Reduction of Na/K-ATPase affects cardiac remodeling and increases c-kit cell abundance in partial nephrectomized mice

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1Department of Medicine, University of Toledo, Toledo, Ohio; 2Department of Physiology Pharmacology, University of Toledo, Toledo, Ohio; and 3Joan C. Edwards School of Medicine, Marshall University, Huntington, West Virginia

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Drummond CA, Sayed M, Evans KL, Shi H, Wang X, Haller ST, Liu J, Cooper CJ, Xie Z, Shapiro JJ, Tian J. Reduction of Na/K-ATPase affects cardiac remodeling and increases c-kit cell abundance in partial nephrectomized mice. Am J Physiol Heart Circ Physiol 306: H1631–H1643, 2014. First published April 18, 2014; doi:10.1152/ajpheart.00102.2014.—The current study examined the role of Na/K-ATPase α₁-subunit in animals subjected to 5/6th partial nephrectomy (PNx) using Na/K-ATPase α₁-heterozygous (α₁⁺⁻⁻) mice and their wild-type (WT) littermates. After PNx, both WT and α₁⁺⁻⁻ animals displayed diastolic dimension increases, increased blood pressure, and increased cardiac hypertrophy. However, in the α₁⁺⁻⁻ animals we detected significant increases in cardiac cell death in PNx animals. Given that reduction of α₁ elicited increased cardiac cell death with PNx, while at the same time these animals developed cardiac hypertrophy, an examination of cardiac cell number, and proliferative capabilities of those cells was carried out. Cardiac tissues were probed for the progenitor cell marker c-kit and the proliferation marker ki-67. The results revealed that α₁⁺⁻⁻ mice had significantly higher numbers of c-kit-positive and ki-67-positive cells, especially in the PNx group. We also found that α₁⁺⁻⁻ mice express higher levels of stem cell factor, a c-kit ligand, in their heart tissue and had higher circulating levels of stem cell factor than WT animals. In addition, PNx induced significant enlargement of cardiac myocytes in WT mice but has much less effect in α₁⁺⁻⁻ mice. However, the total cell number determined by nuclear counting is higher in α₁⁺⁻⁻ mice with PNx compared with WT mice. We conclude that PNx induces hypertrophic growth and high blood pressure regardless of Na/K-ATPase content change. However, total cardiac cell number as well as c-kit-positive cell number is increased in α₁⁺⁻⁻ mice with PNx.

Among these, c-kit-positive cells are the most studied cardiac stem cells; cardiac hypertrophy; apoptosis; ATPases; caspase; cell proliferation; uremic cardiomyopathy; cardiac progenitor cells; cardiotoxic steroids

STUDIES INDICATE THAT renal insufficiency worsens congestive heart failure and is directly associated with morbidity and mortality in congestive heart failure patients, described as uremic cardiomyopathy (UrC) (6, 9, 37, 44). Mortality due to cardiovascular disease was 10 to 30 times higher in patients receiving dialysis than in the general population (31). However, direct mechanisms by which these cardiovascular changes are incurred are not well understood.

One of the major cardiovascular changes observed in patients and experimental models of UrC is cardiac remodeling, including cardiac hypertrophy and dilatation. Increased gravimetric weight of the heart as well as increased blood pressure occurs in both experimental models and patients with UrC (2, 5, 11, 38, 46). Cardiac hypertrophy typically occurs in two phases. First is the compensated phase where cardiomyocytes enlarge and the walls of the heart thicken to preserve cardiac ejection performance in response to systemic pressure or volume overload (5). The second phase called “decompensated” hypertrophy occurs in response to prolonged pressure or volume overload or in response to an illness or chronic disease condition (5). Decompensated hypertrophy is characterized by cardiac myocyte death, increased ventricular dimension, thinning of the walls of the heart, and significant declines in cardiac output (5). More recently, studies found that regeneration of myocardium via stem cell differentiation is also involved in the process of cardiac remodeling (21, 52, 53). The concept that the adult heart is a self-renewing organ has evolved during the past two decades, with reports showing that stem cells can differentiate into cardiac myocytes (3, 21, 30, 52, 53). Several biomarkers have been discovered that identify the resident cardiac stem cells or progenitor cells (3, 22). Among these, c-kit-positive cells are the most studied cardiac stem cells and have been shown to improve cardiac function in clinical trials (4, 36).

We have previously reported that cardiotoxic steroids (CTS) are elevated and contribute to cardiac hypertrophy and fibrosis in rat and mouse models of UrC (2, 46). Conversely, we found that CTS such as marinobufagenin (MBG) induce cardiac myocyte apoptosis when the Na/K-ATPase α₁-subunit is reduced (33). Decreased Na/K-ATPase and increased endogenous CTS have been found in patients with congestive heart failure and chronic kidney diseases (18, 25, 29, 40, 47). Additionally, infusion of MBG into mice with reduced Na/K-ATPase α₁-subunit levels (α₁⁺⁻⁻) resulted in increased myocyte apoptosis, ventricular dilation, decreased cardiac function, and cardiac wall thickness in α₁⁺⁻⁻ mice versus their wild-type (WT) littermates treated in a similar manner (33). These studies illustrate the causative nature of CTS in these deleterious physiological events. Surgical implementation of partial nephrectomy (PNx) in rats and mice has been previously found to emulate the clinical presentation of cardiovascular decline that patients with chronic kidney disease typically have, i.e., high blood pressure, increased diastolic dimension, and ventricular dysfunction (7, 17, 26, 43). To determine the importance of Na/K-ATPase α₁-subunit receptor levels in the development of UrC and survival/regenerative pathways in cardiovascular tissues, we adapted and used a method of segmental infarction via arterial ligation to induce 5/6th PNx in mice expressing reduced levels of Na/K-ATPase α₁-subunit and their WT littermates.
MATERIALS AND METHODS

Animals. Animal experiments were conducted in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals under protocols approved by the Institutional Animal Care and Use Committee at the University of Toledo. Na/K-ATPase α1+/− mice and their WT littermates were generated as previously described (39). Genomic DNA was obtained from tail biopsies and used for PCR-based genotyping. Adult male mice, which were ~2 to 3 mo of age and weighing between 25–27 g, were used for this study. All mice were reared under a 12-h:12-h light-dark cycle, fed standard chow, and were providing water ad libitum. These conditions were used for the entire duration of the experiment.

The two genetic groups (i.e., α1+/− and WT littermates) were each divided into two groups based on surgical intervention: the first group consisted of sham-operated animals that served as controls, whereas, the second group consisted of animals receiving PNx. Surgery was performed on mice anesthetized with 2% isoflurane (anesthesia time of 1-h) and at 8 wk after the completion of the second step of the operation, just before euthanasia. Transthoracic echocardiography measurement, and optical density at 450 nm was measured after addition of 1 ml/well) in an MBG-BSA-coated plate for 1 h. A secondary horseradish peroxidase (HRP)-conjugated anti-mouse antibody was added after washing. 3,3′,5,5′-Tetramethylbenzidine was used to measure color development.

Table 1. Doppler image study in WT and α1+/− mice

<table>
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<tr>
<th>Variables</th>
<th>WT</th>
<th>PNs</th>
<th>α1+/−</th>
<th>PNs</th>
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<tr>
<td>BW, g</td>
<td>30.3 ± 0.7</td>
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<td>31.4 ± 0.6</td>
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<td>HR, beats/min</td>
<td>479 ± 8</td>
<td>523 ± 14</td>
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<td>480 ± 13</td>
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<td>EDD, mm</td>
<td>3.4 ± 0.1</td>
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<td>3.4 ± 0.1</td>
<td>4.3 ± 0.2*</td>
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<tr>
<td>SWT, mm</td>
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<td>2.3 ± 0.1</td>
<td>3.3 ± 0.2*</td>
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<td>PWT, mm</td>
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<td>0.99 ± 0.05</td>
<td>1.12 ± 0.07</td>
<td>0.98 ± 0.12</td>
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<td>SWT, mm</td>
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<td>1.05 ± 0.07</td>
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<td>RWT</td>
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<td>4.4 ± 0.3</td>
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<td>FS, %</td>
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<td>34.6 ± 1.4</td>
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<td>26.0 ± 1.1</td>
<td>31.5 ± 2.9</td>
<td>24.4 ± 1.3</td>
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Values are means ± SE. WT, wild-type; α1+/−; Na/K-ATPase α1-heterozygous mice; BW, body weight; HR, heart rate; EDD, end diastolic dimension; ESD, end systolic dimension; PWT, posterior wall thickness; SWT, anterior wall thickness; RWT, relative wall thickness = (PWT + SWT)/EDD; FS, fraction shortening (%) = (EDD − ESD)/EDD × 100. *P < 0.05, PNX vs. sham at 8 wk.
phorylation at serine 235/236 (pS6; Cat. No. 2211S) as well as biotinylated antibodies to probe for Akt at phosphorylation sites threonine-308 (Cat. No. 5056S) and serine-473 (Cat. No. 5012S) and streptavidin-conjugated with HRP (Cat. No. 3999S) were purchased from Cell Signaling Technologies (Boston, MA). Loading conditions were controlled using a goat anti-actin polyclonal antibody from Santa Cruz Biotechnology (SC-1616). All appropriate HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, and 

/H92511-subunit levels were probed using the 

/H92516F antibody from the Developmental Studies Hybridoma Bank at the University of Iowa.

Histology. LV sections were immediately fixed in 4% formaldehyde buffer solution (pH 7.2) and was paraffin embedded after 48 h of fixation. The tissues were then cut to a thickness of 4 μm and mounted onto microscopy slides. Masson’s trichrome stain for cardiac fibrosis was conducted and computer-aided morphometry used to quantify the percentage of fibrosis in the tissue as previously described (7, 17, 26, 27). For immunostaining, slides were subjected to coimmunofluorescence staining for c-kit (CD117, marker of progenitor cells, Cat. No. AB25022, Abcam) and ki-67 (marker of proliferation, Cat. No. AB16667, Abcam) or c-kit and CD45 (marker for cells of hematopoietic origin, Cat. No. AB64100, Abcam). To count cardiac cell number, slides were stained for cardiac troponin I (cTnI, a cardiomyocyte specific marker, Cat. No. AB47003, Abcam) and 4’,6-diamidino-2-phenylindole (DAPI, a nuclear marker, Cat. No. D1306, Life Technologies). The protocol for these staining procedures is as follows: the mounted paraffin-embedded tissue sections were first deparaffinized with xylene and rehydrated by sequential incubations in ethanol and water. After rehydration, antigen retrieval was performed by boiling the slides in trisodium citrate buffer (pH 6.0; 10 mM sodium citrate, 0.05% Tween-20) for 15 min. After boiling, the slides were allowed to cool for 30 min before being washed twice for 3 min in Tris-buffered saline supplemented with Tween-20, TBST, consisting of 0.05% Tween-20 (pH 7.5), 20 mM Tris, and 500 mM NaCl. The tissue sections were then blocked for 30 min with 1% bovine serum albumin in TBST. Subsequently, the slides were incubated overnight at 4°C with primary antibody solution containing the primary antibodies for c-kit and ki-67, or c-kit and CD45 in 1% BSA-TBST. After primary antibody incubation, the slides were

Fig. 1. Partial nephrectomy (PNx) induces blood pressure (BP) and marinobufagenin (MBG) concentration increases in both wild-type (WT) and Na/K-ATPase α1 heterozygous knockout (α1+/−) mice. BP was measured by tail cuff as described in MATERIALS AND METHODS. Both systolic BP (A and B) and mean BP (C and D) showed significant increases after PNx vs. sham, but there were no significant differences between the WT and α1+/− animals. MBG concentration increases significantly with PNx in both WT and α1+/− animals. E: means ± SE of plasma [MBG] determined by immunoassay. *P < 0.05, significantly different than sham.
washed three times for 5 min each in TBST. The appropriate secondary antibodies were applied, and the slides were incubated for 2 h at room temperature. The concentrations of the secondary antibodies were as follows: 1:100 for streptavidin Alexa Fluor-594 (for c-kit), and 1:50 dilution in 1% BSA-TBST for anti-rabbit Alexa Fluor-488 (for ki-67), and/or 1:50 dilution in 1% BSA-TBST for anti-rat Alexa Fluor-488 (CD45). After incubation with secondary antibody, the slides were washed three times in TBST and mounted with Antifade Gold from Life Technologies. Immunofluorescence was then visualized on a confocal microscope (TCS SP5 LCSM, Leica, Buffalo Grove, IL).

Active caspase 3 was determined by immunohistochemistry. Preservation, mounting, deparaffinization, and antigen retrieval were similarly performed as above, but the level of active caspase 3 was determined using the HRP/3,3'-diaminobenzidine (DAB) detection kit from Abcam, according to the manufacturer’s protocol. Computer-aided morphometry was used to quantify active caspase-3 staining. Additionally, slides were stained with hematoxalin alone to allow visualization of nuclei in ×20 images of each tissue section.

**Terminal deoxynucleotidyl transferase dUTP nick end labeling assay.** Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed using a commercially available kit from Trevigen (Gaithersburg, MD) according to the company’s protocol. Briefly, paraffin-embedded LV tissue was deparaffinized with xylene and rehydrated with sequential incubation with ethanol as detailed above. Rehydrated tissue sections were washed with PBS and incubated with proteinase K for 15 min, washed, and quenched before labeling with biotin-labeled dUTP. The labeling reaction was stopped by adding stop buffer as provided by the kit. The tissue sections were then incubated with HRP-conjugated streptavidin for 10 min, washed, and immersed in DAB solution for color development. These sections were also counterstained with methyl green before mounting. Apoptotic events are presented as the ratio of positive cells (stained with dark brown color) and the total nuclei (stained with green).

**Wheat germ agglutinin staining.** Briefly, LV tissue sections were deparaffinized and rehydrated in four changes of xylene, two changes of 100% ethanol, two changes of 95% ethanol, and two changes of 70% ethanol. After slides were rinsed in running tap water for 5 min, the tissue sections were incubated with solution containing 5 μg/ml of Biocytin (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. The sections were then washed, incubated with proteinase K for 15 min, washed, and quenched before labeling with biotin-labeled dUTP. The labeling reaction was stopped by adding stop buffer as provided by the kit. The tissue sections were then incubated with HRP-conjugated streptavidin for 10 min, washed, and immersed in DAB solution for color development. These sections were also counterstained with methyl green before mounting. Apoptotic events are presented as the ratio of positive cells (stained with dark brown color) and the total nuclei (stained with green).

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**Fig. 2.** PNx induces cardiac hypertrophy and fibrosis in WT and α1+/− mice. Heart weight (HW) and left ventricle weight (LVW) (A and C) were measured after mice were euthanized, and the heart weight/body weight ratio (HW/BW, C) and LVW/BW ratio (D) were calculated based on the final body weight before euthanasia. Both HW/BW and LVW/BW data indicate a significant increase in cardiac hypertrophy. E: Masson’s trichrome staining in left ventricle tissue from experimental mice: representative images from each group taken by an Olympus FSX100 microscope with a ×20 lens (left) and quantification data (n = 8 in each group; right). *P < 0.05, significantly different than sham of same genotype; **P < 0.01, significantly different than sham of same genotype; !P < 0.01, significantly different than PNx of WT.
Oregon Green 488 wheat germ agglutinin (WGA) stain (Life Technologies, Eugene, OR), at 4°C overnight. After incubation with WGA, slides were washed three times with PBS. The slides were allowed to air dry and then mounted with Prolong Gold antifade reagent from Life Technologies. Subsequently, eight H11003 20 photomicrographs were taken in random areas of each of the tissue sections, and the area of 20 cells in each image was quantified using ImageJ (National Institutes of Health).

Measurement of plasma levels of SCF. After euthanasia (i.e., 8 wk after PNx), blood was collected from the animals in EDTA plasma tubes (Fisher, Chicago, IL). Fresh whole blood was centrifuged as 2,000 rpm for 10 min to allow for separation of the blood and collection of the plasma fraction. Plasma samples were flash frozen and stored at −80°C until used in analysis. Circulating levels of SCF were determined using the mouse anti-SCF ELISA from Abcam. Plasma samples isolated from whole blood by centrifugation were diluted four times in dilution buffer provided in the kit, and then portions of the diluted plasma were assayed for SCF content according to the manufacturer’s protocol.

Statistical analyses. Data are presented as means ± SE. Data obtained were analyzed by t-test or by two-way ANOVA followed by pairwise comparisons with Bonferroni’s corrections conducted post hoc, where appropriate. All statistical analyses were performed using Prism 5 (GraphPad).

RESULTS
PNx induces increases in circulating MBG concentrations, high blood pressure, cardiac hypertrophy, and cardiac fibrosis in both WT and α1+/− mice. Echocardiographic data were obtained at baseline (1 day before surgery) and 8 wk after surgery as summarized in Table 1. These data showed that PNx caused enlargement of the LV in both WT and α1+/− mice as indicated by the end-diastolic dimension (EDD) and end-systolic dimension. PNx also slightly decreased the body weight gain in the α1+/− mice compared with their sham-operated controls or to PNx WT mice, but this decrease did not reach statistical significance. Other echo parameters such as posterior wall thickness (PWT), septal wall thickness (SWT), relative wall thickness [calculated by (PWT + SWT)/EDD], and fractional shortening showed no difference between sham-operated and PNx or between WT and α1+/− mice.

In accord with previous studies (17, 27), both WT and α1+/− animals display a significant increase in systolic (Fig. 1, A and B) and mean blood pressure (Fig. 1, C and D) over sham-operated mice, starting 2 wk after PNx and persisting throughout the rest of the experiment. At 8 wk post-PNx, the WT

![Fig. 3](http://ajpheart.physiology.org/)

Fig. 3. PNx promotes cardiomyocyte hypertrophy in WT animals and hyperplasia in α1+/− mice. A: cardiomyocyte cross-sectional area was determined by wheat germ agglutinin staining as illustrated in MATERIALS AND METHODS: representative images from each group (left) and quantification data analyzed by ImageJ from 5 animals in each group (right). Scale bar = 50 μm. B and C: representative frequency distribution curves illustrating the frequency of cells of a given size as a percentage of the total measured cells in the WT (B) and α1+/− (C). ***P < 0.001, significantly different than sham of shared genotype; !P < 0.001, significantly different than WT receiving PNx.)
animals displayed systolic blood pressures that were 20.9 mmHg higher than sham-operated animals, whereas the α₁⁺/− animals displayed systolic blood pressures that were 19.0 mmHg higher than sham-operated animals (Fig. 1). The WT animals displayed slightly higher levels of end-systolic and mean blood pressure in PNx animals than in α₁⁺/− animals subjected to the same surgery, these differences were not significant (P > 0.05).

Additionally, using a competitive ELISA assay, we measured circulating plasma MBG levels in mice of both genetic groups subjected to either sham surgery or PNx. Plasma MBG concentrations were significantly elevated with PNx in both genetic groups versus sham-operated animals (P < 0.01). The level of PNx-induced increase in MBG did not significantly differ between WT and α₁⁺/− animals (Fig. 1E).

To test whether PNx also increased cardiac hypertrophy and fibrosis as we previously observed in rats, the hearts of experimental animals were collected and weighed. LV tissue was also fixed and stained with trichrome to detect fibrosis. As shown in Fig. 2A, hearts collected from PNx-operated animals of both genotypes were significantly larger (P < 0.01) than those collected from sham-operated animals, though the heart weight of α₁⁺/− mice subjected to PNx was smaller than that of the WT mice (159 ± 6 vs. 179 ± 9 mg). Cardiac hypertrophy, as measured by heart weight (HW)-to-body weight (BW) ratio, was significantly increased (P < 0.05) with PNx in both WT (4.91 ± 0.27 vs. 3.9 ± 0.10) and α₁⁺/− (4.64 ± 0.26 vs. 3.8 ± 0.06) mice versus sham-operated controls (Fig. 2B). Mice in both genotypes also displayed significantly (P < 0.01) more LV tissue, with WT PNx-operated animals having 34.1% more LV tissue by weight than sham-operated controls and α₁⁺/− mice having 22.1% more LV tissue versus sham-operated controls (Fig. 2C). Both WT and α₁⁺/− mice receiving PNx exhibit significant (P < 0.01) LV hypertrophy compared with sham-operated controls of the same genotype as indicated by the LVW-to-BW ratio (Fig. 2D). PNx also causes significant increase in fibrosis in both WT and α₁⁺/− mice (Fig. 2E).

PNx induces hypertrophy of cardiac myocytes in WT animals while inducing hyperplasia of cardiac cells in α₁⁺/− mice. Using WGA-based immunofluorescence to show the borders of individual cardiomyocytes in these tissues, we evaluated the cross-sectional area of individual myocytes in LV tissue sec-

**Fig. 4.** PNx induces increased nuclei numbers in α₁⁺/− mice. Left ventricle sections from each experimental group were stained with 4',6-diamidino-2-phenylindole (DAPI; blue) and cardiac troponin I (cTnI, red) as described in MATERIALS AND METHODS. A: representative images of DAPI and cTnI staining. Scale bar = 25 μm. B: quantification data of nuclei number by counting the DAPI-stained particles that overlaid with cTnI staining. C: quantification data for nuclei that are outside of the cTnI staining area. *P < 0.05, PNx vs. sham in α₁⁺/− animals.
tions of both WT and α1+/−, sham-operated and PNx-operated animals. After PNx, the mean area of cardiac myocytes in WT animals significantly increases (P < 0.001) by more than 76% versus sham-operated controls, whereas only 16% increase was observed in α1+/− mice (Fig. 3A). Of particular interest is the finding that the average myocyte size in WT animals and α1+/− mice receiving PNx are significantly different. WT cardiomyocytes average at 509.9 μm² after PNx, whereas those from α1+/− mice are 379.6 μm² (P < 0.001). A comparison of the distribution of the cell sizes for the WT and α1+/− animals reveals that the center of the distribution is shifted in the direction of larger cells with PNx WT animals (Fig. 3B), wherein the α1+/− distribution of the myocytes does not vary from that of sham-operated controls (Fig. 3C).

Since both WT and α1+/− mice exhibit significant cardiac hypertrophy, whereas only WT PNx-operated animals showed a dramatic increase in cell size, we also measured the cell number by counting the number of nuclei in tissue sections from the animals in this experiment. LV sections from these animals were costained with DAPI and cTnI. As shown in Fig. 4A, most DAPI-stained nuclei overlay with cTnI, whereas a small fraction are outside the cTnI staining area. Interestingly, both the nuclei that overlaid with cTnI (Fig. 4B) and those not costained with cTnI (Fig. 4C) are increased in α1+/− mice with PNx compared with their sham-operated controls. However, there is no significant difference between the WT PNx and sham-operated animals. These results indicate that both myocytes (indicated by nuclei overlaying with cTnI, Fig. 4B) and other types of cells (nuclei outside of cTnI staining area, Fig. 4C) may contribute to increased cell number in the heart tissue from α1+/− mice with PNx.

PNx induces myocyte apoptosis that fails to activate Akt/mammalian target of rapamycin (mTOR) survival signaling in α1+/− mice. Previous work by our laboratory and others has shown that reduction of Na/K-ATPase potentiates cell death in cardiac myocytes and in epithelial cells treated with CTS (33, 51). Because of the fact that Na/K-ATPase reduction has been reported in heart failure patients and that PNx induces significant increases in circulating CTS concentrations (2, 7, 17, 27), we sought to determine if PNx elicited increased cell death in α1+/− mice versus WT littermates subjected to the operation. To evaluate the role of cardiac cell death and the level of apoptotic activity in these animals, we used immunohistochemistry to measure the levels of active caspase 3 in heart

![Figure 5](http://ajpheart.physiology.org/)

**Fig. 5.** PNx induces apoptosis in α1+/− mice but not in WT mice. Active caspase-3 levels in mouse heart tissue were measured using immunohistochemical methods as described in MATERIALS AND METHODS. **A.** Left: representative ×20 photomicrographs of active caspase 3 in sham- or PNx-operated animals. **A.** Right: quantification data from 5 to 6 animals in each group. **B.** Left: representative terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) images from sham- and PNx-operated animals. **B.** Right: quantification of TUNEL-positive nuclei. Red arrows indicate TUNEL-positive nuclei. WT sham, n = 6; WT PNx, n = 5; α1+/− sham, n = 6; PNx+/−, n = 6. *P < 0.05, significantly different than sham; †significantly different than WT PNx. Scale bar is 60 μm.
tissues as previously described (33). Our analysis revealed that both WT and α₁⁺⁻ mice receiving sham surgery similarly displayed low levels of active caspase-3 expression, but the PNx surgery in α₁⁺⁻ mice induced significantly higher levels of active caspase 3 (Fig. 5A), indicating that reduction of α₁ potentiates cardiac cell death.

Additional evidence for increased cardiac cell death was observed in LV tissue sections by TUNEL assay. As shown in Fig. 5B, PNx increased TUNEL-positive nuclei in α₁⁺⁻ animals but not in WT animals, which is consistent with the current caspase-3 data and our previous publication (33).

To further study the mechanism of cardiac cell death, we examined Na/K-ATPase content and Akt/mTOR signaling in these mice. The Akt/mTOR pathway has been demonstrated to regulate survival signaling pathways that protect cells from death. We also illustrated in our previous study that reduction of Na/K-ATPase α₁-expression levels were about 40% less in α₁⁺⁻ mice hearts compared with the WT hearts, which is consistent with previous reports (33, 39). PNx further decreased the Na/K-ATPase α₁-levels in α₁⁺⁻ mice. To determine if cardio-protective signaling as mediated through the Akt/mTOR pathway was regulated in these animals, we carried out Western blot analyses of the levels of phosphorylation at residue serine-235/236 of ribosomal S6 protein (S6). S6 protein is a downstream member of the Akt/mTOR pathway and can be activated through phosphorylation by S6 kinase (19). As shown in Fig. 6B, we found that PNx elicits significantly increased levels of phosphorylated S6 protein in WT animals, whereas in α₁⁺⁻ animals there was no induction of S6 phosphorylation when the animals were subjected to PNx. Additionally, we assessed the activation of Akt, an upstream signaling component of S6, by examining the phosphorylation of the protein at two known phosphorylation sites, i.e., threonine-308 and serine-473. We found that in WT animals, PNx induces significant increases in phosphorylation of Akt at both sites (P < 0.01). In α₁⁺⁻ sham-operated animals, the level of Akt phosphorylation at these two sites is significantly higher than that observed in sham-operated WT animals. Interestingly, Akt phosphorylation was significantly downregulated after PNx versus sham-operated controls in α₁⁺⁻ mice (Fig. 6, C and D). These findings are consistent with our previous publication (33), which indicated that Na/K-ATPase reduction attenuates Akt/
mTOR survival signaling. Reduction of Na/K-ATPase increases c-kit cell abundance and proliferation in heart tissue. Our previous work (33, 51) and the above-mentioned data indicate that reductions in Na/K-ATPase α₁-subunit elicit significant increases in cardiac cell death, whereas the results from the current report show little difference in hypertrophy and diastolic dimension obtained with PNx in these mice. We postulated that the α₁⁻/⁻ mice must have a counteracting cellular program which compensates for the increase in cells lost to apoptosis. A pathway that could counteract cell death is the activation of proliferative/regenerative processes; as such, we examined whether reduction of α₁ resulted in an increase in cardiac regenerative activity of the heart. Cardiovascular tissues were probed with an antibody against a specific surface marker for cardiac progenitor cells, i.e., CD117 (c-kit). We found a significantly higher number of c-kit-positive cells in heart tissues isolated from α₁⁻/⁻ mice than those from WT animals (Fig. 7). Additionally, the number of proliferating (i.e., ki-67-positive) c-kit-positive cells also increased significantly with PNx versus sham surgery in animals of both genotypes, with α₁⁻/⁻ animals having a significantly higher number of proliferating cells than WT animals subjected to either sham or

Fig. 7. Heart tissues exhibit more c-kit-positive and ki-67-positive cells in α₁⁻/⁻ mice vs. WT mice. Left ventricle tissues fixed in 4% formaldehyde solution were used for immunofluorescent staining with anti-c-kit antibody and anti-ki-67 antibody as described in MATERIALS AND METHODS. Top: representative images of c-kit (red) and ki-67 (green) staining. DIC, differential interference contrast. Bottom: quantification data from 5–7 animals from each group. *P < 0.05 and **P < 0.01 vs. WT sham. Scale bar is 25 μm.
PNx surgery (Fig. 7). We further confirmed that the c-kit-positive cell population in the heart was composed primarily of cardiac progenitor cells by dual staining with c-kit and CD45. As shown in Fig. 8, 90% of the c-kit-positive cells were CD45 negative, indicating that they are not of hematopoietic origin.

The cytokine SCF binds to and initiates c-kit cell expansion and movement (35, 50). To evaluate whether the reduction in Na/K-ATPase 1-subunit and/or PNx altered heart tissue SCF levels, we used Western blot analysis to probe for SCF in cardiac tissues isolated from the experimental animals. As shown in Fig. 9A, cardiac tissue expression of SCF significantly increases with PNx in 1+/− animals but only slightly in WT animals. Additionally, tissue level expression of SCF is higher in the sham-operated 1+/− animals versus the WT sham-operated animals, but this higher level failed to reach the threshold for significance (Fig. 9A). Locally, the tissue level expression of SCF increased overall in the 1+/− animals with a significant increase occurring between PNx- and sham-operated animals (P < 0.01).

Circulating levels of SCF were also measured using a commercially available ELISA. As shown in Fig. 9B, there is a significant increase in the plasma levels of SCF with PNx in both WT and 1+/− animals (P < 0.01). Additionally, the level of SCF that is observed in 1+/− animals subjected to PNx was 80% higher than 1+/− sham-operated animals and 25% higher than the SCF levels observed in WT PNx-operated animals.

**DISCUSSION**

A major finding of this study is that reduction of the Na/K-ATPase 1-subunit results in increased cardiac cell death in PNx mice while also increasing cardiac progenitor cell abundance. Our data also suggest that reduction of this signaling subunit cause differential mechanisms of hypertrophy after PNx. In WT animals, hypertrophy is mostly attributed to the enlargement of individual cardiomyocytes, whereas when the 1-subunit of Na/K-ATPase is reduced, cardiac hypertrophy is more attributable to increases in cardiac cell number. Since Na/K-ATPase reduction is common in heart failure patients (2), it is clinically significant to further define the mechanisms regulating the balance between cardiac cell death and regeneration.

LV hypertrophy (23) and dilation (12, 34) are both prevalent in patients starting therapy for end-stage renal disease (ESRD), and both are markers of poor prognoses in dialysis patients (13, 41, 42, 49). We and others have demonstrated that Na/K-ATPase and its ligands are important regulators of cell survival and growth, which may contribute to hypertrophic growth or dilatation of the heart (20, 24, 51, 54). In our previous study using heterozygous knockout mice, we observed that genetic reduction of Na/K-ATPase stimulates the expression of pro-apoptotic proteins and potentiates CTS-induced cardiac cell death. It also caused decreased contractile function in these mice. More importantly, our results revealed that in normal cells, there exists a self-protective mechanism that preserves...
the membrane abundance of Na/K-ATPase and protects cells from death. Reduction of Na/K-ATPase attenuates the signaling function related with this self-protection (33). The current study further demonstrates that in chronic kidney disease induced by PNx, there was increased cardiac cell death and an attenuation of survival signaling when Na/K-ATPase is re-induced by PNx, whereas hyperplasia is increased in α1+/− mice. Since c-kit cells were reported to be surrounded by niches (10, 45), it is possible that the proliferation of c-kit cells was a second consequence which resulted from increased myocyte death but promotes c-kit cell abundance. The mechanism of this differential regulation on different cell type is not clear. Ellison et al. (8) demonstrated that c-kit cells are necessary and sufficient for functional cardiac regeneration and repair using rodent models of diffuse myocardial damage. However, Senyo et al. (48) used a pulse-chase method with stable isotope labeling and concluded that the division of preexisting cardiomyocytes rather than stem cells are the source for new heart cells. Our study observed increased cardiac cell numbers along with increased c-kit cells in Na/K-ATPase-deficient mice with PNx. Although no direct evidence showed that the increased c-kit cells differentiate into cardiac cells, it is conceivable that regenerative processes are activated in the α1+/− mice with PNx. This is further supported by increased total cell number in the heart tissue from these animals. Although our data did not directly demonstrate the increased cell number is originated from c-kit cells, it indicates that hyperplasia may be involved in cardiac hypertrophic growth in the α1+/− mice, which is different from the mechanism that causes hypertrophy in WT mice.

It is noted that Na/K-ATPase reduction potentiates myocyte death but promotes c-kit cell abundance. The mechanism of this differential regulation on different cell type is not clear. Since c-kit cells were reported to be surrounded by niches (10, 45), it is possible that the proliferation of c-kit cells was a second consequence which resulted from increased myocyte death. Previous reports have shown that acute myocardial infarction increases SCF expression in heart tissue, which induces c-kit cell proliferation and movement toward the injured area (32, 55). SCF is well documented to stimulate c-kit cells and regulate regenerative processes during heart tissue damage (22, 56). SCF is expressed in cardiac cells in two forms: the transmembrane form and soluble form found in the cytosol (1). It can also be secreted into blood and circulate to and penetrate other tissues. The molecular mechanism that regulates SCF secretion in injured tissue is not fully understood. However, hypoxic stress (28) and activation of the Akt pathway (15, 16) have been reported to increase SCF expression and secretion in mesenchmal stem cells. We measured SCF levels in LV tissue as well as in blood samples from these experimental mice. The results indicate that the level of SCF in heart tissue or in blood samples in α1+/− PNx mice is significantly higher than that of the sham-operated mice. It is also
higher than the WT PNx mice (Fig. 9), which may explain the higher number of c-kit-positive cells in α1/− mice. However, PNx surgery in the WT mice did not significantly increase the c-kit-positive cells despite the increase in SCF levels. These results indicate that SCF may not be the only factor that regulates c-kit cell abundance. It is also noted that c-kit cell number is more related with the tissue SCF level than the circulating levels. Taken together, the results show that Na/K-ATPase may be directly involved in the regulation of proliferative pathways in c-kit cells, while a determination of the specific mechanism(s) by which increased c-kit cell number and SCF levels are incurred, merits further studies.

In summary, we reason that the overall balance between survival signaling and apoptotic events in cardiac myocytes affects the fate of these cells in specific conditions of stress, while the balance of tissue injury and regenerative processes determine whether the affected organ can compensate and maintain relatively normal function in different disease states. It appears that reduction of Na/K-ATPase potentiates cell death but may also induce the activation of regenerative procedures in this organ as an adaptive mechanism. More study is needed to determine the specific pathways and signaling molecules that regulate this process.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
C.A.D., S.T.H., J.L., C.J.C., Z.X., J.I.S., and J.T. conception and design of manuscript; C.A.D. and J.T. edited and revised manuscript; C.A.D. and J.T. approved final version of manuscript.

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