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Cell specific differences in the protein abundances of GAPDH and Na^+,K^+-ATPase in skeletal muscle from aged individuals

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ABSTRACT

Na^+, K^+-ATPase (NKA) isoforms (α_1,α_2,α_3,β_1,β_2,β_3) are involved in the maintenance of membrane potential and hence are important regulators of cellular homeostasis. Given the age-related decline in skeletal muscle function, we investigated whether the natural physiological process of aging is associated with altered abundance of NKA isoforms (α_1,α_2,α_3,β_1,β_2,β_3) or of the commonly used control protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Importantly, measurements were made in both whole muscle or specific fiber types obtained from skeletal muscle biopsies. Seventeen healthy older (AGED, 69.4 ± 3.5 years, mean ± SD) and 14 younger (YOUNG, 25.5 ± 2.8 years) adults underwent a muscle biopsy for biochemical analyses. Comparing homogenates from AGED and YOUNG individuals revealed higher β_3 isoform ($p < 0.05$) and lower GAPDH ($p < 0.05$). Analysis of individual fibers in muscle from YOUNG individuals, showed greater α_3 and β_2 isoforms, and more GAPDH in Type II compared with Type I fibers ($p < 0.05$). In the AGED, GAPDH was higher in Type II compared with Type I fibers ($p < 0.05$), there were no fiber type differences in the NKA isoforms ($p > 0.05$). Compared with the same fiber type in YOUNG, α_1 was greater (Type I) and α_3 lower (Type II), while in both fiber types, β_2 was lower, β_3 greater and GAPDH lower, in muscle from AGED individuals (all $p < 0.05$). Overall, we demonstrate that (i) GAPDH is an inappropriate choice of protein for normalization in all skeletal muscle research and (ii) full understanding of the role of NKA isoforms in human skeletal muscle requires consideration of age and muscle fiber type.

1. Introduction

Aging is associated with a progressive decline in muscle mass, strength and function (Ballak et al., 2014). The loss of muscle strength and function may in part be related to alterations to proteins which regulate skeletal muscle excitability. Any such alterations would have an effect downstream on the capability of the muscle to produce force necessary for work. In skeletal muscle, the Na^+,K^+-ATPase (NKA) is a key protein generating and sustaining trans-membrane Na^+/K^+ gradients, thus playing a critical role in preserving cell excitability (Clausen, 2003).

Previous assessment of data available in the literature deduced that resting membrane potential was lower in skeletal muscle obtained from older humans (61–85 years) compared with that seen in younger individuals (19–40 years)(Forsberg et al., 1997). Another study reported that older adults had increased resting plasma K^+ under basal conditions compared with healthy young adults and, significantly, showed impairments in plasma K^+ regulation during incremental exercise (Ford et al., 1993). These studies suggest that in older adults the overall content of the skeletal muscle NKA may be reduced or that there may be alterations to the specific isoforms, yet this has yet to be thoroughly examined in human skeletal muscle.

Skeletal muscle expresses six NKA isoforms, three catalytic α isoforms (α_{1-3}), three regulatory β isoforms (β_{1-3}), as well as the regulatory FXYD1 protein, phospholemman (Murphy et al., 2004; Benziane et al., 2011; Thomassen et al., 2013). Studies with genetically modified mice have demonstrated isoform-specific functions for NKA α_1 and α_2 in skeletal muscle. The NKA α_1 isoform is important in regulating basal Na^+/K^+ exchange and membrane potential and also influences muscle strength; the NKA α_2 isoform is vital in countering exercise-induced Na^+/K^+ fluxes, enabling exercise performance and resisting muscle fatigue (He et al., 2001; Lingrel et al., 2003; Lingrel et al., 2007; Radzyukevich et al., 2013). The role for the α_3 isoform in skeletal muscle is unclear. Little is known about the function of the individual β isoforms, with no studies on genetically modified animals in the literature. While it is known that β isoforms are necessary for a functional heterodimer, the relative proportions and which specific β isoform is present in a given heterodimer are not clear for human skeletal muscle. To

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date the specific functions of any NKA isoforms in human skeletal muscle are unknown. Investigation of the effects of aging into NKA content in skeletal muscle is not clear. It is accepted that NKA content in whole muscle is best measured utilising the [^{3}H]ouabain binding site technique (Nørgaard et al., 1984; Clausen, 1996). We recently reported that NKA content was 26% lower in older adults aged between 69 and 81 years compared with those aged 55–68 years (Perry et al., 2013). Overall while comparison of [^{3}H]ouabain binding site content in whole muscle between untrained aged and young adults revealed no significant differences (Klitgaard and Clausen, 1989; McKenna et al. 2012), there was 14% non-significant lower content in the muscle from aged compared with young individuals (Klitgaard and Clausen, 1989). Interestingly, Klitgaard and Clausen found that, compared with untrained aged individuals, total muscle ^{3}[H]ouabain binding was greater in similar aged cohorts who swim, run or were strength trained (Klitgaard and Clausen, 1989) consistent with the finding that NKA content in skeletal muscle is highly adaptable to physical training (Green et al., 1993; McKenna et al., 1993).

Studies in rats report age-related increases in NKA α_1 and β_1 isoforms and decreases in β_2 isoforms, in red and white gastrocnemius muscles, when measured by immunocytochemistry (Ng et al., 2003) and Western blotting (Sun et al., 1999). In contrast, the only study to compare NKA isoforms in skeletal muscle homogenates from aged and young individuals, reported lower NKA α_2 and β_3 isoform abundances with aging (McKenna et al. 2012). Importantly, that study normalized to the commonly used housekeeping protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a glycolytic enzyme (McKenna et al. 2012). There is evidence that GAPDH is not an appropriate housekeeping protein in aging, as it was reduced in Type II fibers from aged compared with young rats (Lowe et al., 2000), as well as very recently, also being less in whole muscle homogenates from old compared with young humans (Vigelsø et al., 2015).

An important consideration in skeletal muscle studies is the inherent fiber type heterogeneity present, as muscle is comprised of Type I (slow-twitch) and Type II (fast-twitch) fibers. In muscle from rat (Fowles et al., 2004) and human (Thomassen et al., 2013; Wyckelsma et al., 2015) some NKA isoforms are present in a fiber type dependent manner. Given that aging induces specific loss and atrophy of Type II fibers (Lexell, 1995; Lamboley et al., 2015), we hypothesized that aging has an impact on the abundance of muscle NKA isoforms in a fiber-dependent manner. Hence we investigated NKA isoforms in single fibers isolated from the *vastus lateralis* muscle obtained from healthy young (YOUNG) and aged (AGED) adults. Based on data in human and rat muscle, we hypothesized that NKA isoforms would be present in either lower (α_2 and β_2 isoforms) or higher (α_1 and β_1 isoforms) amounts in whole muscle homogenates from AGED compared with YOUNG individuals. We also investigated NKA content of skeletal muscle from YOUNG and AGED individuals using [^{3}H]ouabain binding site content, which we hypothesized would not be altered in muscle from the AGED compared with YOUNG individuals. Finally, it was hypothesized that GAPDH would be more abundant in Type II compared with Type I fibers in human skeletal muscle, and would be reduced in AGED adults in both fiber types.

2. Experimental procedures

2.1. Participants and experimental design

This study was approved by the Victoria University Human Research Ethics Committee and conforms to the Declaration of Helsinki. After obtaining medical clearance, 31 participants gave written informed consent and commenced pre-screening tests. A total of 17 healthy older adults (AGED, 10 males, 7 females) and 14 healthy YOUNG adults (YOUNG, 6 males, 8 females) finished the study. The physical characteristics of the AGED (mean ± SD) were age 69.4 ± 3.5 years (range 65–76 years), height 170.8 ± 10.4 cm and body mass 75.2 ± 13.0 kg and for the YOUNG were age 25.5 ± 2.8 years, height 173 ± 12.3 cm, and body mass 72.9 ± 15.6 kg. Apart from age ($p < 0.05$), these physical characteristics did not significantly differ between groups ($p > 0.05$).

YOUNG participants were recruited following the AGED so that they could be matched for the self-reported number of hours of physical activity (8.2 ± 4.4 vs. 6.8 ± 3.9 $h \cdot wk^{-1}$, respectively, AGED vs YOUNG, $p = 0.416$) (Lamboley et al., 2015).

2.2. Resting muscle biopsy and single fiber separation

The biopsy procedure was as described (McKenna et al. 2012). After an injection of a local anesthetic into the skin and fascia (Xylocaine 1%, AstraZeneca, Australia) a small incision was made in the *vastus lateralis* muscle and a sample was taken using a biopsy needle with suction. The muscle was rapidly blotted on filter paper to remove excess blood and approximately 15 mg was placed in a petri dish with paraffin oil. Approximately 30–40 single fiber segments (~3–5 mm in length) were separated from the fresh muscle under a dissecting microscope using jeweler's forceps. The remaining muscle was rapidly frozen in liquid nitrogen and stored at −80 °C until later analysis. Single fibers were dissected from the biopsies obtained from 14 AGED and 13 YOUNG individuals. Whole muscle homogenates were prepared initially from 8 AGED and 8 YOUNG individuals, but in some circumstances this was increased to 12 AGED and 12 YOUNG individuals to address the potential issue of a Type 2 error. Further, for examining the NKA β_2 isoform, only the latter 4 AGED samples could be examined due to difficulties with batch-to-batch variation in the antibody during the course of the study and the company discontinuing the line.

2.3. Whole muscle homogenates

A small portion of whole muscle (15–30 mg), was accurately weighed and homogenized on ice (1:20 wt:vol) in Na–EGTA solution (165 mM Na^+, 50 mM EGTA, 90 mM HEPES, 1 mM free Mg^{2+} (10.3 mM total Mg^{2+}), 8 mM total ATP, 10 mM creatine phosphate, pH 7.10) with a protease inhibitor cocktail (PIC, Complete; Roche Diagnostics, Sydney, Australia). Immediately following this, the homogenate was diluted to 33 μg^{-1} µl muscle wet weight using 3× SDS solution (0.125 M Tris–HCl, 10% glycerol, 4% SDS, 4 M urea, 10% mercaptoethanol and 0.001% bromophenol blue, pH 6.8). Finally, samples were further diluted to 2.5 µg wet weight muscle·μl^{-1} samples in 3× SDS solution diluted 2:1 with 1× Tris·Cl (pH 6.8), as previously described (Murphy et al., 2011). Portions from each AGED muscle homogenate were pooled together and the pooled sample used to create the same four point calibration curve for every gel. Use of the same homogenate for all calibration curves across all gels performed for the entire study allowed comparisons of single fibers or whole muscle homogenates across gels. Individually, these homogenates were used to compare the NKA isoform and GAPDH protein abundances in muscle from AGED and YOUNG individuals. In most cases, duplicate or triplicate measurements were obtained for each sample.

2.4. Western blotting

Western blots on single skeletal muscle fiber segments and whole muscle homogenates were performed to determine the relative protein abundances of NKA isoforms (α_{1-3}, β_{1-3}) and GAPDH, as well as myosin heavy chain (MHC) I and II isoforms for fiber type identification. The single fiber Western blotting technique has been previously described (Murphy, 2011; Wyckelsma et al., 2015). Briefly, samples were loaded onto gels with calibration curves (1 to 30 µg total muscle wet weight) derived from addition of graduated amounts of whole human muscle homogenate (Murphy and Lamb, 2013). In some instances half a fiber was loaded onto each of two gels, allowing the entire set of NKA isoforms to be analyzed in the same fibers. Denatured protein samples were separated on 26 well, 4–15% or 10% Criterion TGX Stain Free gels