 Isoform-Specific Regulation of Myocardial Na,K-ATPase α-Subunit in Congestive Heart Failure

Role of Norepinephrine

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Background Myocardial ouabain-binding sites and Na,K-ATPase activity are reduced in congestive heart failure (CHF), but the mechanisms by which CHF reduces the Na,K-ATPase remain unknown. We propose to investigate whether the changes are accompanied by isoform-specific reductions of the Na,K-ATPase α-subunit proteins in CHF and whether similar changes could be produced by exogenous norepinephrine administration.

Methods and Results CHF was induced in dogs by rapid ventricular pacing at a rate of 225 beats per minute for 8 weeks (protocol 1). A second group of dogs were paced at 100 beats per minute and served as controls. In protocol 2, norepinephrine was infused in normal dogs using a subcutaneous osmotic minipump for 8 weeks. The control dogs received normal saline through the pump. Animals were studied after 8 weeks of pacing or norepinephrine infusion. After the baseline hemodynamics and interstitial norepinephrine concentration had been obtained, the hearts were removed for measuring [3H]ouabain-binding sites and Na,K-ATPase α-subunit proteins using isoform-specific monoclonal antibodies.

Results Myocardial [3H]ouabain-binding sites were reduced in dogs with CHF and chronic norepinephrine infusion. The Western blot analysis showed that adult canine hearts possess both α1 and α3 isoforms of the Na,K-ATPase α-subunit but not the α2 isoform protein. CHF and NE infusion had no effect on the Na,K-ATPase α1-subunit protein but did reduce the α3 isoform protein significantly. In addition, there was a significant inverse correlation between the amount of myocardial α3 isoform protein and interstitial norepinephrine content in the dogs. In contrast, the specific activity of the sarcolemmal marker S1-nuclease in the dogs did not differ among the groups of animals.

Conclusions The reduction of myocardial Na,K-ATPase in CHF is limited to the α3 isoform. Furthermore, because similar changes in myocardial ouabain-binding sites and Na,K-ATPase α3 isoform were produced by chronic norepinephrine infusion, the decrease in the Na,K-ATPase in CHF is most likely mediated via excess sympathetic stimulation.

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Key Words • ouabain • heart failure, congestive • norepinephrine • genetics

The integral membrane-bound enzyme Na,K-ATPase is responsible for maintaining cell volume, ionic gradients, and membrane potential in a wide variety of cells.1 It is also recognized as the receptor site for ouabain and other cardiac glycosides, which exert their action on the catalytic α-subunit of the enzyme to inhibit the Na,K-coupled transmembrane transport.2 Na,K-ATPase exists as a heterodimer composed of an α- and a β-subunit.1 The catalytic unit of the enzyme resides in the α-subunit, which consists of three isoforms, each a product of its own gene.3 The three isoforms differ in their antigenicity, migratory properties on SDS-PAGE, distribution in various tissues, level of expression during different developmental stages, binding affinity for ouabain, and susceptibility to the regulatory effects of hormones and pharmacological agents.3 The β-subunit exists in a one-to-one ratio with the α-subunit. Although no enzymatic function has been ascribed for the β-subunit, it is recognized that the β-subunit plays a critical role in the assembly and integration of the mature Na,K-ATPase into the plasma membrane. The β-subunit, by forming a complex with the α-subunit, also has been shown to prevent trypsin-mediated degradation of the α-subunit.1

[3H]Ouabain-binding assay has been used to quantitate the abundance of Na,K-ATPase. It has been shown that myocardial ouabain-binding sites are reduced in patients with dilated cardiomyopathy.4 Similarly, myocardial ouabain-binding sites and Na,K-ATPase activity are reduced in dogs with right heart failure produced by tricuspid avulsion and pulmonary artery constriction5 and in dogs with biventricular failure produced by rapid ventricular pacing.6 The clinical implications of these observations are far-ranging given the multitude of roles that Na,K-ATPase plays in cellular functions and the widespread use of digitalis in the treatment of congestive heart failure (CHF). However, the ouabain-binding method does not distinguish the various isoforms of the Na,K-ATPase. In addition, the ouabain-binding properties and activity of the enzyme are affected by the concentrations of Na+, K+, Ca2+, and Mg2+ in the incubation medium.7 In the present study, we proposed to investigate whether the myocardial Na,K-ATPase

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downregulation in CHF is limited to specific isoforms of the enzyme protein. We induced CHF in dogs using the rapid ventricular pacing technique. Using Western blot analysis, we were able to show that the reduction of myocardial ouabain-binding sites was associated with a decrease in Na,K-ATPase α-subunit protein and that the change in protein expression was isoform specific.

Furthermore, because the reduction of myocardial ouabain-binding sites in right heart failure could be prevented by β-receptor blockade, we speculate that the changes in myocardial Na,K-ATPase in CHF are mediated via the sympathetic stimulation. Thus, we proposed in this study to determine whether norepinephrine infusion reduces myocardial ouabain-binding sites and Na,K-ATPase α-subunit isoforms. A subcutaneous osmotic minipump method was adopted to deliver norepinephrine to normal dogs. Finally, we used an intracoronary norepinephrine tracer technique to measure myocardial interstitial norepinephrine concentration and to determine whether the Na,K-ATPase contents correlated with interstitial norepinephrine concentration in both the pacing-induced CHF and norepinephrine infusion studies.

Methods

Animal Preparation and Study Protocols

The study involved two different animal preparations: protocol 1 for pacing-induced CHF and protocol 2 for norepinephrine infusion experiments. Adult mongrel dogs weighing 18.6 to 29 kg were used. The initial surgery was the same in all dogs, involving general anesthesia with intravenous sodium pentobarbital (25 mg/kg). A left thoracotomy was performed under sterile conditions for the placement of heparin-filled Tygon catheters in the left atrium, main pulmonary artery, and descending thoracic aorta. In addition, an implantable Konigsberg micromamomenter (Konigsberg Instruments, Inc, Pasadena, Calif) was placed in the left ventricle via a stab wound at the left ventricular apex. One week later, the animals were divided into one of the following protocols.

In protocol 1, a custom-designed Medtronic multiprogrammable pacemaker (Medtronic, Minneapolis, Minn) was placed with the dog under local xylocaine anesthesia in a cervical pocket and connected to a bipolar pacing lead positioned in the apex of the right ventricle through the external jugular vein. The animals were randomized to receive either rapid ventricular pacing at a rate of 225 beats per minute (CHF group) or control pacing at a rate of 100 beats per minute (control group). Adequacy of pacing threshold was assessed weekly by ECG recordings. CHF was assessed by development of tachypnea and elevated left atrial pressure. All animals were acclimatized to the laboratory and trained to lie quietly with minimal restraint on a table.

Eight to 12 weeks after implantation of the pacemaker, animals underwent basal hemodynamic and plasma norepinephrine evaluations and subsequently were killed with an intravenous injection of lethal doses of pentobarbital (>100 mg/kg). The heart was excised immediately, and the right and left ventricles were separated and weighed. The left ventricular free wall and interventricular septum were combined for left ventricular weight measurement. Muscle block samples were taken from the left ventricular free wall 3 cm below the atrioventricular groove for measuring specific activity of the plasma membrane marker 5'-nucleotidase, ouabain-binding sites, and tissue protein expression of the Na,K-ATPase α-subunit.

In protocol 2, an Alzet model 2ML4 osmotic minipump (Alza Corp, Palo Alto, Calif) was implanted aseptically in the posterior neck region under local anesthesia using xylocaine. The pumps were used to administer either norepinephrine (0.5 μg · kg⁻¹ · min⁻¹) or sterile normal saline SC. Because each pump could deliver only a constant volume for 4 weeks, a second osmotic pump was implanted 3 to 4 weeks later to ensure constant norepinephrine delivery for 8 weeks. At that time, the dogs were brought to the laboratory for the final basal hemodynamic and norepinephrine measurements and then killed as described in protocol 1.

The study was approved by the University of Rochester Committee on Animal Resources and conformed to the guiding principles of the American Physiological Society and the National Institutes of Health Guide on the Humane Care and Use of Laboratory Animals.

Basal Hemodynamic Measurements

In the paced dogs (protocol 1), the pacemaker was reprogrammed to a subthreshold level and its lowest rate 2 hours before the final hemodynamic measurements. The previously implanted catheters were attached to a Spectramed P23XL (Spectramed, Inc, Oxnard, Calif) and an eight-channel Brush model 448 recorder (Gould, Inc, Instrument Systems Division, Cleveland, Ohio) for measuring heart rate, left atrial pressure, and aortic pressure. The Konigsberg transducer was connected to the Brush recorder for measuring left ventricular pressure and its first derivative (dP/dt) using an electronic differentiator. Cardiac output was measured using indocyanine green (Cardio-Green, Hyoscymus, Westcott and Dunning, Inc, Baltimore, Md) and Lyons model D-014 dye-dilution cardiac output system (Lyons Medical Instrument Corp, Sylmar, Calif). Resting hemodynamic measurements were taken in triplicate 5 minutes apart, and the values were averaged and used for statistical analyses.

Plasma Norepinephrine Concentrations

Arterial blood was collected into ice-chilled glass tubes containing reduced glutathione. The blood was centrifuged, and the plasma was stored at −70°C for subsequent radioenzymatic norepinephrine assay.

Myocardial Membrane Preparation

Muscle blocks (4 g) taken from the left ventricular free walls were trimmed, minced, and homogenized in an ice-cold 50 mmol/L Tris-HCl buffer (pH 7.4 at 22°C). The homogenate was centrifuged at 500g for 15 minutes at 4°C to remove nuclei and unbroken cells. The supernatant was collected and centrifuged again at 40,000g for 15 minutes at 4°C. The pellet then was resuspended in a buffer medium and filtered through a 53-μm nylon mesh. We have shown in pilot studies that the 500g pellets exhibit a much lower specific activity of 5'-nucleotidase (2.90±0.29 nmol adenosine · mg⁻¹ · min⁻¹, n=16) than the 40,000g pellets (16.9±0.73 nmol adenosine · mg⁻¹ · min⁻¹, n=16).

In addition, if the initial step of 500g centrifugation was omitted, the 40,000g pellet would have a much lower specific activity of 5'-nucleotidase (3.68±0.49 nmol adenosine · mg⁻¹ · min⁻¹, n=15), suggesting that the low-speed centrifugation confers enrichment of the plasma membrane to the membrane preparation we used.

Measurement of 5'-Nucleotidase Activity

The activity of 5'-nucleotidase was determined using the method of Panagia et al. Approximately 50 μg of tissue protein was incubated for 20 minutes at 37°C in 0.5 mL of a medium containing 2 mmol/L MgCl₂, 0.02 mmol/L erythro-9-(2-hydroxy-3-nonyl)-adenine, 2 mmol/L dicyclohexylammonium p-nitrophenolphosphate, 0.2 mmol/L AMP, 40 000 dpm [¹⁴C(U)]AMP (561 mCi/mmol; New England Nuclear, Boston, Mass), and 50 mmol/L Tris-HCl, pH 8.0. The reaction was terminated by addition of 100 μL of 0.25 mol/L ZnSO₄ and 100 μL of 0.25 mol/L barium hydroxide. After centrifugation at 800g for 15 minutes at 4°C, a 200-μL aliquot of the supernatant was counted for 14°C radioactivity by liquid scintillation spectrometry. Each assay was performed in triplicate with appropriate concurrent blanks. The enzyme activity is ex-
pressed as nanomoles of adenosine formed per milligram of protein per minute.

**Myocardial Ouabain-Binding Sites**

The ouabain-binding assay was carried out as described previously. Briefly, the pellet was suspended in 50 mmol/L Tris-HCl buffer supplemented with 4 mmol/L MgCl₂ and 1 mmol/L Na₂HPO₄ (Mg-PO₄ buffer, pH 7.4, at 22°C) filtered through a 53-μm nylon mesh. Approximately 70 μg of membrane protein was incubated in triplicate with six concentrations (5 to 100 nmol/L) of [³H]ouabain (23.2 Ci/mmol; New England Nuclear) in the presence of either unlabelled ouabain (final concentration, 1 mmol/L) or vehicle at 37°C for 40 minutes. To separate bound and unbound [³H]ouabain, the samples were diluted with 5 mL ice-cold Mg-PO₄ buffer and immediately filtered through Whatman GF/B filters on a Brandel cell harvester (Biomedical Research and Development Laboratories, Inc, Gaithersburg, Md), followed by three additional washes with the same buffer. The filters were counted for ³H radioactivity by liquid scintillation spectrometry (Tricarb 460 CD, Packard Instrument Co, Inc, Downers Grove, III). Specific binding was defined as the difference between binding of [³H]ouabain in the absence and presence of 1 mmol/L unlabelled ouabain. The Lowry method was used to determine protein concentration in triplicate using bovine serum albumin as a standard.

**Protein Electrophoresis and Western Blotting**

Ventricular muscle samples (0.5 to 1.0 g) were minced and homogenized in 10 vol of a sample buffer (0.32 mol/L sucrose, 1 mmol/L EDTA, pH 7.35) supplemented with 0.5 mmol/L phenylmethylsulfonyl fluoride and 1 μg/mL leupeptin. The homogenate was centrifuged at 500g for 15 minutes at 4°C. The supernatant was collected and spun at 40 000g for 15 minutes at 4°C. The pellet was then resuspended in the sample buffer, and the protein content was determined using bicinchoninic acid (BCA kit, Pierce, Rockford, Ill) with bovine serum albumin as a standard.

Na⁺K-ATPase α isoforms were detected by immunoblotting using isofrom-specific monoclonal antibodies on the SDS-PAGE system. Membrane fractions containing 50 μg of protein were loaded onto 4.5% polyacrylamide stacking gel and a 7.5% polyacrylamide/0.1% SDS resolving gel. The gel system was run at 10 mA for 15 hours, and the proteins were transferred to polyvinylidene difluoride membranes in a transfer buffer containing 10 mmol/L 3-(cyclohexylamino)-1-propanesulfonic acid (pH 11) and 10% methanol at 4°C for 80 minutes at 500 mA constant current. The membranes were then placed in a blocking buffer (50 mmol/L Tris base, 147 mmol/L NaCl, and 1 mg/L thimerosal [pH 7.4], supplemented with 1% nonfat milk) for 1 hour to minimize nonspecific binding and then probed with isofrom-specific monoclonal antibodies for 1.5 hours. The anti-chicken α₁-specific monoclonal antibody (α₁F) was obtained from the Developmental Hybridoma Bank at the University of Iowa. The anti-rat α₁ (McB2) and anti-dog α₁ (XVI F9G10) monoclonal antibodies were kindly provided by Kathleen Sweadner (Harvard University) and Kevin Campbell (University of Iowa), respectively. All three antibodies are mouse IgG in origin. Species specificity testing included dog for all three antibodies. The transfer membranes were then washed four times with the blocking buffer, each for 10 minutes, and incubated with the biotinylated secondary antibody and avidin-biotin–horse-radish peroxidase complex of Vectastain ABC kit (Vector Laboratories, Burlingame, Calif). The membranes were again washed four times with the blocking buffer before reaction with 4-chloro-1-naphthol detection system. The signal intensity of the blue precipitates was quantitated by videodensitometric analysis using a Bio-Image system (Millipore Corp, Ann Arbor, Mich).

To determine the isofrom specificity of the antibodies, we included in the assays membrane fractions taken from the dog kidney, rat brain, and dog brain as positive controls for the α₁, α₂, and α₃ isoforms, respectively. Varying concentrations of the protein standards were run with each experiment to generate a reference standard curve by plotting videodensitometric readings against protein concentration. The isofrom-specific protein contents of the unknown samples were then calculated using the reference curve and expressed in micrograms of reference protein per milligram of sample protein (Fig 1).

**Interstitial Norepinephrine Content**

Interstitial norepinephrine concentration was measured using a modification of the method originally described by Cousins et al. Briefly, it uses a bolus intracoronary injection of a mixture of [³H]albumin, [¹⁴C]sucrose, and [³H]norepinephrine and a timed, continuous sampling of coronary sinus blood (19 mL/min) during the ensuing 60 seconds. Blood samples were also taken simultaneously from the aorta and coronary sinus for norepinephrine measurements. As the total activity of each radioactive compound injected is known, the activity of each species in the coronary sinus can be used to calculate the relative extraction fraction of each isotope at each point in time and the rate constant of the "uptake−1" function. The myocardial interstitial norepinephrine content was then calculated.

**Data Analyses**

The number of maximum binding sites and the apparent dissociation constant (Kᵦ) for [³H]ouabain-binding assays were analyzed using the AccuFit saturation two-site program developed by Hawkin (Lundon Software, Inc, Chargin Falls, Ohio). All results are expressed as mean±SEM. The statistical significance between groups was determined using the Student’s t test for unpaired data. The Pearson product-moment corre-
TABLE 1. Resting Hemodynamics, Plasma Norepinephrine, and Left Ventricular Weight in CHF and Control Dogs

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CHF (n=16)</th>
<th>Control (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, kg</td>
<td>23.0±0.4</td>
<td>24.1±0.5</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>130±4*</td>
<td>98±4</td>
</tr>
<tr>
<td>Left atrial pressure, mm Hg</td>
<td>28.5±1.6*</td>
<td>8.8±0.6</td>
</tr>
<tr>
<td>Plasma NE, ng/mL</td>
<td>0.96±0.13*</td>
<td>0.27±0.05</td>
</tr>
<tr>
<td>Mean aortic pressure, mm Hg</td>
<td>96±2</td>
<td>109±2</td>
</tr>
<tr>
<td>Cardiac output, L/min</td>
<td>2.80±0.26*</td>
<td>3.96±0.26</td>
</tr>
<tr>
<td>LV dP/dt, mm Hg/s</td>
<td>1444±46*</td>
<td>2971±121</td>
</tr>
<tr>
<td>LV weight, g</td>
<td>112±4</td>
<td>102±4</td>
</tr>
</tbody>
</table>

CHF indicates congestive heart failure; bpm, beats per minute; NE, norepinephrine; and LV, left ventricular. Values are mean±SEM.

*P<.001 compared with the control group, as determined by Student’s t test for unpaired data.

Results

Protocol 1: Fast Ventricular Pacing

CHF developed in the fast-paced dogs gradually. At the time of the final hemodynamic studies, the CHF dogs displayed resting tachycardia, increased left atrial pressure, elevated plasma norepinephrine, and reduced cardiac output, mean aortic pressure, and left ventricular dP/dt compared with the control-paced animals (Table 1). There was no difference in body weight or left ventricular weight between the two groups.

The cardiac membrane preparations showed no difference in the specific activity of 5'-nucleotidase between the CHF (13.49±0.89 nmol adenosine·mg⁻¹·min⁻¹) and control (14.08±0.90 nmol adenosine·mg⁻¹·min⁻¹) animals. The radiolabeled ouabain-binding assay revealed a saturable and reversible binding of [3H]ouabain to a homogeneous population of receptors in the membrane preparation. Nonspecific binding accounted for <5% of total binding. The number of myocardial ouabain-binding sites was significantly lower in the CHF dogs than the control-paced dogs (5.10±0.26 versus 7.28±0.36 pmol/mg protein, t=4.74, P<.0001). The dissociation constant did not differ between the two groups (12.3±0.8 versus 15.9±2.1 nmol/L). To determine whether the discarded 500g pellets contained the missing ouabain receptors in CHF, we also prepared a crude membrane 40 000g pellet without the initial step of 500g centrifugation from the hearts of a subset of CHF and control animals. Like the enriched membrane preparation, the crude membrane preparation showed a reduced ouabain-binding site density in CHF (3.83±0.14 pmol/mg, n=7) compared with the control animals (4.57±0.17 pmol/mg, n=7, t=4.155, P=.001). Again, there was no difference in the dissociation constant between the two groups (10.37±0.33 versus 10.49±0.29 nmol/L) using the crude membrane preparation.

Fig 2 illustrates the distribution of the three Na,K-ATPase α-subunit isoforms and the isoform specificity of the antibodies in various canine tissues using the Western blot technique. Canine brain contained proteins of all three α-subunit isoforms, whereas only the α1 isoform was detected in the kidney. The dog heart contained α1 and α2 but no α3 isoform.

Fig 3 represents a Western blot for the myocardial Na,K-ATPase isoforms. This particular blot was reacted in sequence with antibodies against the α1 and α3 isoforms to illustrate the relative migratory positions of the two isoforms. The molecular weight standards (not shown) indicated that the isoforms had molecular weights near 100 kd. The α3 isoform was slightly smaller and migrated farther than the α2 isoform. Also shown in Fig 3 is a reduction of α3 isoform signal in three representative CHF dogs compared with three control animals. In contrast, there was no difference in the abundance of α1 isoform between the CHF and control animals. The same conclusions were reached when the results of all 16 CHF and 17 control dogs were compared (Table 2). In the latter analyses, blots were reacted individually with either α1 or α3 isoform-specific antibody. The values presented in Table 2 indicate the relative abundance of the specific isoform proteins in the two groups of animals. Because the α isoform proteins were standardized by two different reference organ proteins, the measurements do not provide the relative abundance of α1 and α3 isoforms in the canine myocardium.

Fig 3. Illustrations of the relative changes of myocardial Na,K-ATPase α1 (bottom bands) and α3 (upper bands) isoforms in three fast-paced congestive heart failure (CHF) and three control-paced (Sham) dogs. Each lane contained 50 μg of membrane protein.
Table 2. Myocardial Na,K-ATPase α-Subunit Isoforms in CHF and Control Dogs

<table>
<thead>
<tr>
<th>Na,K-ATPase Isoform</th>
<th>CHF (n=16)†</th>
<th>Control (n=17)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>α₁</td>
<td>147±15</td>
<td>154±9</td>
</tr>
<tr>
<td>α₃</td>
<td>25±4*</td>
<td>61±4</td>
</tr>
</tbody>
</table>

CHF indicates congestive heart failure. Values are mean ±SEM.

*P<.0001, compared with the control animals, as determined by Student’s t test for unpaired data.

†Values in μg reference per mg protein.

Protocol 2: Norepinephrine Infusion

At the doses administered, norepinephrine did not produce significant hypertension in the dogs over the 8 weeks of infusion. Despite the marked elevations of plasma norepinephrine, there were no changes in body weight, left atrial pressure, mean aortic pressure, left ventricular dp/dt, and cardiac output between the two groups (Table 3). Basal heart rate, however, was reduced significantly in the norepinephrine-infused dogs compared with the saline-infused dogs.

Fig 4 shows the effects of norepinephrine infusion on myocardial ouabain-binding sites and Na,K-ATPase isoform contents. As occurred in CHF dogs, myocardial ouabain receptor density was reduced in dogs after chronic norepinephrine infusion. There was no difference in the Kᵦ between the norepinephrine- and saline-infused dogs (13.2±1.6 versus 12.6±1.6 nmol/L). Concurrent with the change in ouabain receptor density was a reduction in the myocardial Na,K-ATPase α₃ isoform of the norepinephrine-infused animals. In contrast, the α₁ isoform protein did not differ for the two groups. Nor did the specific activity of 5’-nucleotidase differ significantly between the two groups (14.21±1.71 versus 14.15±0.88 nmol adenosine·mg⁻¹·min⁻¹).

Correlation Between Na,K-ATPase Isoforms and Intersitial Norepinephrine Concentrations

Myocardial interstitial norepinephrine concentrations were increased in CHF (1.12±0.15 ng/mL) and norepinephrine-infused (1.78±0.43 ng/mL) dogs compared with their respective sham-operated (0.20±0.07 ng/mL) and saline-infused (0.25±0.07 ng/mL) control groups. Fig 5 shows a negative correlation between the abundance of myocardial Na,K-ATPase α₁ isoform and myocardial interstitial norepinephrine concentration in the CHF and norepinephrine infusion experiments (r= .57, P<.001). The coefficient of determination (r²) of .32 suggests that in this study population, 32% of the variation in myocardial Na,K-ATPase α₁ isoform could be attributed to a linear association with interstitial norepinephrine concentration. In contrast, there was no significant correlation between interstitial norepinephrine concentration and Na,K-ATPase α₃ isoform content.

Discussion

This study demonstrates the isoform-specific nature of downregulation of cardiac Na,K-ATPase in CHF. A marked reduction in the α₁ isoform of the catalytic subunit was found with no alteration in the α₃ isoform in cardiac tissue from dogs with pacing-induced CHF. We provided further evidence for the role of norepinephrine in mediating this downregulation by showing a similar isoform-specific reduction of the Na,K-ATPase in dogs with elevated circulating norepinephrine levels from chronic norepinephrine infusion without heart failure. Furthermore, a significant inverse correlation was found between myocardial interstitial norepinephrine concentration and the α₁ isoform protein content in the CHF and norepinephrine infusion studies.
In a previous study of right heart failure,5 we have shown that ouabain-binding sites are reduced not only in the hypertrophied failing right ventricle but also in the left ventricle, which has a normal or reduced filling pressure. These changes of ouabain-binding sites differ from those of myocardial β-receptor density, which is reduced only in the failing right ventricle of our right heart failure animals23 or patients with pulmonary hypertension.24 The findings suggest that the changes in ouabain-binding sites probably are related to a systemic process that affects both the failing and nonfailing ventricles. Likewise, ouabain-binding sites have been reported to be decreased not only in the failing myocardium but also in skeletal muscle of CHF patients.25

It has been speculated that the reduction of ouabain-binding sites in hypertrophied hearts is due to a relative decrease of sarcolemmal fractions in the tissue with increased cell mass.26 However, because neither left ventricular weight nor 5'-nucleotidase activity changed significantly in the CHF or norepinephrine-infused animals, the reductions of myocardial ouabain-binding sites and Na,K-ATPase α3 isoform probably cannot be explained by dilution or differences in the amount of sarcolemmal fraction in the myocardial membrane preparations of our present study. Furthermore, because the α3 isoform content did not change in the same membrane preparations, results of our study indicate that the α3 isoform was affected selectively by CHF and norepinephrine infusion and could not be accounted for by variations in sarcolemmal contents among the experimental groups.

Our present study is the first to describe alterations of Na,K-ATPase isoforms in a canine model of heart failure. Isoform-specific changes of myocardial Na,K-ATPase also have been described in animals in response to developmental growth, physiological stimuli, and other pathological states. In rodents, there is a selective increase in the α2 isoform and decrease in the α3 isoform during normal postnatal development to young adulthood; the α1 isoform remains unchanged.27 Thyroid hormone28 and insulin29 stimulate Na,K-ATPase enzyme activity and number in an isoform-specific manner. Cultured rat cardiocytes exposed to T3 displayed a preferential increase in the α1 and α2 isoform mRNAs. The α2 isoform mRNA initially increased but later decreased.30 A reduction of the α2 and α3 isoform mRNA levels and an increase in the α1 mRNA have been reported in the hypertrophied rat left ventricle.31 An isoform shift was also suggested in another study of cardiac hypertrophy,32 in which myocardial ouabain-binding characteristics and Na,K-ATPase activity changed to those resembling neonatal form. In addition, dogs sustaining myocardial infarctions demonstrated a preferential reduction of the α1 isoform.33 As in our present study, the α1 isoform did not change significantly in the ischemic canine myocardium. Furthermore, the absence of α2 isoforms is not unique to the dog heart; the α2 isoform is also absent in the sheep, chicken, and guinea pig hearts.

Prior studies have shown conflicting results of the effects of β-adrenergic agonists on myocardial Na,K-ATPase. In one study, isoproterenol was administered to rats at a dosage of 2 mg·kg⁻¹·d⁻¹ for 7 days, and this resulted in an increase in Na,K-ATPase activity and ouabain-binding sites.34 In contrast, a higher dosage of isoproterenol (5 mg·kg⁻¹·d⁻¹) for 7 days decreased myocardial Na,K-ATPase activity.35 Norepinephrine also appears to exert a dose-dependent differential effect on myocardial Na,K-ATPase. Using guinea pig ventricular tissue slices, Wanless et al36 found that at the physiological concentration, norepinephrine stimulated Na,K-ATPase activity, but at the high doses it inhibited the Na,K-ATPase activity. The effects of norepinephrine on Na,K-ATPase also appear to be specific to the high-affinity isoform.34 Thus, it appears that our results of norepinephrine infusion in intact dogs are consistent with the effects of chronic high-dose norepinephrine. Further studies are needed of the time course or dose dependency of the effect of norepinephrine on the Na,K-ATPase isoform changes.

The dose of norepinephrine chosen for our studies was the same as that used previously in studying the effects of norepinephrine on the regulation of myocardial β-adrenoceptor-coupled adenylate cyclase in dogs.7 As demonstrated previously,7,37 despite the marked elevations in plasma norepinephrine, chronic norepinephrine infusion produced no significant increases in mean aortic pressure, left ventricular dp/dt, left atrial pressure, or left ventricular weight in dogs. Cardiac output also did not differ between the norepinephrine- and saline-infused animals. We have also found no histological evidence of hemorrhage or necrosis in the myocardium of the norepinephrine-infused animals (unpublished observations). However, basal heart rate was reduced after chronic norepinephrine infusion, probably because norepinephrine could either sensitize the aortic baroreceptor directly or increase sympathetic tone enough to cause baroreflex-mediated slowing of the heart rate.

Our findings that norepinephrine infusion decreased myocardial ouabain-binding sites in dogs with normal left atrial pressure suggest that elevated filling pressure is not a prerequisite for the decrease in myocardial ouabain-binding sites. Results of our study indicate that an increase in interstitial norepinephrine to levels seen in CHF is sufficient to produce a decrease in myocardial ouabain-binding sites and the Na,K-ATPase α3 isoform protein level. However, only one third of the variations of myocardial Na,K-ATPase could be explained by the differences in interstitial norepinephrine concentrations in our present study, probably because significant biological individual variabilities exist in the relation between myocardial interstitial norepinephrine and Na,K-ATPase. A stronger correlation might exist between the two variables if interstitial norepinephrine and Na,K-ATPase could be measured serially in the same animals.

Our findings of norepinephrine infusion confirm the acute inhibitory effect of high-dose norepinephrine on Na,K-ATPase.34 They also are consistent with the observations that chronic administration of β-adrenergic agonists reduces the density of ouabain-binding sites in guinea pig skeletal muscle.38 The latter findings suggest that the effects of norepinephrine on ouabain-binding sites are not limited to the ventricular myocardium. However, whether the β-adrenergic agonists also produce isoform-specific changes on skeletal muscle Na,K-ATPase is not known.

Relatively little is known of the mechanisms by which norepinephrine modulates the molecular changes of the Na,K-ATPase enzyme. β-Adrenoceptors appear to play a significant role in mediating the regulatory effects of norepinephrine since β-blockade prevents most, if not
all, of the effects of norepinephrine. Norepinephrine increases Na+ influx into the cells and may act to alter the Na,K-ATPase activity by changing intracellular Na+ and K+ concentrations. Norepinephrine may also increase intracellular cAMP and inhibit Na,K-ATPase through a protein kinase A pathway involving phospholipase A2 and arachidonic acid release. Phospholipase A2 has been shown to inhibit myocardial Na,K-ATPase activity and decrease the ouabain-binding sites by accumulation of phospholipids and nonesterified fatty acids and membrane phospholipid depletion. Furthermore, Na,K-ATPase can be inhibited by oxygen free radicals and oxidants. One possible mechanism of action of β-adrenoceptor-blocking agents in preventing the downregulation of Na,K-ATPase may be related to their antioxidant properties.

The physiological and pharmacological significance of the reduction of Na,K-ATPase and α2 isomeric-specific downregulation of Na,K-ATPase in CHF has not been established. It has been shown that the positive inotropic effect of ouabain is well preserved in the isolated muscle preparations of failing human hearts. In a canine right heart failure model, we also have demonstrated that despite a 38% to 50% decrease in ouabain-binding sites, the failing myocardium retains the same responsiveness as the normal heart to the positive inotropic effect of acetylcholine. However, because Na,K-ATPase affects resting membrane potential and transmembrane fluxes of various ions, changes in myocardial Na,K-ATPase like those occurring in CHF may influence important cardiac function and lead to certain functional alterations, such as potentiation of the arrhythmogenic effect of cardiac glycosides. The increase in arrhythmogenic transient depolarization that occurs in isoproterenol-induced cardiac hypertrophy could also be caused by the reduced myocardial ouabain-binding sites. Recently, using in situ hybridization techniques, Zahler et al have shown in adult rat hearts that the mRNAs for the α1 and α2 isoforms of the Na,K-ATPase are preferentially localized to the cardiac conduction system. Additional studies are warranted to delineate the anatomic distribution of Na,K-ATPase isoforms in other species and to determine whether a decrease in myocardial α2 Na,K-ATPase contributes to the development of cardiac dysfunction or the arrhythmogenic responses of the heart to cardiac glycosides in patients with CHF.

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