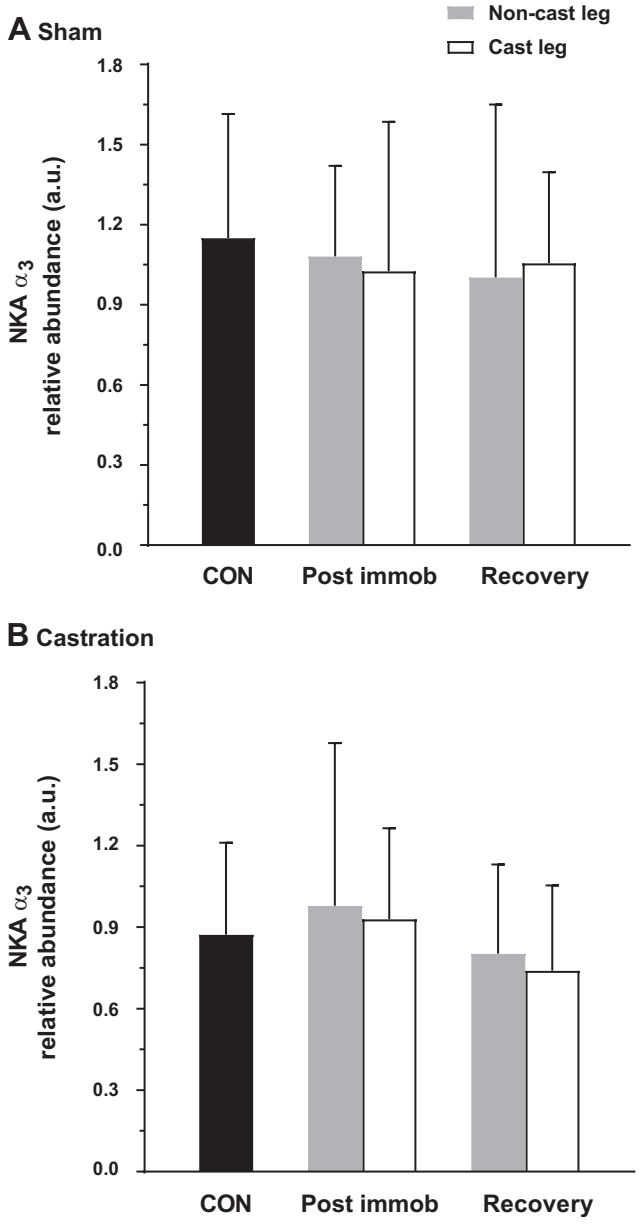

Fig. 7. Effects of 10 days of hindlimb immobilization and castration in rats on soleus muscle Na^+,K^+-ATPase (NKA) α_3 isoform protein abundances from the sham group (*A*) and the castration group (*B*). Values are means ± SD in arbitrary units (a.u.); $n = 7–8$ per group. Samples were compared by one-way ANOVA. CON, nonimmobilization control group; post immob, 0 days following immobilization; recovery, 14 days of recovery after immobilization.

post surgery, NS, $P = 0.51$; $d = 0.021$), but was greater than castration nonimmobilized recovery (31 days post surgery, $P = 0.020$, $d = 1.639$).

Other NKA isoforms. There were no differences between the sham control and the castration control nonimmobilized legs (7 days 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 days or 31 days post surgery, although a tendency was noted to a lower α_3 in the castration recovery noncast 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 days and subsequent 14 days of 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, although muscle OB had returned to control levels in sham animals after 14 days of recovery, this remained depressed in the castration group. Despite the reduction with

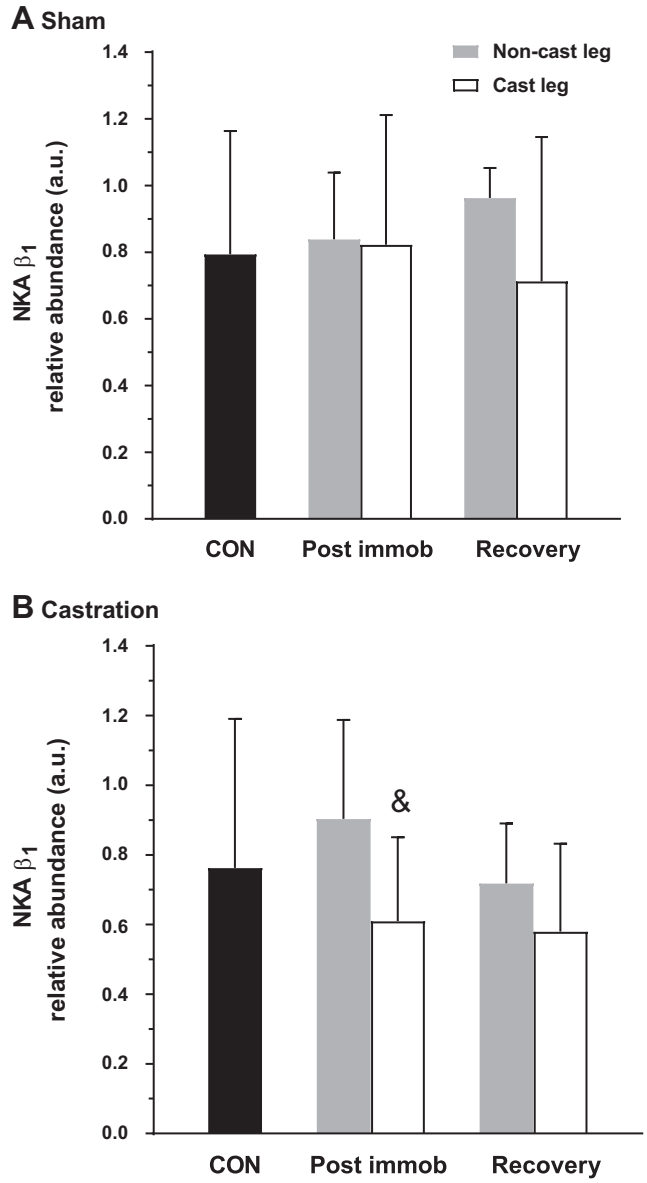

Fig. 8. Effects of 10 days of hindlimb immobilization and castration in rats on soleus muscle Na^+,K^+-ATPase (NKA) β_1 isoform protein abundance from the sham group (*A*) and the castration group (*B*). Values are means ± SD in arbitrary units (a.u.); $n = 7–8$ per group. Samples were compared by one-way ANOVA. &Cast leg abundance is less than noncast leg abundance; $P < 0.05$. CON, nonimmobilization control group; post immob, 0 days following immobilization; recovery, 14 days of recovery after immobilization.

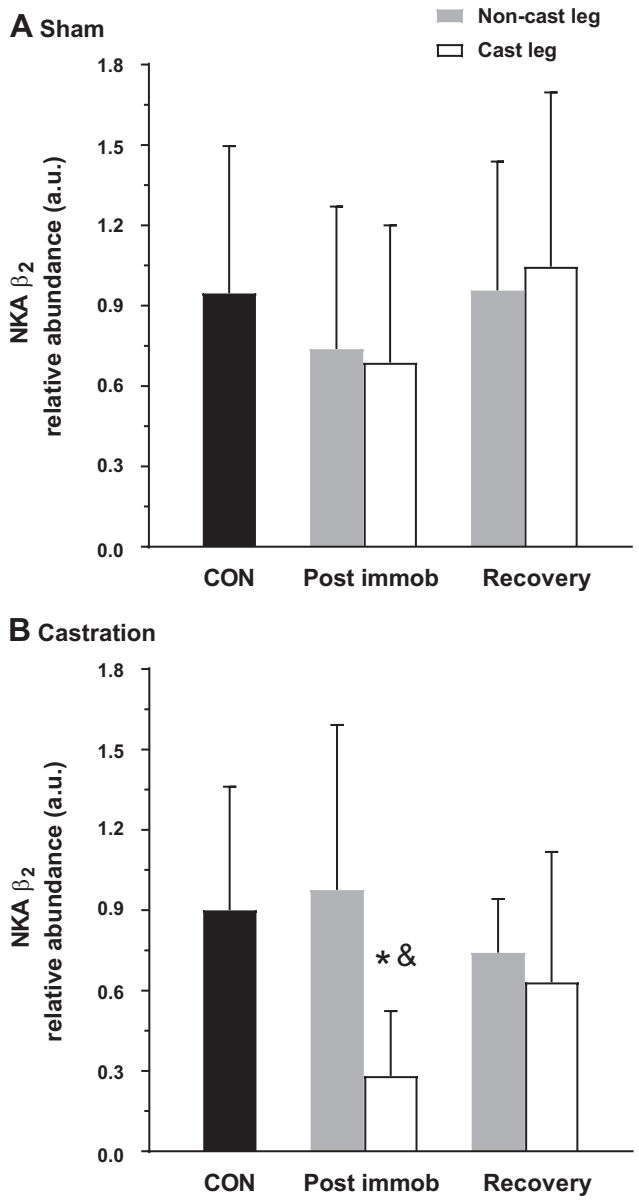

Fig. 9. Effects of 10 days of hindlimb immobilization and castration in rats on soleus muscle Na^+,K^+-ATPase (NKA) β_2 isoform protein abundances from the sham group (*A*) and the castration group (*B*). Values are means ± SD in arbitrary units (a.u.); $n = 7–8$ per group. Samples were compared by one-way ANOVA, *cast leg abundance is less than nonimmobilization control group abundance; $P < 0.05$. &Cast leg abundance is less than noncast leg abundance; $P < 0.05$. CON, nonimmobilization control group; post immob, 0 days following immobilization; recovery, 14 days of recovery after immobilization.

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,$\beta\beta_1$, and β_2 isoform abundances following the hindlimb immobilization, with NKA α_2 remaining suppressed at 14 days in the castration group. Third, although testosterone suppression per se did not initially adversely affect the muscle OB or NKA isoform abundances in the nonimmobilized muscle 7 days post surgery, a depressive effect on OB was manifest both 17 days and 31 days post surgery; furthermore, the α_2 isoform abundance was also lower 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 7 days post surgery. This was despite substantial 31% and 58% reductions in muscle mass and force after immobilization compared with nonimmobilized control animals, respectively, and 37% and 62% reductions compared with the noncast leg. Importantly, however, when the control sham nonimmobilized legs were compared with the castration nonimmobilized legs, muscle OB was depressed after the 10-day period associated with immobilization and also the further 14-day recovery, with α_2 also depressed in the longer recovery period. This implies that androgen depletion treatments that markedly suppress testosterone levels, such as those applied to prostate cancer patients, might also be expected to reduce muscle NKA, although this remains to be determined in human muscle.

In animals that underwent sham surgery, hindlimb immobilization for 10 days reduced muscle OB by 34% in rat soleus muscle. 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, which is characterized by greatly reduced muscle activity, in humans, 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 14 days post immobilization, which is 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 of 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 nonimmobilized muscle, indicating a systemic effect of testosterone suppression. This may therefore be important during muscle contractions, potentially impairing cellular Na^+ efflux, K^+ influx, and 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, composing ~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 is thought to be low (22, 24). In rat muscle, the standard ouabain binding site method employed here detects the α_2 isoform because of its high affinity

for ouabain (8). Thus, OB depressed with immobilization and testosterone suppression is expected to represent primarily the 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, which remained 42% depressed after 14 days of recovery, compared with nonimmobilized control muscles. These reductions coincided with the acute and sustained reductions in muscle OB. A further interesting finding was that the decrease in NKA α_2 isoform protein abundance after immobilization (60%) was twice the decline in OB (32%). However, the NKA α_2 isoform was not reduced in the sham group following hindlimb immobilization despite the substantial reduction of [^{3}H]ouabain binding in the same group, although a moderate effect size was found after immobilization even after 14 days of recovery. This lack of decrease in NKA α_2 was surprising and may be due to the semiquantitative, nonmolar, and variable nature of Western blot analysis, making it difficult to detect the changes. This finding appears to contrast with recent studies where short-term hindlimb suspension in the rat for 6–12 h and for 1–3 days initially increased the NKA α_2 protein but depressed the associated electrogenic activity (29–32). Whether this discrepancy simply reflects a time-course effect of an initial increase followed by reductions is unclear.

The qualitative disparity between the reductions in muscle OB and the NKA α_2 isoform after immobilization and recovery in the castrated animals as well as the lack of change in NKA α_2 isoform after immobilization in sham animals may reflect biological and methodological factors. First, the [^{3}H]ouabain binding site content method detects binding in functional pumps (i.e., $\alpha\beta$ complexes), whereas the Western blot measures all isoforms present in the tissue. It is therefore possible that a considerable fraction of the α_2 isoforms are not present as functional pump complexes, which might change with the immobilization intervention. However, a closer correspondence between findings using the different methods was found in recovery. We recently observed a similar discrepancy after injury and chronic inactivity in humans, in whom the muscle α_2 isoform abundance was 63% lower in the knee-injured leg than the noninjured leg, which was associated with ~20% lower muscle OB (51). In contrast, after short-term inactivity alone, which was induced by unilateral lower limb suspension, both the α_2 isoform abundance and muscle OB were unchanged (53). The [^{3}H]ouabain binding technique is based on intact portions of muscle tissue and is a fully quantitative method, whereas the Western blot analysis is based on a standardized total protein concentration from a muscle homogenate. Discrepancies in α_2 isoform findings between the groups may also in part reflect the variable nature of Western blot analysis of NKA proteins (7).

Regardless, this sustained reduction in the muscle OB and α_2 isoform with castration and immobilization may contribute to impaired muscle excitability and contractility. The NKA α_2 isoform plays a major role in Na^+/K^+ transport during muscle contractions (11); partial global knockout of NKA α_2 caused impaired contractile force in mouse EDL muscle (44), whereas 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. To our knowledge, 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 because of 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 h and 1–3 days in rat soleus muscle (31, 32).

To the best of our knowledge, this is the first study showing that the NKA α_3 isoform abundance was not affected by hindlimb immobilization or testosterone reduction. A tendency toward lowered α_3 with testosterone suppression per se was, however, evident in nonimmobilized muscles compared with 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 days of unilateral lower limb suspension (51, 53). However, in type I single muscle fibers, 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 group 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 only 26% compared with the noncast leg, whereas the β_2 isoform was 71% and 65% lower than in the noncast leg and control group, respectively. This suggests that reductions 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 fibers, such as in soleus muscle, whereas the β_2 isoform is more abundant in muscles rich in fast-twitch fibers, 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 stabilizing the movement of the α isoform from the endoplasmic (sarcoplasmic) reticulum to the plasma membrane (39, 59). Therefore, downregulation in NKA β_1 and β_2 isoform abundances with castration and immobilization is likely to be functionally important and may reflect reduced skeletal muscle NKA activity, thereby potentially adversely impacting muscle NKA activity and fatigue (39). We are unclear as to why reductions in β_1 and β_2 isoform did not occur with immobilization in the sham group.

Reductions in testosterone have been reported to lead to increased total fat mass (37). An increased fat infiltration into muscle might then artifactually lower the [^{3}H]ouabain binding site content, since this is expressed per gram of muscle. It seems unlikely that this effect could, however, account for the large reductions evident in OB after immobilization in the castrated rats (−34%)because similar reductions also occurred

in the sham group (−26%, −34%) when testosterone was normal. Thus, this reduction is unlikely to be due to a fat infiltration artifact. In addition, future studies should measure fat-free mass to correct for any such effect of the measured [^{3}H]ouabain binding site content. This effect would probably be less influential on Western blot measurements of isoform abundances because these are conducted on samples of known protein content, but a possible small effect cannot be excluded.

Mechanisms of NKA Downregulation in Muscle with Castration and Immobilization

The mechanisms underlying the decrease in muscle OB and the α_2 isoform in skeletal muscle with castration and immobilization cannot be ascertained from the results of this study. One possibility is that the contractile-induced increases in muscle intracellular [Na^+] (13, 49) are greatly lessened with immobilization simply because of reduced muscle contractions. However, increased intracellular Na^+ content was not associated with elevated NKA mRNA (46). Although no initial effects of testosterone were observed herein on muscle OB or any α or β isoforms in the nonimmobilization control muscle, depression of OB was evident in subsequent measures in nonimmobilized muscles, and a lowered α_2 was also evident in recovery; this also means that recovery of already depressed muscle OB and α_2 isoform was absent in castrated rats. The possible mechanism of testosterone suppression effects on recovery of already lowered abundance of NKA might also occur via muscle Na^+ ion regulation, as testosterone regulates intracellular Na^+ in other tissues (14, 38). Reduced muscle protein synthesis was found with androgen deprivation therapy (23). This suggests one possible mechanism responsible for the lack of recovery of OB after castration, but no evidence exists to support or refute this. Further research is warranted on testosterone effects on intracellular ions, fluid dynamics, and NKA regulation in skeletal muscle, including signaling mechanisms such as ERK1/2. A possible mechanism for decreased [^{3}H]ouabain binding site content is via impairment or lesser activity of signaling pathways via the non-ion-transducing role of NKA. This may occur as result of direct-protein interactions between NKA and its neighboring proteins, which trigger a signaling cascade culminating in decreased NKA gene transcription (2, 67, 68). Because 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 that 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 patients with prostate cancer undergoing androgen deprivation therapy, in patients with type 2 diabetes, and in the elderly.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.M.A., E.D.H., A.C.B., and A.H. performed experiments; M.M.A., A.C.P., and M.J.M. analyzed data; M.M.A., A.C.P., and M.J.M. interpreted results of experiments; M.M.A. prepared figures; M.M.A. drafted manuscript; M.M.A., E.D.H., A.C.B., A.C.P., A.H., and M.J.M. edited and revised manuscript; A.C.P. and M.J.M. approved final version of manuscript.

REFERENCES

1. **Aoki MS, Lima WP, Miyabara EH, Gouveia CH, Moriscot AS.** Deleteriuos effects of immobilization upon rat skeletal muscle: role of creatine supplementation. *Clin Nutr* 23: 1176–1183, 2004. doi:10.1016/j.clnu.2004.03.004.
2. **Aperia A, Akkuratov EE, Fontana JM, Brismar H.** Na+-K+-ATPase, a new class of plasma membrane receptors. *Am J Physiol Cell Physiol* 310: C491–C495, 2016. doi:10.1152/ajpcell.00359.2015.
3. **Bøttger P, Tracz Z, Heuck A, Nissen P, Romero-Ramos M, Lykke-Hartmann K.** Distribution of Na/K-ATPase alpha 3 isoform, a sodium-potassium P-type pump associated with rapid-onset of dystonia parkinsonism (RDP) in the adult mouse brain. *J Comp Neurol* 519: 376–404, 2011. doi:10.1002/cne.22524.
4. **Brown M.** Skeletal muscle and bone: effect of sex steroids and aging. *Adv Physiol Educ* 32: 120–126, 2008. doi:10.1152/advan.90111.2008.
5. **Brown M, Fisher JS, Hasser EM.** Gonadectomy and reduced physical activity: effects on skeletal muscle. *Arch Phys Med Rehabil* 82: 93–97, 2001. doi:10.1053/apmr.2001.18697.
6. **Cairns SP, Lindinger MI.** Do multiple ionic interactions contribute to skeletal muscle fatigue? *J Physiol* 586: 4039–4054, 2008. doi:10.1113/jphysiol.2008.155424.
7. **Christiansen D, Bishop DJ, Broatch JR, Bangsbo J, McKenna MJ, Murphy RM.** Cold-water immersion after training sessions: effects on fiber type-specific adaptations in muscle K^+ transport proteins to sprint-interval training in men. *J Appl Physiol (1985)* 125: 429–444, 2018. doi:10.1152/japplphysiol.00259.2018.
8. **Clausen T.** Na+-K+ pump regulation and skeletal muscle contractility. *Physiol Rev* 83: 1269–1324, 2003. doi:10.1152/physrev.00011.2003.
9. **Clausen T, Nielsen OB, Clausen JD, Pedersen TH, Hayward LJ.** Na+,K+-pump stimulation improves contractility in isolated muscles of mice with hyperkalemic periodic paralysis. *J Gen Physiol* 138: 117–130, 2011. doi:10.1085/jgp.201010586.
10. **Cohen J.** *Statistical Power Analysis for the Behavioral Sciences*. Mahwah, NJ: Lawrence Erlbaum Associates, 1988.
11. **DiFranco M, Hakimjavadi H, Lingrel JB, Heiny JA.** Na,K-ATPase α2 activity in mammalian skeletal muscle T-tubules is acutely stimulated by extracellular K+. *J Gen Physiol* 146: 281–294, 2015. doi:10.1085/jgp.201511407.
12. **Ditor DS, Hamilton S, Tarnopolsky MA, Green HJ, Craven BC, Parise G, Hicks AL.** Na+,K+-ATPase concentration and fiber type distribution after spinal cord injury. *Muscle Nerve* 29: 38–45, 2004. doi:10.1002/mus.10534.
13. **Everts ME, Clausen T.** Activation of the Na-K pump by intracellular Na in rat slow- and fast-twitch muscle. *Acta Physiol Scand* 145: 353–362, 1992. doi:10.1111/j.1748-1716.1992.tb09375.x.
14. **Farnsworth WE.** Androgen regulation of prostatic membrane ATPase. *Biol Reprod* 3: 218–222, 1970. doi:10.1093/biolreprod/3.2.218.

15. **Feldman HA, Longcope C, Derby CA, Johannes CB, Araujo AB, Coviello AD, Bremner WJ, McKinlay JB.** Age trends in the level of serum testosterone and other hormones in middle-aged men: longitudinal results from the Massachusetts male aging study. *J Clin Endocrinol Metab* 87: 589–598, 2002. doi:10.1210/jcem.87.2.8201.
16. **Fowles JR, Green HJ, Ouyang J.** Na+-K+-ATPase in rat skeletal muscle: content, isoform, and activity characteristics. *J Appl Physiol (1985)* 96: 316–326, 2004. doi:10.1152/japplphysiol.00745.2002.
17. **Galvão DA, Spry NA, Taaffe DR, Newton RU, Stanley J, Shannon T, Rowling C, Prince R.** Changes in muscle, fat and bone mass after 36 weeks of maximal androgen blockade for prostate cancer. *BJU Int* 102: 44–47, 2008. doi:10.1111/j.1464-410X.2008.07539.x.
18. **Galvão DA, Taaffe DR, Spry N, Joseph D, Turner D, Newton RU.** Reduced muscle strength and functional performance in men with prostate cancer undergoing androgen suppression: a comprehensive cross-sectional investigation. *Prostate Cancer Prostatic Dis* 12: 198–203, 2009. doi:10.1038/pcan.2008.51.
19. **Geering K.** Functional roles of Na,K-ATPase subunits. *Curr Opin Nephrol Hypertens* 17: 526–532, 2008. doi:10.1097/MNH.0b013e3283036cbf.
20. **Guerra M, Rodriguez del Castillo A, Battaner E, Mas M.** Androgens stimulate preoptic area Na+,K+-ATPase activity in male rats. *Neurosci Lett* 78: 97–100, 1987. doi:10.1016/0304-3940(87)90568-4.
21. **Hansen O.** The α1 isoform of Na+,K+-ATPase in rat soleus and extensor digitorum longus. *Acta Physiol Scand* 173: 335–341, 2001. doi:10.1046/j.1365-201X.2001.00910.x.
22. **Hansen O, Clausen T.** Quantitative determination of Na+-K+-ATPase and other sarcolemmal components in muscle cells. *Am J Physiol Cell Physiol* 254: C1–C7, 1988. doi:10.1152/ajpcell.1988.254.1.C1.
23. **Hanson ED, Nelson AR, West DWD, Violet JA, O'Keefe L, Phillips SM, Hayes A.** Attenuation of resting but not load-mediated protein synthesis in prostate cancer patients on androgen deprivation. *J Clin Endocrinol Metab* 102: 1076–1083, 2017. doi:10.1210/jc.2016-3383.
24. **He S, Shelly DA, Moseley AE, James PF, James JH, Paul RJ, Lingrel JB.** The α(1)- and α(2)-isoforms of Na-K-ATPase play different roles in skeletal muscle contractility. *Am J Physiol Regul Integr Comp Physiol* 281: R917–R925, 2001. doi:10.1152/ajpregu.2001.281.3.R917.
25. **Herbst KL, Bhasin S.** Testosterone action on skeletal muscle. *Curr Opin Clin Nutr Metab Care* 7: 271–277, 2004. doi:10.1097/00075197-200405000-00006.
27. **Järvinen MJ, Lehto MU.** The effects of early mobilisation and immobilisation on the healing process following muscle injuries. *Sports Med* 15: 78–89, 1993. doi:10.2165/00007256-199315020-00002.
28. **Jebens E, Steen H, Fjeld TO, Bye E, Sejersted OM.** Changes in Na+, K(+)-adenosinetriphosphatase, citrate synthase and K+ in sheep skeletal muscle during immobilization and remobilization. *Eur J Appl Physiol Occup Physiol* 71: 386–395, 1995. doi:10.1007/BF00635871.

28a. **Kim JC, Kang YS, Noh EB, Seo BW, Seo DY, Park GD, Kim SH.** Concurrent treatment with ursolic acid and low-intensity treadmill exercise improves muscle atrophy and related outcomes in rats. *Korean J Physiol Pharmacol* 22: 427–436, 2018. doi:10.4196/kjpp.2018.22.4.427.

29. **Kjeldsen K, Richter EA, Galbo H, Lortie G, Clausen T.** Training increases the concentration of [^{3}H]ouabain-binding sites in rat skeletal muscle. *Biochim Biophys Acta* 860: 708–712, 1986. doi:10.1016/0005-2736(86)90570-5.
31. **Kravtsova VV, Matchkov VV, Bouzinova EV, Vasiliev AN, Razgovorova IA, Heiny JA, Krivoi II.** Isoform-specific Na,K-ATPase alterations precede disuse-induced atrophy of rat soleus muscle. *BioMed Res Int* 2015: 720172, 2015. doi:10.1155/2015/720172.
32. **Kravtsova VV, Petrov AM, Matchkov VV, Bouzinova EV, Vasiliev AN, Benziane B, Zefirov AL, Chibalin AV, Heiny JA, Krivoi II.** Distinct α2 Na,K-ATPase membrane pools are differently involved in early skeletal muscle remodeling during disuse. *J Gen Physiol* 147: 175–188, 2016. doi:10.1085/jgp.201511494.
33. **Krawiec BJ, Frost RA, Vary TC, Jefferson LS, Lang CH.** Hindlimb casting decreases muscle mass in part by proteasome-dependent proteolysis but independent of protein synthesis. *Am J Physiol Endocrinol Metab* 289: E969–E980, 2005. doi:10.1152/ajpendo.00126.2005.
35. **Kujala UM, Orava S, Järvinen M.** Hamstring injuries. Current trends in treatment and prevention. *Sports Med* 23: 397–404, 1997. doi:10.2165/00007256-199723060-00005.
36. **Kutz LC, Mukherji ST, Wang X, Bryant A, Larre I, Heiny JA, Lingrel JB, Pierre SV, Xie Z.** Isoform-specific role of Na/K-ATPase α1 in skeletal muscle. *Am J Physiol Endocrinol Metab* 314: E620–E629, 2018. doi:10.1152/ajpendo.00275.2017.
37. **Kvorning T, Andersen M, Brixen K, Madsen K.** Suppression of endogenous testosterone production attenuates the response to strength training: a randomized, placebo-controlled, and blinded intervention study. *Am J Physiol Endocrinol Metab* 291: E1325–E1332, 2006. doi:10.1152/ajpendo.00143.2006.
38. **Lao L, Franklin RB, Costello LC.** High-affinity L-aspartate transporter in prostate epithelial cells that is regulated by testosterone. *Prostate* 22: 53–63, 1993. doi:10.1002/pros.2990220108.
39. **Lavoie L, Levenson R, Martin-Vasallo P, Klip A.** The molar ratios of α and β subunits of the Na+-K+-ATPase differ in distinct subcellular membranes from rat skeletal muscle. *Biochemistry* 36: 7726–7732, 1997. doi:10.1021/bi970109s.
40. **Leichtnam M-L, Rolland H, Wüthrich P, Guy RH.** Testosterone hormone replacement therapy: state-of-the-art and emerging technologies. *Pharm Res* 23: 1117–1132, 2006. doi:10.1007/s11095-006-0072-5.
41. **Leivseth G, Clausen T, Everts ME, Bjordal E.** Effects of reduced joint mobility and training on Na,K-ATPase and Ca-ATPase in skeletal muscle. *Muscle Nerve* 15: 843–849, 1992. doi:10.1002/mus.880150714.
42. **Leivseth G, Reikerås O.** Changes in muscle fiber cross-sectional area and concentrations of Na,K-ATPase in deltoid muscle in patients with impingement syndrome of the shoulder. *J Orthop Sports Phys Ther* 19: 146–149, 1994. doi:10.2519/jospt.1994.19.3.146.
43. **Lin X, Hanson E, Betik AC, Brennan-Speranza TC, Hayes A, Levinger I.** Hindlimb immobilization, but not castration, induces reduction of undercarboxylated osteocalcin associated with muscle atrophy in rats. *J Bone Miner Res* 31: 1967–1978, 2016. doi:10.1002/jbmr.2884.
44. **Lingrel J, Moseley A, Dostanic I, Cougnon M, He S, James P, Woo A, O'Connor K, Neumann J.** Functional roles of the α isoforms of the Na,K-ATPase. *Ann N Y Acad Sci* 986: 354–359, 2003. doi:10.1111/j.1749-6632.2003.tb07214.x.
45. **McKenna MJ, Bangsbo J, Renaud J-ME.** Muscle K+, Na+, and Cl disturbances and Na+-K+ pump inactivation: implications for fatigue. *J Appl Physiol (1985)* 104: 288–295, 2008. doi:10.1152/japplphysiol.01037.2007.
46. **Murphy KT, Macdonald WA, McKenna MJ, Clausen T.** Ionic mechanisms of excitation-induced regulation of Na+-K+-ATPase mRNA expression in isolated rat EDL muscle. *Am J Physiol Regul Integr Comp Physiol* 290: R1397–R1406, 2006. doi:10.1152/ajpregu.00707.2005.
47. **Murphy KT, Snow RJ, Petersen AC, Murphy RM, Mollica J, Lee J-S, Garnham AP, Aughey RJ, Leppik JA, Medved I, Cameron-Smith D, McKenna MJ.** Intense exercise up-regulates Na+,K+-ATPase isoform mRNA, but not protein expression in human skeletal muscle. *J Physiol* 556: 507–519, 2004. doi:10.1113/jphysiol.2003.054981.
48. **Murphy RM, Lamb GD.** Important considerations for protein analyses using antibody based techniques: down-sizing Western blotting up-sizes outcomes. *J Physiol* 591: 5823–5831, 2013. doi:10.1113/jphysiol.2013.263251.
49. **Nielsen OB, Clausen T.** Regulation of Na(+)-K+ pump activity in contracting rat muscle. *J Physiol* 503: 571–581, 1997. doi:10.1111/j.1469-7793.1997.571bg.x.
50. **Nørgaard A, Kjeldsen K, Clausen T.** A method for the determination of the total number of 3H-ouabain binding sites in biopsies of human skeletal muscle. *Scand J Clin Lab Invest* 44: 509–518, 1984. doi:10.3109/00365518409083604.
51. **Perry BD, Levinger P, Morris HG, Petersen AC, Garnham AP, Levinger I, McKenna MJ.** The effects of knee injury on skeletal muscle function, Na+, K+-ATPase content, and isoform abundance. *Physiol Rep* 3: e12294, 2015. doi:10.14814/phy2.12294.
52. **Perry BD, Levinger P, Serpiello FR, Caldow MK, Cameron-Smith D, Bartlett JR, Feller JA, Bergman NR, Levinger I, McKenna MJ.** The effects of osteoarthritis and age on skeletal muscle strength, Na+-K+-ATPase content, gene and isoform expression. *J Appl Physiol (1985)* 115: 1443–1449, 2013. doi:10.1152/japplphysiol.00789.2013.
53. **Perry BD, Wyckelsma VL, Murphy RM, Steward CH, Anderson M, Levinger I, Petersen AC, McKenna MJ.** Dissociation between short-term unloading and resistance training effects on skeletal muscle Na+,K+-ATPase, muscle function, and fatigue in humans. *J Appl Physiol (1985)* 121: 1074–1086, 2016. doi:10.1152/japplphysiol.00558.2016.
54. **Petersen AC, Murphy KT, Snow RJ, Leppik JA, Aughey RJ, Garnham AP, Cameron-Smith D, McKenna MJ.** Depressed Na+-K+-

ATPase activity in skeletal muscle at fatigue is correlated with increased Na+-K+-ATPase mRNA expression following intense exercise. *Am J Physiol Regul Integr Comp Physiol* 289: R266–R274, 2005. doi:10.1152/ajpregu.00378.2004.

55. **Powers SK, Kavazis AN, DeRuisseau KC.** Mechanisms of disuse muscle atrophy: role of oxidative stress. *Am J Physiol Regul Integr Comp Physiol* 288: R337–R344, 2005. doi:10.1152/ajpregu.00469.2004.
56. **Radzyukevich TL, Neumann JC, Rindler TN, Oshiro N, Goldhamer DJ, Lingrel JB, Heiny JA.** Tissue-specific role of the Na,K-ATPase α2 isozyme in skeletal muscle. *J Biol Chem* 288: 1226–1237, 2013. doi:10.1074/jbc.M112.424663.
57. **Remmers DE, Cioffi WG, Bland KI, Wang P, Angele MK, Chaudry IH.** Testosterone: the crucial hormone responsible for depressing myocardial function in males after trauma-hemorrhage. *Ann Surg* 227: 790–799, 1998. doi:10.1097/00000658-199806000-00002.
58. **Schatzl G, Madersbacher S, Thurridl T, Waldmüller J, Kramer G, Haitel A, Marberger M.** High-grade prostate cancer is associated with low serum testosterone levels. *Prostate* 47: 52–58, 2001. doi:10.1002/pros.1046.
59. **Scheiner-Bobis G.** The sodium pump. Its molecular properties and mechanics of ion transport. *Eur J Biochem* 269: 2424–2433, 2002. doi:10.1046/j.1432-1033.2002.02909.x.
60. **Sejersted OM, Sjøgaard G.** Dynamics and consequences of potassium shifts in skeletal muscle and heart during exercise. *Physiol Rev* 80: 1411–1481, 2000. doi:10.1152/physrev.2000.80.4.1411.
61. **Sheng-qi L, Bin-Yan Z, Ling-Long W.** A successive obervation of Na+ K+ ATPase activity in male immature castrated rats. *Bing Du Xue Bao*, 1994.
62. **Sinha-Hikim I, Artaza J, Woodhouse L, Gonzalez-Cadavid N, Singh AB, Lee MI, Storer TW, Casaburi R, Shen R, Bhasin S.** Testosterone-induced increase in muscle size in healthy young men is associated with muscle fiber hypertrophy. *Am J Physiol Endocrinol Metab* 283: E154–E164, 2002. doi:10.1152/ajpendo.00502.2001.
63. **Stanworth R, Jones T.** Testosterone in obesity, metabolic syndrome and type 2 diabetes. *Front Horm Res* 37: 74–90, 2009. doi:10.1159/000176046.
64. **Stellato RK, Feldman HA, Hamdy O, Horton ES, McKinlay JB.** Testosterone, sex hormone-binding globulin, and the development of type 2 diabetes in middle-aged men: prospective results from the Massachusetts male aging study. *Diabetes Care* 23: 490–494, 2000. doi:10.2337/diacare.23.4.490.
65. **Traish AM, Saad F, Guay A.** The dark side of testosterone deficiency: II. Type 2 diabetes and insulin resistance. *J Androl* 30: 23–32, 2009. doi:10.2164/jandrol.108.005751.
66. **Vingren JL, Kraemer WJ, Ratamess NA, Anderson JM, Volek JS, Maresh CM.** Testosterone physiology in resistance exercise and training: the up-stream regulatory elements. *Sports Med* 40: 1037–1053, 2010. doi:10.2165/11536910-000000000-00000.
67. **Xie Z, Askari A.** Na(+)/K(+)-ATPase as a signal transducer. *Eur J Biochem* 269: 2434–2439, 2002. doi:10.1046/j.1432-1033.2002.02910.x.
68. **Xie Z, Cai T.** Na+-K+--ATPase-mediated signal transduction: from protein interaction to cellular function. *Mol Interv* 3: 157–168, 2003. doi:10.1124/mi.3.3.157.
69. **Zemková H, Teisinger J, Almon RR, Vejsada R, Hník P, Vyskočil F.** Immobilization atrophy and membrane properties in rat skeletal muscle fibres. *Pflugers Arch* 416: 126–129, 1990. doi:10.1007/BF00370233.
70. **Zhang L, Morris KJ, Ng Y-C.** Fiber type-specific immunostaining of the Na+,K+-ATPase subunit isoforms in skeletal muscle: age-associated differential changes. *Biochim Biophys Acta* 1762: 783–793, 2006. doi:10.1016/j.bbadis.2006.08.006.