Influence of chronic and acute spinal cord injury on skeletal muscle 
Na\(^+\)-K\(^+\)-ATPase and phospholemman expression in humans

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Submitted 7 December 2011; accepted in final form 19 January 2012

Boon H, Kostovski E, Pirkmajer S, Song M, Lubarski I, Iversen PO, Hjeltnes N, Widegren U, Chibalin AV. Influence of chronic and acute spinal cord injury on skeletal muscle Na\(^+\)-K\(^+\)-ATPase and phospholemman expression in humans. Am J Physiol Endocrinol Metab 302: E864–E871, 2012. First published January 24, 2012; doi:10.1152/ajpendo.00625.2011.—Na\(^+\)-K\(^+\)-ATPase is an integral membrane protein crucial for the maintenance of ion homeostasis and skeletal muscle contractibility. Skeletal muscle Na\(^+\)-K\(^+\)-ATPase content displays remarkable plasticity in response to long-term increase in physiological demand, such as exercise training. However, the adaptations in Na\(^+\)-K\(^+\)-ATPase function in response to a suddenly decreased and/or habitually low level of physical activity, especially after a spinal cord injury (SCI), are incompletely known. We tested the hypothesis that skeletal muscle content of Na\(^+\)-K\(^+\)-ATPase and the associated regulatory proteins from the FXYD family is altered in SCI patients in a manner dependent on the severity of the spinal cord lesion and postinjury level of physical activity. Three different groups were studied: 1) six subjects with chronic complete cervical SCI, 2) seven subjects with acute, complete cervical SCI, and 3) six subjects with acute, incomplete cervical SCI. The individuals in groups 2 and 3 were studied at months 1, 3, and 12 postinjury, whereas individuals with chronic SCI were compared with an able-bodied control group. Chronic complete SCI was associated with a marked decrease in [\(^3\)H]ouabain binding site concentration in skeletal muscle as well as reduced protein content of the α1-, α2-, and β1-subunit of the Na\(^+\)-K\(^+\)-ATPase. In line with this finding, expression of the Na\(^+\)-K\(^+\)-ATPase α1- and α2-subunits progressively decreased during the first year after complete but not after incomplete SCI. The expression of the regulatory protein phospholemman (PLM or FXYD1) was attenuated after complete, but not incomplete, cervical SCI. In contrast, FXYD5 was substantially upregulated in patients with complete SCI. In conclusion, the severity of the spinal cord lesion and the level of postinjury physical activity in patients with SCI are important factors controlling the expression of Na\(^+\)-K\(^+\)-ATPase and its regulatory proteins PLM and FXYD5. FXYD proteins; sodium pump; physical inactivity; paralysis

THE Na\(^+\)-K\(^+\)-ATPase IS AN INTEGRAL MEMBRANE PROTEIN that is crucial for the maintenance of ion homeostasis, cell volume, and muscle contractibility. It is composed of two polypeptide subunits, a catalytic 112-kDa α-subunit (α1-, α2-, and α3-isoforms) and a 35- to 60-kDa glycosylated β-subunit (β1-, β2-, and β3-isoforms) (6, 35). The relative abundance of each isoform and Na\(^+\)-K\(^+\)-ATPase activity is regulated in a muscle- and fiber type-specific manner (29, 59, 62). Among the regulating factors of Na\(^+\)-K\(^+\)-ATPase are hormones, ions (Na\(^+\) and K\(^+\)), and phospholemman (PLM; FXYD1), a transmembrane protein of the FXYD domain-containing ion transport regulator family (4, 8, 51, 56).

Skeletal muscle Na\(^+\)-K\(^+\)-ATPase content displays remarkable plasticity in response to an increased long-term physiological demand such as exercise training (31). Acute exercise in untrained subjects increases mRNA expression of Na\(^+\)-K\(^+\)-ATPase α- and β-subunit isoforms (47, 50). Furthermore, both Na\(^+\)-K\(^+\)-ATPase content and activity are increased after endurance training (3, 17, 18), and [\(^3\)H]ouabain binding (reflecting Na\(^+\)-K\(^+\)-ATPase content) is higher in endurance-trained males than in sedentary controls (45). Such increases in content and maximal activity of the Na\(^+\)-K\(^+\)-ATPase are involved in the maintenance of membrane excitability and ion homeostasis and thus may increase fatigue resistance (40). Conversely, the adaptations in Na\(^+\)-K\(^+\)-ATPase function that occur in response to suddenly decreased and/or habitually low levels of physical activity are incompletely known. In animals, immobilization decreases the Na\(^+\)-K\(^+\)-ATPase content by up to 25% in guinea pig hindlimb muscle (33) and in soleus muscle of young rats (65). Furthermore, muscle inactivity induced by denervation, plastr immobilization, or denervation reduced the [\(^3\)H]ouabain binding site concentration by 20–30% (31). The effects of different levels of physical inactivity on Na\(^+\)-K\(^+\)-ATPase content or isoform expression in humans are incompletely described. However, shoulder immobilization leads to a profound reduction in [\(^3\)H]ouabain binding sites in the deltoid muscle (34), thus indicating a reduced Na\(^+\)-K\(^+\)-ATPase content upon physical inactivity.

Cervical spinal cord injury (SCI) leads to varying degrees of physical inactivity (depending on the extent and severity of the lesion) and thus functions as a model to study the effects of an acute or chronic lack of physical activity. The paralysis and physical inactivity associated with SCI causes profound changes in skeletal muscle metabolism and morphology below the injury level that include atrophy, fiber type transformation, reduced oxidative capacity, and reduced fiber size (2, 32, 36). These metabolic and morphological changes may lead to reduced basal metabolic rate (1, 7, 44) and impair whole body glucose homeostasis and may account partly for the increased risk of type 2 diabetes mellitus and cardiovascular diseases in these individuals (64). Expression of Na\(^+\)-K\(^+\)-ATPase is decreased in people with a low fitness level, insulin resistance, or...
type 2 diabetes mellitus, which provides evidence of a role for Na\(^+\)–K\(^+\)-ATPase protein abundance level in insulin resistance and accompanying low physical activity (12, 15, 17). Additionally, total Na\(^+\)–K\(^+\)-ATPase content, as measured by ouabain binding, is decreased in chronic SCI (14). However, the extent to which severity of the spinal cord lesion and postinjury level of physical inactivity determine Na\(^+\)–K\(^+\)-ATPase subunit expression and its regulatory proteins remains to be determined.

The degree of physical inactivity after SCI depends on the level and extent of the lesion. In contrast to complete lesions, sensory and/or motor function below the level of injury may be preserved after an incomplete lesion. Thus, patients with sufficient motor function of the lower limbs may be able to perform mild weight-bearing exercises. Such varying degrees of physical inactivity, depending on the severity of the lesion, allow for the identification of a possible differential response of Na\(^+\)–K\(^+\)-ATPase abundance to varying levels of physical inactivity.

In this study, we tested the hypothesis that skeletal muscle expression of Na\(^+\)–K\(^+\)-ATPase and the associated regulatory proteins from the FXYD family are altered in SCI patients in a manner dependent on the severity of the spinal cord lesion and postinjury level of physical activity. Moreover, several hormones involved in long-term regulation of Na\(^+\)–K\(^+\)-ATPase were measured to provide additional insight into mechanisms controlling Na\(^+\)–K\(^+\)-ATPase expression following SCI.

MATERIALS AND METHODS

*Ethical approval.* The procedures of the study were explained to the subjects, and all subjects gave their written informed consent. The Regional Committee for Medical Ethics at Helse Sør-Øst, Norway, and the Regional Ethics Committee at Karolinska Institutet, Sweden, approved the study protocol, and the investigation conforms to the principles outlined in the Declaration of Helsinki.

*Subjects.* Subjects’ characteristics of the spinal cord injured and control groups are described in Table 1. Three groups of subjects with SCI were recruited for this study: (1) six males with complete lesions of the cervical spinal cord, (2) seven subjects (6 male, 1 female) with acute, complete cervical SCI, and (3) six males with acute, incomplete lesions of the cervical spinal cord. Spinal cord injuries are classified by the American Spinal Injury Association (ASIA) impairment scale, which indicates the level of remaining sensory and/or motor function below the spinal level of injury. Therefore, ASIA classification is an indication of the completeness of injury and its functional consequences. In short, an ASIA-A injury indicates no preserved motor or sensory function, ASIA-B indicates preserved sensory function but no preserved motor function, and ASIA-C and ASIA-D injuries indicate preserved sensory function with varying degrees of loss of motor function.

Subjects in groups 2 and 3 were studied throughout the first year after SCI, with biopsies taken 1, 3, and 12 mo after injury. Eight healthy males were recruited as control subjects for group 1.

*Study protocol.* All spinal cord-injured subjects underwent a thorough clinical examination, including routine blood chemistry analysis as well as dual-energy X-ray absorptionmetry scans at time of biopsy (group 1) or at 3 and 12 mo after injury (groups 2 and 3). Skeletal muscle biopsies were obtained under local anesthesia from the vastus lateralis and immediately frozen in liquid nitrogen, as described previously (27).

*Muscle lysis preparation and Western blot analysis.* All muscle samples were freeze-dried before microscopical removal of fat, blood, and connective tissue. Cleared muscle fibers were immersed and homogenized with a motor-driven pestle in 500 μl of ice-cold buffer containing 137 mM NaCl, 1 mM MgCl\(_2\), 2.7 mM KCl, 1 mM EDTA, 20 mM Tris, pH 7.8, 5 mM Na pyrophosphate, 10 mM NaF, 1% Triton X-100, 100 mM (vol/vol) glycerol, 0.2 mM phenylmethanesulfonfyl fluoride, 0.5 mM Na\(_3\)VO\(_4\), and 1× protease inhibitor cocktail set 1 (Calbiochem-EMD Biosciences, San Diego, CA), followed by end-over-end rotation at 4°C for 60 min. Insoluble material was removed by centrifugation at 12,000 × g for 10 min at 4°C, followed by collection of the supernatant. Only negligible amounts of Na\(^+\)–K\(^+\)-ATPase subunits (1–2% of initial homogenate) were detected by Western blot in the final 12,000-g pellet, dissolved in 3.7% SDS and 6 M urea, indicating efficient recovery of the proteins in the supernatant. Expression of Na\(^+\)–K\(^+\)-ATPase β-subunits was determined after den glycosylation of the protein. To remove N-glycan chains, 10 μl of lysate was incubated at 37°C for 60 min with peptide N-glycosidase F (P0704, New England Biolabs, Ipswich, MA), as described (17). Protein concentration was determined using a commercially available protein assay (Pierce BCA protein assay kit; Thermo Scientific, Rockford, IL). Sample dilutions were adjusted to yield equal protein concentration. Aliquots for Western blot analysis were suspended in Laemml buffer and separated by SDS-PAGE, and proteins were transferred to polyvinylidene difluoride membranes. Membranes were blocked with 7.5% nonfat dry milk in Tris-buffered saline containing 0.1% (vol/vol) Tween (TBS-T) for 1–2 h at room temperature. Membranes were incubated overnight at 4°C with primary antibodies.

### Table 1. Subjects’ characteristics

<table>
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<tr>
<th>Variable</th>
<th>Control</th>
<th>Chronic SCI</th>
<th>Complete SCI</th>
<th>Incomplete SCI</th>
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<tbody>
<tr>
<td>Males/females</td>
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<td>6/0</td>
<td>6/1</td>
<td>6/0</td>
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<tr>
<td>Age, y</td>
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<td>44 ± 3</td>
<td>33 ± 4</td>
<td>49 ± 5*#</td>
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<td>BMI kg/m(^2)</td>
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<td>Month 3</td>
<td>24.2 ± 1.1</td>
<td>24.6 ± 1.24</td>
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<tr>
<td>Month 12</td>
<td>25.0 ± 1.7</td>
<td>25.4 ± 1.21</td>
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<td>ASIA level</td>
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<td>A</td>
<td>C/D</td>
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<td>Na(^+), mmol/l</td>
<td>140 ± 2</td>
<td>138 ± 1</td>
<td>141 ± 1</td>
<td>141 ± 1</td>
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<tr>
<td>Month 1</td>
<td>141 ± 1</td>
<td>141 ± 1</td>
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<td>Month 3</td>
<td>141 ± 1</td>
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<td>Month 12</td>
<td>142 ± 1</td>
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<tr>
<td>K(^+), mmol/l</td>
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<td>4.30 ± 0.12</td>
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<td>4.30 ± 0.09</td>
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<td>Month 3</td>
<td>4.00 ± 0.09†</td>
<td>4.20 ± 0.17</td>
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<td>Glucose</td>
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<td>mmol/l</td>
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<td>4.63 ± 0.18</td>
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<td>4.74 ± 0.07</td>
<td>5.15 ± 0.09#</td>
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<td>Insulin, pmol/l</td>
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<td>Month 1</td>
<td>45 ± 6</td>
<td>45 ± 9</td>
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<td>Month 3</td>
<td>26 ± 3</td>
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<td>Month 12</td>
<td>62 ± 20</td>
<td>47 ± 17</td>
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<td>Cortisol, nmol/l</td>
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<td>Month 1</td>
<td>427 ± 63</td>
<td>485 ± 78</td>
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<td>Month 3</td>
<td>361 ± 65</td>
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<td>Month 12</td>
<td>347 ± 31</td>
<td>460 ± 77</td>
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<td>TSH, mIU/l</td>
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<tr>
<td>Month 1</td>
<td>1.07 ± 0.17</td>
<td>1.56 ± 0.33</td>
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<tr>
<td>Month 3</td>
<td>1.75 ± 0.22</td>
<td>1.45 ± 0.18</td>
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<tr>
<td>Month 12</td>
<td>1.26 ± 0.11†</td>
<td>1.29 ± 0.13</td>
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<tr>
<td>T(_4), pmol/l</td>
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<tr>
<td>Month 1</td>
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<tr>
<td>Month 3</td>
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<td>14.8 ± 0.6</td>
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<td>Month 12</td>
<td>15.0 ± 0.7</td>
<td>14.9 ± 0.8</td>
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</table>

Values indicate means ± SE. SCI, spinal cord injury; BMI, body mass index; ASIA, American Spinal Injury Association; T\(_4\), thyroxine. *P < 0.05 vs. control. †P < 0.05 vs. complete SCI (same time point). ‡P < 0.05, change over time.
directed against α1-, α2-, α3-, β1-, β2-, and β3-subunits of the Na+-K+-ATPase, total PLM, phospho-PLM Ser68, and phospho-PLM Ser63. The antibody against total PLM was acquired from ProteinTech Group (Chicago, IL). Antibodies against phospho-PLM Ser68 and phospho-PLM Ser63 were kindly donated by Dr. J. Cheung (Thomas Jefferson University, Philadelphia, PA). Polyclonal antibodies against the β2-subunit of Na+-K+-ATPase were kindly provided by Dr. P. Martín-Vasallo (University of La Laguna, Tenerife, Spain). The antibody against FXYD5 was kindly provided by Dr. Haim Garty (Weizmann Institute of Science, Rehovot, Israel). Na+-K+-ATPase α1 antibody was purchased from Cell Signaling Technology (Danvers, MA), Na+-K+-ATPase α3 antibody from Millipore (Billerica, MA), Na+-K+-ATPase α3 antibody from Affinity Bioreagents/AH Diagnostics, Na+-K+-ATPase β1 monoclonal antibody from ThermoFisher (Waltham, MA), and Na+-K+-ATPase β3 and GAPDH antibody from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were of analytical grade (Sigma, St. Louis, MO), unless otherwise specified. Membranes were washed with TBS-T, which was followed by incubation with the appropriate horseradish peroxidase-conjugated anti-IgG antibody. Antibody-bound protein was detected using enhanced chemiluminescence (Amersham, Arlington, IL) and quantified by densitometry using Quantity One Software (Bio-Rad, Hercules, CA). Ponceau staining was used to assess equal protein loading, as validated recently (53). Moreover, in line with our previous study (36), we did not detect any difference in GAPDH protein expression between the groups studied (data not shown).

[^3H]ouabain binding. Measurements of skeletal muscle[^3H]ouabain (Amersham) binding site concentration were performed as described (30, 55). Biopsy samples (3–5 mg wet wt) were washed twice for 10 min at 4°C in a buffer containing 10 mM Tris·HCl, 250 mM sucrose, 3 mM MgSO4 and 1 mM Na2VO4, pH 7.4. Subsequently, specimens were equilibrated twice at 37°C for 60 min (120 min in total) in the same buffer containing[^3H]ouabain (2 μCi/ml) and unlabeled ouabain to a final concentration of 1 μM. After incubations, a washout in ice-cold unlabeled buffer for 2 h with a change of medium every 30 min was performed to eliminate nonspecific[^3H]ouabain binding. Samples were then blotted on dry filter paper, weighed, and soaked with 0.5 ml of 5% trichloroacetic acid overnight in minivials. The following day, 3 ml of scintillation fluid was added, and samples were counted in a tritium channel. The amount of[^3H]ouabain uptake and retained by samples was calculated on the basis of sample wet weight and the specific radioactivity of incubation medium and samples and was expressed as picomoles per gram wet weight.[^3H]ouabain binding site concentration was measured in triplicates for each muscle.

Blood chemistry analysis. All blood samples were analyzed for insulin, cortisol, free thyroxine (T4) and thyroid-stimulating hormone (TSH) using commercial glucose assays (PerkinElmer Life Sciences, Turku, Finland, and Immulite 2000; Siemens Healthcare Diagnostics, Los Angeles, CA) at the Aker University Hospital, Oslo, Norway. Plasma [K+] and [Na+] measurements were performed using a Vitros 250 Chemistry System (Ortho Clinical Diagnostics; Johnson & Johnson).

Statistics. Data are presented as means ± SE. Data were evaluated using IBM SPSS Statistics Version 18 (IBM, Chicago, IL). A Mann-Whitney U-test was performed to identify differences between SCI and control subjects for variables with single measurements. One-way repeated measures ANOVA was performed to identify changes in protein expression levels during the first year after SCI. When a significant F ratio was found, Tukey’s least significant difference post hoc test was performed to identify the exact location of the differences. P values <0.05 were considered to indicate statistical significance.

RESULTS

Subjects’ characteristics and plasma chemistry. Subjects’ characteristics as well as plasma hormone, glucose, and cation concentrations are given in Table 1. Body mass index between groups was comparable and increased slightly over time in subjects with SCI. Dual-energy X-ray absorptiometry scans performed at 3 and 12 mo postinjury indicated no changes in whole body muscle mass or fat percentage from 3 to 12 mo in any group (data not shown). When only muscle and adipose tissue in the lower limbs were considered, a nonsignificant trend toward an increased fat percentage in the lower limbs of the subjects with an incomplete injury was observed (P = 0.08). For practical reasons, body composition measurements were not obtained in these subjects at 1 mo postinjury. To determine whether potential changes in Na+-K+-ATPase protein expression were associated with changes in plasma values of Na+ and K+, plasma cation concentrations were measured at all time points postinjury. In the first year after complete injury, plasma K+ concentration decreased from 4.3 ± 0.3 to 4.0 ± 0.2 mmol/l (P < 0.05), and a trend toward an increase in plasma Na+ levels was observed (P = 0.08). However, these parameters were unaltered during the first year after incomplete injury. Plasma levels of glucose, insulin, cortisol, TSH, and free T4 were not different between the groups, nor did they display deviation from normal reference values.

Expression of the Na+-K+-ATPase and associated regulatory proteins in chronic SCI. Protein expression of the catalytic α1- and α2-subunits of the Na+-K+-ATPase was reduced in study participants with a chronic complete cervical SCI (α1 75% lower than in able-bodied control subjects, α2 52% lower than controls; Fig. 1, A and B). β1-Subunit expression was 38% lower with a chronic complete cervical SCI compared with control subjects (P < 0.05; Fig. 1D). Protein expression of the α3-, β2-, and β3-subunits was unaltered between the two groups (Fig. 1, C, E, and F).

Expression of the regulatory PLM protein (a transmembrane protein of the FXYD family) was reduced by 52% in study participants with a chronic, complete cervical SCI compared with control subjects (Fig. 2A). However, changes in total PLM expression were not accompanied by similar alterations in the levels of phospho-PLM Ser68 and phospho-PLM Ser63 (Fig. 2, B and C, respectively). The apparent increase in the ratio of phosphorylated PLM to total PLM could represent a compensatory response (i.e., disinhibition of Na+-K+-ATPase) in chronic SCI. In contrast, expression of another member of the FXYD family, FXYD5, was increased substantially in individuals with chronic SCI (Fig. 2D).

[^3H]ouabain binding in chronic SCI. A marked decrease in[^3H]ouabain binding site concentration, reflecting reduced Na+-K+-ATPase content, was found in subjects with chronic complete cervical SCI vs. able-bodied control subjects (Fig. 3).
creased, this resulted in an apparently increased ratio of phospho-PLM Ser68 to total PLM. Phosphorylation of PLM at Ser 63 tended to decrease over time, but changes were not statistically significant (Fig. 5C). In contrast to the markedly changed expression patterns in chronic (Fig. 1) and recent complete SCI, the expression of the Na+/H+-ATPase subunits and total PLM expression were unaltered during the first year after incomplete cervical SCI (Figs. 4 and 5). Moreover, there was no significant change in the levels of phospho-PLM Ser68 and phospho-PLM Ser63 (Fig. 5, E and F).

**DISCUSSION**

In this study, we report that skeletal muscle expression of Na+/K+-ATPase and the associated regulatory proteins from the FXYD family are altered in SCI patients in a manner dependent on the severity of the spinal cord lesion and postinjury level of physical activity. Na+/K+-ATPase protein expression is profoundly reduced in an isoform-specific manner after complete but not incomplete cervical SCI in humans. Moreover, whereas total PLM protein expression was reduced with simultaneous increase in phospho-PLM Ser68 level, total FXYD5 protein was substantially upregulated in complete SCI. The level of remaining neuromuscular activity appears to be an important determinant of the extent of adaptive changes in Na+/K+-ATPase since concentrations of hormones, which are normally involved in long-term regulation of Na+/K+-ATPase, did not differ between individuals with complete vs. incomplete SCI. The expression of Na+/K+-ATPase is increased significantly in response to physical activity and/or training (12, 20–22, 31, 40, 41, 45, 46, 61). However, our study is unique since we report isoform- and subunit-specific changes in Na+/K+-ATPase protein expression in relation to the extent and severity of the SCI and postinjury physical inactivity level in humans. In individuals with chronic complete SCI, a marked decrease was found in both Na+/K+-ATPase content (as reflected by ouabain binding) and in protein expression of the α1-, α2-, and β1-subunits of the Na+/K+-ATPase. Our findings are consistent with animal studies investigating the effect of...
SKELETAL MUSCLE Na⁺⁻K⁺-ATPase AFTER SPINAL CORD INJURY

Fig. 4. Protein expression of Na⁺⁻K⁺-ATPase α-subunit isoforms in subjects with complete and incomplete cervical SCI at months 1, 3, and 12 postinjury. Results (means ± SE) are expressed in AU; n = 6–7. A: Na⁺⁻K⁺-ATPase-α1 in complete SCI. B: Na⁺⁻K⁺-ATPase-α2 in complete SCI. C: Na⁺⁻K⁺-ATPase-α1 in incomplete SCI. D: Na⁺⁻K⁺-ATPase-α2 in incomplete SCI. *P < 0.05, change over time.

immobilization or denervation on Na⁺⁻K⁺-ATPase content. Leg immobilization in sheep for 9 wk is associated with a 39% reduction in Na⁺⁻K⁺-ATPase abundance in the vastus lateralis muscle (28). In rats, [³H]ouabain binding capacity in soleus muscle is decreased 22% after 7 days of denervation (11). In humans, Na⁺⁻K⁺-ATPase content is decreased in several conditions associated with physical inactivity, such as type 2 diabetes, McArdle’s disease, and chronic obstructive pulmonary disease (12, 15, 19, 25). In humans with complete thoracic SCI (T4–T10), total Na⁺⁻K⁺-ATPase content, as measured by the [³H]ouabain binding technique, is reduced compared with controls (14). Our results are consistent with these data but provide additional mechanistic insight, which indicates that the observed decrease in Na⁺⁻K⁺-ATPase content after SCI is isoform specific involving α1-, α2-, and β1- but not α3-, β2-, or β3-subunits. Furthermore, we demonstrate that these changes in Na⁺⁻K⁺-ATPase expression are rapidly established after the injury, because the extent of the decline in α-subunit expression in the first year after injury (50–80% in the 1st year) closely mirrors the difference in α-subunit expression between healthy controls and subjects with chronic SCI (50–75%).

A different approach to normalization of protein expression data (see MATERIALS AND METHODS) could theoretically affect the analysis of Western blots. However, this seems unlikely since measurements of [³H]ouabain binding provided essentially the same result.

Total protein expression of PLM was decreased in individuals with SCI, which was accompanied by an upregulation of phosphorylation of PLM at serine residue 68 after complete but not incomplete cervical SCI. PLM is a regulatory protein of the Na⁺⁻K⁺⁻ATPase expressed in skeletal muscle (16, 52). Phosphorylation of PLM leads to an increase of Na⁺⁻K⁺⁻ATPase activity primarily by increasing the affinity of the Na⁺⁻K⁺⁻ATPase for intracellular Na⁺ (13, 56, 57). The decreased PLM expression in complete SCI with concomitant increase in its phosphorylation may be a compensatory mechanism to overcome the decrease in Na⁺⁻K⁺⁻ATPase expression by increasing its activity. We have previously provided evidence that acute endurance exercise and a 10-day training program increase PLM phosphorylation at Ser⁶³ and Ser⁶⁸ without altering total PLM abundance (5). Since exercise increases Ser⁶³ and Ser⁶⁸ PLM phosphorylation in the exercised but not in the rested leg, these data suggested that phosphorylation of PLM may be due to a direct effect from neuromuscular activity rather than systemic or circulating factors. Expression of another member of the FXYD family, FXYD5, was markedly increased in chronic SCI patients. FXYD5, like PLM, is a partner protein of the Na⁺⁻K⁺⁻ATPase, but its upregulation increases Na⁺⁻K⁺⁻ATPase activity (38). This increase in FXYD5 expression may provide a mechanism to compensate for the decrease in Na⁺⁻K⁺⁻ATPase content in a manner analogous to the increased PLM phosphorylation. Furthermore, FXYD5 is a marker of dedifferentiation of cells, which may suggest that the muscle fibers demonstrate a dysfunctional structural organization (48).

A decreased expression, indicating reduced Na⁺⁻K⁺⁻ATPase activity in skeletal muscle, might decrease plasma Na⁺ levels and increase plasma K⁺ level. However, K⁺ plasma concentration decreased during the first 12 mo after complete cervical SCI and was at the low end of the normal range (3.6–5.5 mmol/l). Moreover, subjects with chronic SCI had completely normal K⁺ plasma concentration (Table 1). This suggests that mechanisms other than Na⁺⁻K⁺⁻ATPase are more essential in long-term maintenance of plasma cation concentration. Since tetraplegic individuals can rely less on the sympathetic nervous system (54), they are likely more dependent on the renin-angiotensin-aldosterone (RAA) system for regulation of blood pressure. This may lead to hyperactivation of the RAA system and increased aldosterone levels (24), which in turn would increase excretion of K⁺ by the kidney and explain the attenuated plasma K⁺ levels after complete SCI. Thus Na⁺⁻K⁺⁻ATPase expression in individuals with complete SCI seems sufficient to meet the demand and to maintain ionic homeostasis under basal conditions. Nevertheless, a functional deficit due to decreased Na⁺⁻K⁺⁻ATPase expression could perhaps become evident under conditions of increased ionic flux. In fact, a rapid and time-dependent increase in plasma potassium concentration is evident in tetraplegic patients during electrically stimulated leg cycling (ESLC) (43). Moreover, severe, life-threatening hyperkalemia can be triggered in SCI individuals by the depolarizing neuromuscular blocking agent succinylcholine (23, 49, 63). Although this is probably due mainly to accentuated potassium release (39), reduced Na⁺⁻K⁺⁻ATPase expression could plausibly worsen the situation, especially since skeletal muscle normally possesses the largest pool of Na⁺⁻K⁺⁻ATPase in the body (9).

A comparison between the effects of complete vs. incomplete SCI, as categorized according to the ASIA impairment scale, was undertaken to elucidate the factors that may be...
involved in the changes in Na\textsuperscript{+}-K\textsuperscript{+}-ATPase expression after SCI. The expression of PLM, as well as Na\textsuperscript{+}-K\textsuperscript{+}-ATPase \( \alpha_1 \) and \( \alpha_2 \)-subunits, decreased progressively during the first year after complete but not incomplete SCI. Consequently, the level of (especially voluntary) neuromuscular activity after SCI appears to be an important determinant of the changes in Na\textsuperscript{+}-K\textsuperscript{+}-ATPase and ion homeostasis. Neuromuscular activity is different between complete and incomplete SCI, and reduced neuromuscular activity is an important determinant of protein expression of myosin heavy-chain isoforms (27, 60). All of our subjects with incomplete SCI had an ASIA function score of C or D, indicating preserved motor and sensory function below the level of injury. Although pulse signal patterns and/or motor unit size may have changed, these patients still exhibited neuromuscular activity and were capable of exerting mild weight-bearing and walking exercises. The importance of neuromuscular activity on muscle properties after SCI is further stressed by evidence that spasms and ESLC may protect against whole muscle and/or type I fiber atrophy (26, 58) as well as increased expression of key metabolic enzymes (27). Moreover, ESLC improves inactivity-associated changes in exercise performance capacity, as evident in increased \( \dot{V}O_2 \text{max} \) and decreased fatigue (42).

Other than neuromuscular activity, several hormones, including thyroid hormones and cortisol, can regulate long-term Na\textsuperscript{+}-K\textsuperscript{+}-ATPase expression (10). However, TSH, \( T_4 \), and cortisol as well as insulin and plasma glucose did not differ between individuals with complete vs. incomplete cervical SCI and remained within the normal reference range. Therefore, hormonal changes are unlikely to explain the differential expression of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in subjects with complete vs. incomplete SCI. This view is supported indirectly by our observation that local (contraction-related) factors are predominant in the regulation of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase during exercise, since we have observed an increase in Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity only in the exercised and not in the rested leg, thus excluding a major role for systemic factors (5).

In conclusion, complete, but not incomplete, cervical SCI in humans causes a severe distortion in the expression and regulation of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase. Differences in residual neuromuscular activity determined by the extent of SCI may play an important role in decreasing Na\textsuperscript{+}-K\textsuperscript{+}-ATPase content and its regulatory proteins after cervical SCI.

ACKNOWLEDGMENTS

We thank Drs. Anna Krook and Juleen Zierath for helpful discussion and critical reading of the manuscript. We greatly acknowledge the generous gift of the FXYD5 antibody by Dr. Haim Garty (Weizmann Institute of Science, Rehovot, Israel).

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GRANTS

This work was supported by grants from the Netherlands Organization for Scientific Research, the Throne Holst Foundation of the University of Oslo, the Norwegian South-Eastern Health Authority, the Swedish Research Council, the Novo-Nordisk Foundation, and the Commission of the European Communities (EUGENEHEART and EXGENESIS).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.
AUTHOR CONTRIBUTIONS
H.B., E.K., S.P., M.S., I.L., and N.H. performed the experiments; H.B., E.K., S.P., M.S., I.L., P.O.I., U.W., and A.V.C. analyzed the data; H.B., E.K., S.P., I.L., P.O.I., N.H., U.W., and A.V.C. interpreted the results of the experiments; H.B. and U.W. prepared the figures; H.B., S.P., and U.W. drafted the manuscript; E.K., P.O.I., N.H., U.W., and A.V.C. did the conception and design of the research; U.W. and A.V.C. edited and revised the manuscript; A.V.C. approved the final version of the manuscript.

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