Na\textsuperscript{+}-K\textsuperscript{+}-ATPase properties in rat heart and skeletal muscle 3 mo after coronary artery ligation

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This study was designed to determine whether chronic heart failure (CHF) results in changes in Na\textsuperscript{+}-K\textsuperscript{+}-ATPase properties in heart and skeletal muscles of different fiber-type composition. Adult rats were randomly assigned to a control (Con; \(n = 8\)) or CHF (\(n = 8\)) group. CHF was induced by ligation of the left main coronary artery. Examination of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity (means ± SE) 12 wk after the ligation measured, using the 3-O-methylfluorescein phosphatase assay (3-O-MFPase), indicated higher (\(P < 0.05\)) levels in soleus (Sol) (250 ± 13 vs. 179 ± 18 nmol·mg protein\(^{-1}\)·h\(^{-1}\)) and lower (\(P < 0.05\)) levels in diaphragm (Dia) (200 ± 12 vs. 272 ± 27 nmol·mg protein\(^{-1}\)·h\(^{-1}\)) and left ventricle (LV) (760 ± 62 vs. 992 ± 16 nmol·mg protein\(^{-1}\)·h\(^{-1}\)) in CHF compared with Con, respectively. Na\textsuperscript{+}-K\textsuperscript{+}-ATPase protein content, measured by the \[^{3}H\]ouabain binding technique, was higher (\(P < 0.05\)) in white gastrocnemius (WG) (166 ± 12 vs. 135 ± 7.6 pmol/g wet wt) and lower (\(P < 0.05\)) in Sol (193 ± 20 vs. 260 ± 8.6 pmol/g wet wt) and LV (159 ± 10 vs. 221 ± 10 pmol/g wet wt) in CHF compared with Con, respectively. Isoform content in CHF, measured by Western blot techniques, showed both increases (WG; \(P < 0.05\)) and decreases (Sol; \(P < 0.05\)) in \(\alpha_1\). For \(\alpha_2\), only increases [red gastrocnemius (RG), Sol, and Dia; \(P < 0.05\)] occurred. The \(\beta_1\)-isoform was decreased (LV, Sol, RG, and WG; \(P < 0.05\)) in CHF, whereas the \(\beta_1\) was both increased (WG and Dia; \(P < 0.05\)) and decreased (Sol and LV; \(P < 0.05\)). For \(\beta_3\), decreases (\(P < 0.05\)) in RG were observed in CHF, whereas no differences were found in Sol and WG between CHF and Con. It is concluded that CHF results in alterations in Na\textsuperscript{+}-K\textsuperscript{+}-ATPase that are muscle specific and property specific. Although decreases in Na\textsuperscript{+}-K\textsuperscript{+}-ATPase content would appear to explain the lower 3-O-MFPase in the LV, such does not appear to be the case in skeletal muscles where a dissociation between these properties was observed.

chronic heart failure; sodium-potassium-adenosinetriphosphatase content; activity; isoforms

ALTHOUGH ABNORMALITIES in the ability of the heart to expel blood remain the fundamental disorder in chronic heart failure (CHF), it is now recognized that chronic adjustment to CHF results in a complex pathophysiological syndrome, culminating in a wide range of disturbances (10). These disturbances would appear to be mediated in large part by chronically exaggerated neurohormonal responses involving increased activation of adrenergic, renin-angiotensin-aldosterone, and vasopressin pathways (10). The cumulative effect of these neurohormonal alterations include, in part, excessive vasoconstriction and redistribution of blood flow, increased afterload on the heart, and fluid and electrolyte retention (10). Intrinsic alterations in skeletal muscle composition have also been identified and may contribute to dyspnea and exercise intolerance, two cardinal clinical manifestations of CHF (9, 15, 33, 46).

Numerous studies have been conducted examining skeletal muscle in CHF; however, the specific intrinsic abnormalities and the mechanisms underlying exercise intolerance remain unclear (33, 46). One inviting possibility to explain exercise intolerance in CHF, given the complex neurohormonal changes observed, is failure in the ability of the muscle to respond to neural activation as a result of disturbances in membrane excitability (7, 34). Disturbances in membrane excitability are intimately dependent on the active transport of two ions, namely K\textsuperscript{+} and Na\textsuperscript{+}, which is governed by the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase (Na\textsuperscript{+}-K\textsuperscript{+} pump) (40). Inappropriate regulation of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity, leading to excessive loss of K\textsuperscript{+} from the cell and gain of Na\textsuperscript{+} into the cell, could alter depolarization and action potentials in the sarcolemma and T tubules, resulting in disturbances in Ca\textsuperscript{2+} signaling and contractility (40). The Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity is subject to complex neurohormonal regulation, both acutely (52) and chronically (16).

The Na\textsuperscript{+}-K\textsuperscript{+}-ATPase is an integral membrane protein that catalyzes the chemical hydrolysis of ATP to the vectorial transport of Na\textsuperscript{+} out of and K\textsuperscript{+} into the cell. This active transport mechanism restores ionic gradients for the maintenance of membrane potential and “excitability” in response to repetitive action potentials (8). The effectiveness with which the normal pump is able to perform these functions is dependent on the protein and isoform content of the enzyme and the precise control of the regulatory signals that allow for appropriate recruitment of catalytic activity (8).

The Na\textsuperscript{+}-K\textsuperscript{+}-ATPase is an oligomer containing at least two principle polypeptides that constitute a functional \(\alpha\)-\(\beta\)-heterodimer. There are presently four isoforms of the \(\alpha\)-subunit (\(\alpha_1, \alpha_2, \alpha_3, \alpha_4\)) and three isoforms of the \(\beta\)-subunit (\(\beta_1, \beta_2, \beta_3\)) that have been cloned and sequenced (3). In mammalian skeletal muscle, \(\alpha_1\beta_1, \alpha_2\beta_1, \alpha_3\beta_2, \text{and } \alpha_4\beta_2\) appear to represent the major combinations. The isoforms appear to be distributed in a fiber-specific manner with \(\alpha_1\beta_1\) and \(\alpha_3\beta_2\) predominating in slow-twitch oxidative (SO)-based muscles, such as the soleus (Sol), and \(\alpha_1\beta_2\) and \(\alpha_2\beta_2\) predominating in fast-twitch glycolytic (FG)-based muscles, such as the white gastrocnemius (WG) (26, 27, 51). Fast-twitch oxidative-glycolytic fibers (FOG), prominent in the red gastrocnemius (RG), appear to contain all four combinations (51). The existence of the \(\beta_3\)-isoform in skeletal muscle remains controversial. In two
recent papers (22, 39), β₃ has been detected in adult rats. However, in previous work very little (1) or no (53) β₃ has been found in adult rat muscle. The different isoform combinations may have functional implications, given their specific kinetic characteristics and their different affinities for cations (Na⁺, K⁺) and ATP (3, 11). These differences may be important for adapting the muscle Na⁺-K⁺-ATPase activity to the specific challenges encountered in CHF.

In rats with experimentally induced CHF, determination of Na⁺-K⁺ pump content using [³H]ouabain binding indicated a significant decrease in maximal binding capacity (β_max) in muscles composed primarily of SO and FOG but not muscles composed of FG fibers (38, 45). These changes occurred in the absence of changes in the dissociation constants (K_d) (38, 45). Interestingly, changes in muscle Na⁺-K⁺-ATPase content in humans with CHF are uncertain because both a reduction (42) and no change (19) have been reported. Only two studies have examined isoform distribution in skeletal muscle of CHF animals. These studies, conducted on muscles composed of a predominance of FOG and FG fibers, indicate changes only in α2 of FOG-based muscle (22, 34). Species differences and/or differences in CHF severity could explain, in part, the differences in the responses observed (38). These studies not withstanding, it remains to be established how CHF alters the content and expression of the Na⁺-K⁺-ATPase isoforms across muscles of different fiber-type composition and how the maximal activity of the enzyme is affected. It is possible that a dissociation between total Na⁺-K⁺ pump content and Na⁺-K⁺-ATPase activity may occur, depending on the isoform expression and regulatory factors (8, 11, 52).

The purpose of this study was to determine the effects of experimentally induced CHF on the content, isoform, and activity characteristics of the Na⁺-K⁺-ATPase in skeletal muscles of different fiber-type composition. We have hypothesized that Na⁺-K⁺-ATPase content and activity would be lower but only in muscles composed of a predominance of SO and FOG fibers. The changes in Na⁺-K⁺-ATPase isoform content and activity would also be accompanied by a decreased expression in both α (α₁ and α₂)- and β (β₁, β₂, and β₃)-isoforms.

METHODS

Experimental Model

Myocardial infarction was induced in male Sprague-Dawley rats (250–300 g) by occlusion of the left coronary artery essentially as described by Musch et al. (37). The animals were prepared for surgery using ketamine (100 mg/ml) and xylazine (20 mg/ml) and sterile water in ratio of 1:1.5, respectively, which was administered intraperitoneally (3.0 ml/kg) as the anesthetization cocktail. In brief, the procedure for coronary artery ligation included making a cranialocaudal incision in the chest, through the skin and pectoral muscles, transaction of the third and fourth ribs, blunt dissection of the intercostal muscles, opening of the pericardium, and exteriorization of the heart. The left anterior descending coronary artery was identified, a 5-0 silk suture was used to tie the ligature, and the heart was replaced in the thorax. The air in the thoracic cavity was gently squeezed out while the thoracic wall and skin incision was closed. The animal was resuscitated using positive-pressure ventilation and placed in an oxygen-rich environment. All surgical procedures and post-surgery recovery occurred at the supplier (Charles River Canada, St. Constant, Quebec, Canada) according to Charles River’s Institutional Animal Care and Use Committee protocols. After 5 days of recovery, the CHF and age- and weight-matched controls (Con) were shipped to the Animal Care Facility at the University of Waterloo (Waterloo, Ontario, Canada). During residence at the University of Waterloo, the care and use of animals were according to protocols approved by the University of Waterloo, Office of Research Ethics (Animal Care), following the guidelines of the Canadian Council on Animal Care.

While at the University of Waterloo, the animals were housed in individual cages, provided with water and rat chow ad libitum, and maintained in a 12:12-h light-to-dark cycle. Twelve weeks after the ligation, both CHF and Con animals were anesthetized with pentobarbital sodium (Somnotol; 5 mg/100 g body wt) and prepared for tissue sampling. After deep anesthesia, a number of muscles were rapidly removed, sectioned into portions for the measurement of different properties, and rapidly frozen in liquid N₂. For the Na⁺-K⁺-ATPase measurements, the Sol and portions of the RG and WG were used. The heart was rapidly removed, dissected into right ventricle (RV) and left ventricle (LV), weighed, separated into a number of samples, and frozen in liquid N₂. During sampling, care was taken to avoid the infarcted area. A portion of the costal diaphragm (Dia) was also removed and prepared for storage. The last step in the sampling procedure involved removing the lungs and recording their weight.

For assessment of the severity of CHF in the myocardial infarcted rats, we have used the ratios of LV/body mass, RV/body mass, and lung/body mass (13, 38).

Our rationale for selecting the specific locomotor muscles was that they are largely composed of different fiber types on the basis of myosin isoform-based histochemistry. As such, the Sol primarily consists of myosin heavy chain (MHC) I, the RG of MHC Ila, and the WG of MHC Iib (12). This classification provides for one type of fiber (I slow) and two subtypes of type II (fast) fibers (48). Fiber-type classification, on the basis of MHC isoform composition, should not be confused with other classification systems such as the oxidative and glycylotic potential. In general, the oxidative potential is of the order RG > Sol > WG in rat muscles (12).

Analytical Protocols

To characterize the Na⁺-K⁺-ATPase, we have measured the content, isoform composition, and maximal activity in the different tissues.

Na⁺-K⁺-ATPase. The measurement of Na⁺-K⁺-ATPase content was based on the vanadate-facilitated [³H]ouabain binding procedure, as previously described (42, 44). In brief, this procedure involves dissection of the muscle in small samples (2–8 mg), prewashing 2× for 10-min periods in a Tris-sucrease buffer (10 mM Tris-HCl, 3 mM MgSO₄, 1 mM Tris-vanadate, and 250 mM sucrose) containing sodium metavanadate (NaVO₃) at 0–4°C (pH 7.2–7.4). This protocol was followed by incubating the samples in a Tris-sucrease buffer with (1 μM) [³H]ouabain (1.8 μCi/ml) and unlabeled ouabain (1 μM final concentration) for 2 × 60 min at 37°C. After removal of the unbound ouabain (washing 4× for 30 min in ice-cold buffer), the samples were blotted dry, weighed, and immersed in 1 ml of 5% trichloroacetic acid for 16 h at room temperature. Small aliquots of the sample (0.5 ml) were counted for ³H activity in a scintillation mixture. [³H]ouabain binding capacity was corrected (×1.05) for loss of specifically bound [³H]ouabain during washout (44). No correction was made for nonspecific uptake and retention of ³H, which was estimated at <3% (H. Green, unpublished observations). The isotopic purity of the [³H]ouabain was 99% as determined by the supplier (New England Nuclear-DuPont Canada).

K⁺-dependent 3-O-MFase activity. Maximal enzyme activity in whole skeletal muscle homogenates was determined by the method of Huang and Askari (25) as modified by Fraser and McKenna (17) and Barr et al. (2). Briefly, homogenates were prepared from frozen tissue (5% wt/vol) at 0–4°C for 2 × 20 s at 25,000 rpm (Polytron) in a buffer containing 250 mM sucrease, 2 mM EDTA, 1.25 mM EGTA, 5 mM NaN₄, and 10 mM Tris (pH 7.40). Homogenates were freeze-thawed four times and diluted 1:5 in cold homogenate buffer. Ho-
mogenates (30 μl) were incubated for 5 min at 37°C in a buffer containing 5 mM MgCl₂, 1.25 mM EDTA, 100 mM Tris base (pH 7.4), 1 mM EGTA, and 5 mM NaN₃. Activity was determined during the linear increase in signals measured via spectrophotometry by the addition of 160 μM 3-O-methylfluorescein phosphate (3-O-MFP) followed by 10 μM KCl. K⁺-dependent 3-O-MFPase activity was determined by subtracting activity after addition of KCl from activity before the addition of KCl, as previously outlined (17). We have previously shown (H. Green, unpublished observations), as have others (17), that specific activity can be completely abolished by the addition of 2 mM ouabain. Protein content of the homogenate was determined by the method of Lowry as modified by Schacterle and Pollack (47).

**Na⁺-K⁺-ATPase isoforms.** Western blot analysis was used to quantitate subunit protein levels of the Na⁺-K⁺-ATPase, namely α (α₁, α₂) and β (β₁, β₂, β₃)-isoforms. The β₂-isoform was only measured in Sol, RG, and WG. For homogenate preparation, frozen tissue was placed in a buffer (5% w/vol) at 0–4°C containing 25 mM sucrose, 2 mM EDTA, 5 mM NaN₃, and 10 mM Tris (pH 7.40) and homogenized for 2 × 20 s at 25,000 rpm (Polytron). After preparation of the homogenates, several aliquots were obtained and quick frozen in liquid N₂ until analysis. Samples from each homogenate prepared from each muscle were electrophoresed on separate 7.5% SDS-polyacrylamide gels (Mini-Protein II, Bio-Rad) as described by Laemmli (31). For analysis of α-subunit isoforms, equal amounts of homogenates (30 μg) were electrophoresed, whereas for the β-subunits, equal amounts of homogenate preparations (50 μg) were deglycosylated with N-glycosidase F (Boehringer Mannheim, Indianapolis, IN) overnight at room temperature and electrophoresed.

Gels were electrophoretically transferred to polyvinylidene difluoride membranes (membranes, Bio-Rad). The nonspecific binding sites were blocked with 5 or 7.5% BSA in Tris-buffered saline (TBS, pH 7.4). Incubated using the primary antibodies diluted in 5% BSA (Boehringer Mannheim, Indianapolis, IN) overnight at room temperature and electrophoresed. For any particular isoform, an equal amount of homogenate sample from each homogenate prepared from each muscle were electrophoresed. A total of 11 samples (5 Con and 5 CHF in RG, WG, and Dia) were incubated for 5 min at 37°C in a buffer containing 20 mM imidazole buffer with 0.2% BSA. Enzyme activity was determined by subtracting activity after addition of KCl from activity before the addition of KCl, as previously outlined (17). We have previously shown (H. Green, unpublished observations), as have others (17), that specific activity can be completely abolished by the addition of 2 mM ouabain. Protein content of the homogenate was determined by the method of Lowry as modified by Schacterle and Pollack (47).

**Results**

**Indexes of CHF**

There were no differences in either total body mass or in the LV and RV between Con and CHF groups (Table 1). However, when the ratios of LV/body mass and RV/body mass were calculated, higher ratios where found in CHF rats compared with Con. An increase of ~49% was observed in the lung-to-body mass ratio in CHF compared with Con group.

**Na⁺-K⁺-ATPase Content**

Na⁺-K⁺-ATPase content (β₃max), as assessed with the [³H]ouabain binding technique, was altered in CHF in a muscle-specific manner (Fig. 1). Decreases in Na⁺-K⁺-pump content ranging from 26 to 28% were found in the Sol and LV with CHF. In contrast, CHF resulted in an ~23% increase in Na⁺-K⁺-pump content in WG. No differences were observed between Con and CHF in Na⁺-K⁺-pump activity in the Dia and RG.

**Na⁺-K⁺-ATPase Activity**

Maximal Na⁺-K⁺-ATPase activity, estimated using the 3-O-MFPase assay, was also altered in specific muscles (Fig. 2). For the Sol, the activity was 39% higher in CHF compared with Con, whereas in the LV and Dia, the activities were 23 and 36% lower, respectively, in CHF compared with Con. There were no differences in 3-O-MFPase activity between Con and CHF in RG and WG.

<table>
<thead>
<tr>
<th>Body Mass, g</th>
<th>LV, g</th>
<th>RV, g</th>
<th>LV/Vbody Mass, mg/g</th>
<th>RV/Vbody Mass, mg/g</th>
<th>Lung/Vbody Mass, mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td>629 ± 30</td>
<td>1.06 ± 0.07</td>
<td>0.387 ± 0.1</td>
<td>1.68 ± 0.04</td>
<td>0.61 ± 0.04</td>
</tr>
<tr>
<td>CHF</td>
<td>575 ± 42</td>
<td>1.02 ± 0.11</td>
<td>0.453 ± 0.1</td>
<td>1.86 ± 0.07*</td>
<td>0.79 ± 0.05*</td>
</tr>
</tbody>
</table>

Values are means ± SE for 8 rats in each group. Con, control; CHF, chronic heart failure; LV, left ventricle; RV, right ventricle; LV/body mass, ratio of LV to body mass; RV/body mass, ratio of RV to body mass; lung/body mass, ratio of lung to body mass. *Significantly different from CHF, P < 0.05.
The relative changes in α (α1 and α2) and β (β1, β2, and β3)-isoform distribution were assessed using Western blot analysis (Figs. 3 and 4). The changes in specific isoforms were, in large part, specific to the muscle sampled. For LV, CHF resulted in decreases in both the β1- and β2-isoforms but no change in the α1- and α2-isoforms. For Dia, the only changes observed were in α3 and β1, where the levels were higher in CHF compared with Con. The β3-isoform was not measured in LV and Dia due to tissue limitations. For the RG, increases in α2 and decreases in β2 and β3 were observed. The WG also displayed decreases in β2 in CHF, but, in addition, increases in α1 and β1 also occurred. For the Sol, α2 increased while α1, β1, and β2 all decreased in CHF. No changes in β3 with CHF were observed in either the WG or Sol. Typical autoradiograms of the relative subunit abundance between CHF and Con for each of the muscles examined are provided in Fig. 5.

Oxidative Potential

The maximal activity of CS was used as a measure of the effects of CHF on muscle oxidative potential. For all of the muscles examined, CHF was without effect in altering maximal CS activity (Fig. 6).

DISCUSSION

In this study, we have found that experimentally induced CHF in rats results in intrinsic changes in muscle at the level of the Na\(^+\)-K\(^+\)-ATPase pump. We have also found that the changes that occur are specific to both the muscle and the property assessed. As an example, Na\(^+\)-K\(^+\)-ATPase content, measured by the [\(^3\)H]ouabain binding procedure, was decreased in the Sol and LV, increased in the WG, and unchanged in RG and Dia. Although changes in Na\(^+\)-K\(^+\)-ATPase content would be expected to be accompanied by changes in maximal catalytic activity, as measured by 3-O-MFPase, this was only observed in LV and Dia, where decreases in activity were found, and in Sol, where increases occurred. The increase in activity observed in the Sol in CHF was opposite of the change in Na\(^+\)-K\(^+\)-ATPase content. For WG, no changes occurred in Na\(^+\)-K\(^+\)-ATPase activity; however, an increase in Na\(^+\)-K\(^+\)-ATPase content occurred in CHF. Collectively, these findings suggest multiple levels of control, both with respect to Na\(^+\)-K\(^+\)-ATPase protein expression and the regulatory factors governing the kinetic behavior of the enzyme. Our findings do not support our hypothesis because muscles with a high oxidative potential (Sol, RG) showed a differential response to CHF with either no change (RG) or a decrease (Sol) in Na\(^+\)-K\(^+\)-ATPase content and either no change (RG) or an increase (Sol) in Na\(^+\)-K\(^+\)-ATPase activity. In addition, the changes that we have observed in α- and β-isoform distribution do not support our hypothesis because both increases and decreases occurred, depending on the muscle and the isoform. Our findings of the effects of CHF on Na\(^+\)-K\(^+\)-ATPase content (β\(_{\text{max}}\)) are similar to what has been reported previously in the Sol muscles of rats with CHF (22, 38, 45). Interestingly, the absolute values we report for β\(_{\text{max}}\) in Sol in Con rats and rats with severe CHF are nearly identical to those previously published (38). In contrast, although we found no effect of CHF on β\(_{\text{max}}\) in RG, reductions in this muscle have been reported in rats with severe but not moderate CHF (38). In the case of the WG, increases in β\(_{\text{max}}\) with CHF were found in our study, but in rats with both moderate and severe CHF the change was not significant (38). Because β\(_{\text{max}}\) of the Na\(^+\)-K\(^+\)-ATPase is positively related to the muscle oxidative potential (5), sampling site discrepancies in gastrocnemius muscle control and CHF rats, either in our study or in the study by Musch.
et al. (38), would appear to represent one probable explanation for the contradictory results. Not to be denied as well is the potential influence of the age of the animal and the duration of experimental CHF. In contrast to Musch et al. (38), who studied CHF animals 24 wk after surgery, our protocol extended for only 12 wk.

Given the changes in muscle Na$^+$-K$^+$-ATPase content between our study and a previous study in rats with both moderate and severe CHF (38), an important issue is whether our animals displayed moderate or severe CHF. Similar to the study by Helwig et al. (22) and Musch et al. (38), we report increases in the ratios of LV/body mass, RV/body mass, and lung/body mass of a similar magnitude. These ratios appear to offer a reasonable measure of the severity of CHF as assessed by more direct techniques, such as left ventricular end-diastolic pressure (LVEDP) and myocardial infarct size (13, 38, 45). In previous research involving experimental CHF in rats, the oxidative potential, as measured by CS, has been reported to decrease in Sol, RG, and WG in severe but not moderate stages of the disease (13). In this study, we could find no alterations in CS in any of the muscles examined. A limitation in our study was the omission of measures of LVEDP, which would have

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**Fig. 4.** Na$^+$-K$^+$-ATPase isoforms as measured by Western blotting techniques in selected locomotor muscles of Con rats (solid bars) and rats with CHF (open bars). Values are means ± SE with CHF relative to Con; n = 8 per group. *Significantly different from Con, P < 0.05.

**Fig. 5.** Representative immunoblots of the expression of the Na$^+$-K$^+$-ATPase subunit isoforms in muscles of Con rats and rats with CHF. Blots are from LV, Dia, RG, WG, and Sol for Con and CHF. For the α-subunits (α1 and α2), 30 μg of homogenate were loaded per lane, whereas for β-subunits (β1, β2, and β3) 50 μg of homogenate were loaded per lane. For β-subunits, measurements were performed after removal of sugar residues. Scanning density is calculated relative to a known standard (STD) of brain tissue for each plot. The molecular mass (MM) markers in kDa are also provided. Values for Con and CHF were initially calculated relative to brain standards.
homogenates does present other obstacles, particularly with the measurement of the Na\(^{+}\)-K\(^{+}\)-ATPase activity, given the relatively low activity of the enzyme and the need to selectively inhibit other ATPases in the cell (21). As a consequence of these problems, an alternative procedure has been developed, which is not based on the hydrolytic activity of the enzyme per se but on the K\(^{+}\)-dependent phosphatase activity of the enzyme (43). Measurement of the phosphatase activity is based on the K\(^{+}\)-dependent hydrolysis of the chromogenic substrate 3-O-MFPase, which substitutes for the aspartylphosphate intermediate of the ATPase, to represent the terminal step in ATP hydrolysis (24). Although, the 3-O-MFPase activity can be inhibited by ouabain, the possibility exists that the catalytic activity of the phosphatase may not directly relate to the Na\(^{+}\)-K\(^{+}\)-ATPase activity per se and/or that differences in this relationship may exist between muscles of different fiber-type composition. At present, few studies have addressed this issue.

The Na\(^{+}\)-K\(^{+}\)-ATPase content (\(\beta_{\text{max}}\)) was assessed using the vanadate-facilitated [\(^{3}H\)]ouabain binding technique (44). This is a highly sensitive technique based on the binding affinity of the \(\alpha\)-isoform for ouabain that under normal circumstances allows for the accurate quantification of the pump content. In this study, we have measured \(\beta_{\text{max}}\) based on saturation binding of ouabain with the enzyme. However, it is widely recognized that at least two distinct binding affinities exist on the basis of \(\alpha\)-subunit composition (3). Although previous studies have reported contradictory results with respect to \(K_d\) in heart with CHF (14, 28), it is possible that in our CHF model changes in \(K_d\) also accompanied the widespread alterations that occurred in \(\beta_{\text{max}}\). To some extent, differences between muscles in the \(\beta_{\text{max}}\) response to CHF could be explained by the species employed. In rodents, the \(\alpha_1\)-subunit possesses a very low affinity for ouabain, and consequently, this subunit is not measured (20). In muscles where \(\alpha_1\)-isoform content changed with CHF, as in the Sol and WG, the effect would be to overestimate (Sol) or underestimate (WG) the changes that occurred. In general, however, the skeletal muscle in healthy rats express <15% of the \(\alpha_1\)-isoform (20).

The third property of the Na\(^{+}\)-K\(^{+}\)-ATPase enzyme that was investigated, namely the subunit isoform composition, was measured using Western immunoblot techniques. With use of appropriate antibodies, we were able to quantitate the relative abundance of the \(\alpha_1\)- and \(\alpha_2\)-isoforms and the \(\beta_1\)-, \(\beta_2\)-, and \(\beta_3\)-isoforms in whole muscle homogenates. In our hands, we were able to resolve clear bands of expected molecular mass, without the use of antiproteolytic agents. The relative abundance of each isoform was calculated in reference to a known standard of brain tissue that expresses all the Na\(^{+}\)-K\(^{+}\)-ATPase isoforms, and the relative changes in CHF muscles were, in turn, referenced to the Con. As in previous studies, we have found that the isoform distribution in rats is highly dependent on the fiber-type composition of the muscle (4, 55). The SOL, which is composed essentially of SO fibers, contains an abundance of \(\alpha_1\beta_1\) and \(\alpha_2\beta_1\)-heterodimers. In contrast, the WG, which contains primarily FG fibers, contains a predominance of \(\alpha_2\beta_2\)-heterodimers, whereas the FOG-based RG has plentiful amounts of all four complexes, namely \(\alpha_1\beta_1\), \(\alpha_2\beta_1\), \(\alpha_1\beta_2\), and \(\alpha_2\beta_2\). The heart mainly consists of the \(\alpha_1\beta_1\) and \(\alpha_2\beta_1\)-subunit combinations (36). The \(\beta_3\)-isoform was not probed for at the time of this study because previous investigators have concluded that little of this isoform exists in the skeletal muscle.

![Graph](https://jap.physiology.org/doi/10.2203/jappl.05.02.03)
muscles of adult rats (1, 53). However, recent studies have reported the presence of β3 in a variety of muscles (22, 39). On the basis of these developments, we reprobed for β3 in the Sol, RG, and WG, where sufficient tissue remained. Changes in β3 were only observed in RG where lower values were found in CHF. Our results on the effects of CHF on isoform distribution indicate that all isoforms examined are altered, with the changes in specific isoforms dependent on the fiber-type composition of the muscle. At present, it is not clear whether the alterations in specific subunits changed in CHF occur as a result of increased degradation or reduced synthesis. Moreover, our analyses preclude rigorously identifying the effects of CHF on the distribution of specific heterodimers.

When comparing the CHF with the Con group, both within a given property and between properties, it is important that the values be expressed per unit protein. This was possible for the measurements of Na⁺-K⁺-ATPase activity. Our measurements indicated no differences between groups for any of the muscles studied. However, for the assessment of pump content using the [³H]ouabain binding procedure, for technical reasons, the values are expressed per unit wet weight. Increases in muscle water content could, in theory, lower the value obtained and contribute to the discordant results observed between pump content and activity. At present, it is not clear whether differences in water content occurred between groups.

Of fundamental importance is the functional significance of the alterations in isoform distribution in CHF on Na⁺-K⁺-ATPase activity and membrane excitability. The α-isoform is clearly recognized as the catalytic subunit, the activity of which appears to be dependent on the specific α-isoform and the accompanying β-isoform component (3). On the basis of different combinations of α- and β-isoforms expressed in Xenopus oocytes, Crambert et al. (11) have concluded that the α1-isoforms in muscle can effectively respond to different physiological conditions involving housekeeping functions. The α2- and α3-isoforms, on the other hand, appear dispensable to responding to challenges other than housekeeping functions (11, 35). In training, as an example, the α2 appears to be upregulated in a variety of muscles in the rat (39). The increase in α2 is also accompanied by decreases in β1, whereas increases in α1 appear restricted to specific muscles.

Although decreases in Na⁺-K⁺-ATPase content in the LV have frequently been reported in CHF (7, 14, 28, 42), only a few studies have investigated additional properties. Schwinger et al. (48a) compared tissue extracted from LV in patients with and without myocardial failure. These investigators found that in patients in myocardial failure, reductions were observed in pump content (βmax), maximal activity, and α3-, α7-, and β3-isoforms but not the β2-isoform. Unlike skeletal muscle, the heart expresses the α7-isoform and very little of the β2-isoform (36). In an additional study, using rapid ventricular pacing to induce CHF in dogs, reductions in [³H]ouabain binding sites in LV were accompanied by reductions in α7- but not α1-isoform content (28). Using a rat model to induce experimental CHF similar to what we have employed, Dixon et al. (14) have found pronounced reductions in Na⁺-K⁺-ATPase activity that were accompanied by increases in [³H]ouabain binding sensitivity (Ko) but not maximal binding (βmax). These findings support the results of Semb et al. (49), who reported that the function of the Na⁺-K⁺-pump was compromised even though the number of pumps per cell was maintained.

The locomotor muscles examined, namely the Sol, WG, and RG, displayed highly discordant responses between the Na⁺-K⁺-ATPase properties that were examined. In the Sol, CHF animals exhibited reduced Na⁺-K⁺-ATPase content in association with reductions in α1-, β1-, and β2-, increases in α2-, and no change in β3-subunit composition. Because β2 is only minimally expressed in Sol, these results suggest a decrease in α1β1- and an increase in α2β1-heterodimers in CHF. Unexpectedly in the Sol, Na⁺-K⁺-ATPase activity was substantially increased in CHF. It would appear, on the basis of the changes in Na⁺-K⁺-ATPase content and subunit composition, that an adaptation in some regulatory process would have to be involved in mediating the increase in Na⁺-K⁺-ATPase activity in CHF. It is possible that the increase in activity could have been mediated by increases in enzyme phosphorylation, secondary to alterations in one or more of the signaling events affecting protein kinase A (PKA) or protein kinase C (PKC). Enzyme phosphorylation induced by both PKA and PKC has been shown to elevate Na⁺-K⁺-ATPase activity in muscle (52). The possibility of defective signaling also extends to the Dia, where a decrease in 3-O-MFPase activity was observed in CHF, despite an unchanged Na⁺-K⁺-ATPase content as measured by the [³H]ouabain binding assay. Interestingly, in the Dia, α2- and β1-isoform composition was increased in CHF, whereas α1 and β2 remained unchanged.

In the WG, we have found that CHF increased Na⁺-K⁺-ATPase content but did not change maximal 3-O-MFPase activity. CHF also resulted in an increase in the composition of the α1- and β1-, a decrease in the β2-, but no change in the α2- and β2-isoform content of the WG. In a recent study (22), no differences in either the α- or β-subunit composition in WG were observed between CHF and sham controls. In normal muscle, α2β2-heterodimers predominate in WG. In CHF, it would appear that α1β1-complexes are increased. This could imply structural changes to the enzyme as previously observed for muscle Ca²⁺-ATPase with aging (57) and contractile activity (29). Structural changes could occur in the region of the adenine nucleotide binding site as a consequence of oxidation and/or nitrosylation (29, 57). Increases in muscle reactive oxygen species and nitric oxide have been previously documented in selected skeletal muscles in CHF (6, 41, 56). The adenine nucleotide region is extremely sensitive to oxidation/nitrosylation-induced damage (30).

The one muscle that appeared to be relatively immune to the effects of CHF was the RG. In this muscle, no changes resulted in either the Na⁺-K⁺-ATPase content or activity in CHF. However, an isoform shift was found that was characterized by an increase in α2- and a decrease in β2- and β3-subunit composition. These findings are at odds with a previous study (22) where the isoform changes in CHF in RG muscle included only reductions in α2. Healthy RG muscles in rats contain a predominance of the α1β1- and α2β1-heterodimers. The isoform shift that was observed in CHF in the muscle would appear to have little functional significance because Na⁺-K⁺-ATPase activity was unaltered.

The changes observed in the Dia with CHF are unique compared with the other skeletal muscles. In this muscle, no changes were observed in Na⁺-K⁺-ATPase content; however, the maximal catalytic activity of the enzyme decreased. Changes in these properties were accompanied by increases in α2- and β1-content but not in α1- and β2-content. These findings
are similar to what have been observed in the heart in CHF (14, 49), where pump content appears protected, but the maximal activity of the pump and its Na⁺ pumping rate are compromised.

In this study, unlike most previous studies employing experimentally induced CHF, which use sham-generated animals as the control, we have used, as a control, animals who were not subject to any surgical procedure. Although previous approaches using sham-operated animals supposedly allow isolation of the effects of CHF per se, it is possible that the effects are superimposed on changes occurring during the surgery. By using animals not subjected to surgical intervention, we were able to compare the effects of CHF on healthy controls. However, to do this, it must be demonstrated that the sham-operated animals are in fact similar to the healthy controls. We have investigated this issue (H. Green, unpublished observations) in separate groups of animals. We have, compared a wide range of properties, including body, LV, RV, and lung mass as well as the ratio of organ mass to body mass and found no differences between groups. As well, we have found that maximal Na⁺-K⁺-ATPase activity was not different either in the LV (1,440 ± 144 vs. 1,660 ± 27 nmol·mg protein⁻¹·h⁻¹) or the Sol (560 ± 32 vs. 585 ± 12 nmol·mg protein⁻¹·h⁻¹) between healthy and sham-operated animals. Similarly, no differences existed in the Na⁺-K⁺-ATPase content between the LV (162 ± 19 vs. 161 ± 17 pmol/g wet wt) and Sol (210 ± 16 vs. 233 ± 24 pmol/g wet wt). Although it was unexpected that the Na⁺-K⁺-ATPase activity would be generally higher in the healthy and sham-operated animals than observed in the control animals used to compare with CHF, the protein content of the Na⁺-K⁺-ATPase, as reflected by the [³H]ouabain binding assay, was also higher. These differences probably reflect differences between litters. Finally, we have also compared the Sham and Con groups on the mitochondrial enzyme CS, which is known to be a sensitive indicator of the level of physical activity. No differences were found between groups in the maximal activity of this enzyme, regardless of the muscle studied.

A significant and long-standing issue is whether the intrinsic alterations observed in skeletal muscle in CHF are subnormal due to differences in activity levels or are abnormal as a result of pathological changes (33). Although a previous study has indicated no differences in activity level between caged sham-operated and experimentally induced CHF in rats (50), the possibility remains that habitual levels of contractile activity are different between the groups, even in the individually housed rats. Potential differences in physical activity levels are particularly important in understanding the role of CHF on muscle Na⁺-K⁺-ATPase, because of the highly adaptable nature of the enzyme to alterations in contractile activity (18). However, decreases in activity levels in CHF would not explain our results because decreases in both Na⁺-K⁺-ATPase content and activity would be expected (18). In this study, only in the case of the WG was Na⁺-K⁺-ATPase content increased, and the increase was not accompanied by changes in enzyme activity levels. In contrast, increases in Na⁺-K⁺-ATPase activity were observed in Sol, whereas Na⁺-K⁺-ATPase content declined. The physiologic significance of these changes remains to be determined.

Another issue of importance in terms of the interpretation of our findings is whether the changes observed in CHF are extrinsic, mediated by changes in muscle mass, or intrinsic, induced by alterations in the expression of specific proteins. The experimental CHF model is known to result in significant hypertrophy of the LV, which could have impacted our findings. Changes in fiber cross-sectional area can also occur in skeletal muscle, depending on the severity of the disease and the muscle studied (13). In this regard, the trend that we have observed toward increased body masses in CHF compared with the Con could have resulted in a disproportionate change in LV-to-body mass ratio that was independent of CHF. However, this does not appear to be the case, since increases in LV mass parallel increases in body mass (58).

In summary, this study adds to the growing body of literature that implicates changes in skeletal muscles as well as the heart in experimentally induced CHF. Our results clearly demonstrate that changes in the catalytic activity can become uncoupled from the content and isoform composition of the Na⁺-K⁺ pump in a muscle-specific manner. Yet to be identified are the mechanisms involved and the functional significance of the changes observed. Although it is tempting to speculate that changes in Na⁺-K⁺-ATPase activity may result in decreased Na⁺ and K⁺ transport across the sarcolemma and T tubule, and decreased sensitivity to Na⁺ and K⁺, resulting in a loss of membrane excitability, these possibilities remain to be investigated.

GRANTS

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