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Corin-deficient W-sh mice poorly tolerate increased cardiac afterload

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Abstract

C57BL/6-Kit^{W-sh/W-sh} mice are generally regarded as a mast cell-deficient model, as they lack the necessary kit receptor for mast cell development. Further characterization of this strain, however, indicates that C57BL/6-Kit^{W-sh/W-sh} mice also have a disruption in the *Corin* gene. Corin is a transmembrane serine protease critical for processing atrial natriuretic peptide (ANP) from pro-ANP through proteolytic cleavage. Pro-ANP is produced, stored and released by cardiac myocytes in response to atrial stretch and the stress generated by increased afterload such as increased ventricular pressure from aortic stenosis or myocardial infarction. ANP inhibits the effects of the renin-angiotensin system to preserve homeostasis under conditions of increased hemodynamic load, and changes in the level of its activating enzyme Corin have been observed during the progression to heart failure.

Here, we investigate the effect of increased hemodynamic load on Corin-deficient C57BL/6-Kit^{W-sh/W-sh} mice. Ten-week old male mice were subjected to transverse aortic constriction for 8 wks and were monitored for changes in cardiac structure and function by echocardiography. Hearts were collected 8 wks after surgery for molecular and histological analyses.

Corin-deficient C57BL/6-Kit^{W-sh/W-sh} mice developed rapidly progressive and substantial left ventricular dilation, hypertrophy, and markedly impaired cardiac function during the 8 wks after surgery, compared to wildtype mice. Concomitant with this we observed increased levels of ANP transcript, but a lack of prepro-ANP or pro-ANP protein in heart tissue extracted from Corin-deficient mice. Surprisingly, fibrosis was not increased in Corin-deficient mice when compared to wildtype mice.

These data indicate that Corin's involvement in ANP processing is a key element in the heart's response to increased hemodynamic load. Further, C57BL/6-Kit^{W-sh/W-sh} strain is an effective model for investigating the involvement of Corin and, conversely, a less than optimal model for investigating mast cell, and immunological, functions in certain cardiovascular pathologies.

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Keywords

Corin; Cardiac hypertrophy; atrial natriuretic peptide; ANP; mast cell; W-sh mice

1.0 Introduction

Corin is the physiological pro-ANP convertase, responsible for the proteolytic maturation of pro-ANP to biologically active ANP [1]. The C57BL/6-Kit^{W-sh/W-sh} strain is generally regarded as a model for the disruption of mast cell development, nonetheless the *Corin* gene is also disrupted in this strain, and is believed to be the major cause of the phenotype seen in this study [2, 3].

ANP is synthesized in atrial myocytes as a propeptide, stored, and then released from intracellular granules after stimulation of the myocyte by stretch stress such as increased afterload. Immediate activation of pro-ANP to active ANP occurs by the transmembrane serine protease Corin. If ANP is not proteolytically cleaved by Corin, it remains biologically inactive in a pro-ANP form [4-6]. The process of producing active ANP is critical for maintaining hemodynamic balance through the regulation of blood pressure. In addition, ANP also inhibits myocyte growth, fibroblast proliferation and collagen deposition [7, 8] all through the natriuretic peptide receptor 1 (NPR1). Multiple studies have detected increased levels of circulating ANP isoforms in plasma samples from patients with congestive heart failure, and more recent clinical studies indicate that plasma concentrations of Corin, as well as processing of ANP, are decreased during end-stage heart failure [9-12]. These and other studies highlight the importance of Corin activity during the transition to heart failure, and led us to investigate the C57BL/6-Kit^{W-sh/W-sh} strain as a model for ANP dysregulation, through disruption of Corin, in cardiac pressure-overload.

We report that the Corin deficiency in C57BL/6-Kit^{W-sh/W-sh} mice leads to immediate significant cardiac hypertrophy and contractile dysfunction in response to pressure overload, but not exaggerated levels of fibrosis. It is unlikely that the cardiac hypertrophy and contractile dysfunction observed is a result of the Kit receptor mutation and lack of mast cells in the C57BL/6-Kit^{W-sh/W-sh} mice, as multiple studies have shown that cardiac mast cells participate in the induction, and not protection from cardiac hypertrophy [13, 14]. Although *in vivo* studies of ANP-null mice have shown exaggerated hypertrophy and fibrosis when exposed to pressure or volume overload for one week, the specific role of Corin in regulating fibrosis has not been investigated [15, 16]. Our findings indicate that Corin activity is critical in compensatory hypertrophy and regulating heart function prolonging the transition to heart failure.

2.0 Materials and methods

2.1 Animal preparations

Ten-week old male C57BL/6 (WT) and Corin-deficient C57BL/6-Kit^{W-sh/W-sh} (Wsh) [3, 8] (Jackson Labs) mice were used in this study. Mice were housed under a 12-h light/dark cycle and fed with standard diet and water *ad libitum*. Eight weeks after surgery mice were euthanized by CO₂ asphyxiation for histological and molecular analysis. All animal procedures were approved by the Institute of Animal Care and Use Committee at the University of Hawai'i.

2.2 Transverse Aortic Constriction (TAC)

TAC is a standard procedure for increasing left ventricular afterload, as previously described by Rockman *et al* [17, 18]. Briefly, the left side of the chest was depilated with Nair and a baseline 2-D echocardiogram was obtained as described below. Mice were then deeply anesthetized with a mixture of ketamine and xylazine. The transverse aorta between the brachiocephalic and left carotid arteries was banded using 6-0 silk ligature around the vessel and a blunt 26-gauge needle, after which the needle was withdrawn. Sham operated mice underwent identical procedure without constriction of the aorta.

2.3 Doppler Echocardiography

Doppler echocardiography was performed one-week post TAC to measure level of constriction under light Ketamine/Xylazine anesthesia. Doppler was performed using Visualsonics Vevo 770 system. In the parasternal short-axis view, the pulsed wave Doppler sample volume was placed in the transverse aorta just proximal and distal to the site of banding. Peak velocity was traced using Vevo 770 software and pressure gradient was calculated using the simplified Bernoulli equation.

2.4 Transthoracic echocardiography

Echocardiography was performed on all animals from each group. Unanaesthetized mice were handled in a supine position, depilated with Nair and echocardiography was performed using a Visualsonics Vevo 770 system and a 30-MHz probe. Measurements were taken from 2-D M-mode images of the left ventricle at the papillary muscles in both diastole and systole using the leading-edge-to-leading-edge convention adopted by the American Society of Echocardiography [19]. Systolic and Diastolic left ventricular internal diameter (LVID), left ventricular posterior wall (LVPW) dimensions were obtained from 2D images and percent ejection fraction (%EF) was measured from M-mode tracings.

2.5 Histology

Hearts were perfused with phosphate-buffered saline and 10% formalin in situ, then removed and fixed overnight in 10% formalin at 4°C. Hearts were subsequently embedded in paraffin and cut in sagittal sections that were mounted on glass slides and stored at -80°C until use. Collagen volume fraction was determined by analysis of picrosirius stained sections. Paraffin-embedded heart sections cut to 5µm in thickness were deparaffinized, stained with Weigert's Hematoxylin before staining with picrosirius red (0.1% Sirius Red in picric acid). Sections were subsequently washed and dehydrated before image analysis. To determine cardiomyocyte size, paraffin-embedded heart sections were deparaffinized and permeabilized before staining with wheat germ agglutinin (WGA). WGA-Alexa488 (Invitrogen) conjugate was used at a concentration of 50µg/ml to identify sarcolemmal membranes. Sections were counter stained with Hoechst nuclear stain (33342) at 1µg/ml and mounted with crystalmount. Sections were then measured for cardiac myocyte cross-sectional area (described below).

2.6 RT-PCR

For RNA extraction, hearts were collected from mice and total RNA was isolated from homogenized hearts with Trizol (Molecular Research Center) and further purified with a RNA isolation kit (Mo Bio Laboratories, Inc) according to the manufacturer's protocol. Single-stranded cDNA was synthesized from 1µg of total RNA using a cDNA synthesis kit (Qiagen). The mRNA levels of ANP, Corin, collagen I and cyclophilin (housekeeping gene) were quantified by RT-PCR in triplicate with QuantiTect SYBR Green (Qiagen, Hilden, Germany) in an Opticon device (MJ Research, Waltham, MA). RNA levels were quantitated relative to cyclophilin and/or sham-treated animals as internal controls. The following

primers were used, ANP: forward primer, 5'AGA AAC CAG AGA GTG GGC AGA G 3'; reverse primer, 5' CAA GAC GAG GAA GAA GCC CAG 3'; Collagen I: forward primer, 5' GAC CGA TGG ATT CCA GTT CG 3'; reverse primer, 5' TGT GAC TCG TGC AGC CAT CC 3'; Corin: forward primer, 5' GGG TTT CCT TCA GCG TTC GGG TCA 3'; reverse primer, 5' CAA CGC ACT CAG GGA AGG CGA G 3' and Cyclophilin: forward primer, 5' CAA AGT TCC AAA GAC AGC AGA AAA C 3'; reverse primer, 5' GGC ACA TGA ATC CTG GAA TAA TTC 3'.

2.7 Western blot analysis

Hearts were collected and protein extracts were prepared from homogenized heart tissue using lysis buffer. Total protein concentrations were determined by the bicinchoninic acid (BCA) colorimetric method and analyzed by spectrophotometer. Twenty µg of total protein was subjected to electrophoresis through sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE). Gel contents were transferred to polyvinylidene fluoride (PVDF) membranes at 1.4 amps for 3.5 hours. Membranes were probed overnight at 4°C with primary antibodies to ANP (Santa Cruz) and GAPDH (Calbiochem). Protein was visualized utilizing secondary antibodies designed for a Li-Cor Odyssey infrared scanner with the reagents and recommended protocol of the manufacturer (Li-Cor Biosciences).

2.8 Image Analysis

Fluorescent and bright field images from sham and TAC-treated hearts were obtained in a blinded fashion on a Zeiss Axioscope at 20×/40× magnification. Three heart sections from each animal were imaged at five images per section. Quantification of picrosirius staining was performed using Image J software (NIH) excluding perivascular tissue. Collagen fibers were highlighted to determine the pixel count in each field as a percentage of overall number of pixels for a ratio of red-stained collagen fiber to total tissue area. Cardiomyocytes from WGA stained sections were traced to determine area of individual myocytes, in a double blind manner. All other images were captured and analyzed in a single blind fashion.

2.9 Statistics

Statistical significance of echocardiography data was evaluated using 2-way ANOVA, Bonferroni post hoc test, and linear regression. Histology and molecular data were evaluated using 2-tailed Student's t-test. Evaluations were performed using PRISM software (La Jolla, CA) with $p < 0.05$ regarded as significant. We show data as mean $\pm$ SEM.

3.0 Results

Briefly, to investigate the effect of increased hemodynamic load on Corin-deficient C57BL/6-Kit^{W-sh/W-sh} mice, ten-week old male mice were subjected to transverse aortic constriction for 8 weeks and were monitored for changes in cardiac structure and function by echocardiography. Hearts were collected 8 wks after surgery for molecular and histological analysis. Data indicates that C57BL/6-Kit^{W-sh/W-sh} mice developed rapidly progressive and substantial left ventricular dilation, hypertrophy, and markedly impaired cardiac function during the 8 wks after surgery, compared to wildtype mice. Concomitant with this we observed increased levels of ANP transcript, but a lack of Corin processed ANP protein in heart tissue extracted from Corin-deficient mice. Fibrosis was not greater in Corin-deficient mice when compared to wildtype mice.

3.1 Confirmation of aortic constriction by Doppler echocardiography

The extent of aortic constriction was determined by Doppler echocardiography one-week after TAC. Peak velocity was measured immediately distal to the site of TAC. Pressure was

sufficient in TAC-treated mice to induce a hypertrophic response seen by significant changes in heart weight. Heart weight in TAC-treated mice was significantly greater in both WT ($p = 0.015$) and Wsh mice ($p = 0.001$) compared to sham-treated mice (Table 1).

3.2 Corin-deficient Kit^{W-sh/W-sh} mice

contain an inversion upstream of the kit regulatory site that disrupts *Corin* and the *c-kit* receptor [2, 3], and have been used as a model for investigating the effects of mast cell activation on health and disease [20, 21]. We confirmed undetectable levels of Corin mRNA in heart tissue of Wsh mice (Figure 1A). Conversely, levels of ANP mRNA from TAC-treated Wsh mice showed an increase in transcript levels of ANP compared to WT, but less in sham-treated mice (Figure 1B). Further, levels of ANP mRNA from TAC-treated Wsh mice showed more than a two increase in transcript levels of ANP ($p = 0.04$) compared to WT when using sham as an internal control (Figure 1C). Although it would appear that corin-deficiency regulates a significant change in ANP transcription in hearts from TAC Wsh mice, protein expression of pre and or pro-ANP (Figure 1D), indicates less than detectable concentrations of prepro-ANP and or pro-ANP protein in sham or TAC treated Wsh mice compared to Sham and TAC treated WT mice. It is unclear why this would occur, multiple factors could be speculated on, the Western blot sensitivity could be the major cause of this result, as previous studies have had to utilize HPLC and ELISA to detect pro-ANP in *Corin*^{-/-} mice [6].

3.3 Analysis of cardiac hypertrophy by heart weight

Eight weeks after TAC treatment the heart weight/body weight (HW/BW) ratio was 32.7% greater in TAC-treated Wsh mice compared to WT controls ($p = 0.02$) (Figure 2A). When heart weight was normalized to tibia length (HW/TL), a similar difference (33.1%) between TAC-treated WT and Wsh mice was also significant ($p = 0.015$) (Figure 2B). Additionally, HW/BW and HW/TL were significantly increased compared to sham-treated in both WT ($p < 0.05$) and Wsh mice ($p < 0.01$); a less severe hypertrophic response was seen in WT mice.

3.4 Corin-deficient Kit^{W-sh/W-sh} mice develop decompensated heart failure in response to pressure overload

The moderate pressure gradient obtained by 26 gauge-TAC impaired left ventricular (LV) function in Wsh mice and caused LV dilation while having less effect on WT mice. Moderate and severe LV dilation was observed in M-mode images in WT and Wsh mice at 8 wks post TAC (Figure 2C). Echocardiographic evaluation of the M-mode images from baseline to 8 wks showed LV dilation in Wsh mice beginning at 2 wks, which achieved statistical significance compared to WT controls at 6 wks (3.78 ± 0.25 , 3.13 ± 0.15) ($p = 0.03$) (Figure 2D). Sham-operated mice showed no significant change in LV dilation from baseline to 8 wks ($P = 0.583$); sham Wsh mice were significantly different from TAC Wsh mice at 6 and 8 wks ($p < 0.05$) (Figure 2D). Analysis of heart function, as measured by percent ejection fraction (%EF), indicated a decline in function starting at 2 wks in Wsh mice with a significant difference of 33.24% from WT mice at 8 wks post TAC ($p < 0.05$) (Figure 2E). Sham-operated WT and Wsh mice exhibited no change in LV function from baseline to 8 wks ($p = 0.767$); sham-operated Wsh mice were significantly different from TAC-treated mice at 6 and 8 wks ($p < 0.05$) (Figure 2E).

3.5 Hearts from Corin-deficient Kit^{W-sh/W-sh} mice develop enhanced cardiomyocyte hypertrophy

Cardiomyocytes in isolated heart sections from TAC-treated Wsh mice show dramatic signs of cellular hypertrophy (Figure 3A top). Quantification of myocyte area (pixel²) confirmed greater hypertrophy in TAC-treated mice compared to sham-treated mice in both Wsh (6764

± 237.3 , 3680 ± 192.7) ($p < 0.0001$) and WT mice (4991 ± 430.6 , 3312 ± 143.9) ($p < 0.01$); significantly more severe hypertrophy in Wsh mice 8 wks post TAC than in WT mice ($p = 0.007$) (Figure 3B).

3.6 Corin-deficient $\text{Kit}^{\text{W-sh/W-sh}}$ hearts have similar interstitial fibrosis compared to wild-type mice

Isolated heart sections stained red for collagen fibers did not show differences in collagen deposition between WT and Wsh mice 8 wks post TAC (Figure 3A bottom). The collagen fraction was greater in TAC-treated compared to sham-treated mice in both Wsh and WT mice ($p = 0.004$, $p < 0.0001$), although there was no significant difference between TAC Wsh and TAC WT mice (Figure 3B). RNA isolated from heart tissue confirmed transcript levels of collagen I were increased in TAC Wsh and TAC WT mice compared to sham (Figure 3C), and slightly higher in TAC-treated Wsh mice compared to WT. Further, there was no significant change in mRNA transcript levels of collagen between TAC-treated Wsh and WT hearts when using sham as an internal control (Figure 3D).

4.0 Discussion

Corin is critical for processing pro-ANP to active ANP in cardiac myocytes, and thereby regulates blood pressure and intravascular volume by natriuresis. In addition to the role that ANP has in endocrine regulation of heart function, it is an important inhibitor of myocyte growth via the natriuretic peptide receptor [7]. Here we show that Corin is essential in regulating compensatory hypertrophy and Corin-deficient $\text{Kit}^{\text{W-sh/W-sh}}$ mice are highly susceptible to cardiac hypertrophy and LV structural and functional changes in a model of cardiac pressure overload.

$\text{Kit}^{\text{W-sh/W-sh}}$ mice deficient in Corin and lacking active ANP exhibit rapid structural and functional changes in the myocardium represented by LV dilation and decreased fractional shortening (Figure 2). Compensatory hypertrophy during increased hemodynamic load is an important response to minimize cardiac stress and maintain cardiac function, yet the degree of cellular hypertrophy and cardiac dysfunction in Corin-deficient $\text{Kit}^{\text{W-sh/W-sh}}$ mice suggests that there is greater wall stress due to a disruption of ANP processing.

Previous studies of mice lacking Corin or ANP indicate they have increased body weight compared to WT mice [6], possibly due to salt and water retention in these mice. Here the mice lacking functional Corin exhibited a slight increase in body weight compared to WT mice (Table 1), and baseline echocardiographic data did not indicate a difference in cardiac function or structure in ten-week old mice. Therefore, the lack of Corin in $\text{Kit}^{\text{W-sh/W-sh}}$ mice does not present a cardiac phenotype until the heart is stressed.

Increased hemodynamic load causes myocyte growth and enlargement of the heart, in addition to remodeling of the myocardium with collagen deposition and matrix degradation [22]. Such remodeling can help to compensate for increased load; though a result of this adaptive response is increased wall thickness and contractile dysfunction. This increase in interstitial collagen may contribute to the 'tipping point' from compensatory hypertrophy to decompensation [22]. Active ANP is thought to be anti-fibrotic by inhibiting TGF- β -mediated deposition of extracellular matrix by cardiac fibroblasts [8]. The anti-fibrotic effect of active ANP was illustrated by a study in which fibrosis and expression of ECM proteins increased significantly one-week post TAC in ANP-null mice compared to controls [23, 24]. Here we show there is no significant difference in the level of fibrosis between TAC-treated WT and Corin-deficient $\text{Kit}^{\text{W-sh/W-sh}}$ mice. It is possible that at earlier time-points after banding $\text{Kit}^{\text{W-sh/W-sh}}$ mice may have shown differences in the level of interstitial collagen deposition; yet at 8 wks the amount of collagen deposition was no longer different.

Additionally, it is unknown whether ANP and TGF- β expression levels were temporally affected in this study. The role of Corin and the effect of the kit mutation on temporal and spatial expression of ANP and TGF- β and rate of collagen deposition may warrant further study.

We cannot rule out that elements of the pathology observed in the Kit^{W-sh/W-sh} mice might be due to disruption of the kit receptor. Other studies however, utilizing other mice with mast cell deficiency (W/W^v), and rats treated with inhibitors of mast cell degranulation, have shown that cardiac mast cells participate in the induction, and not protection, from cardiac hypertrophy and cardiac fibrosis. [13, 14]. This suggests that the Kit receptor mutation, and mast cell deficiency, in the Kit^{W-sh/W-sh} mice, is likely to be overwhelmed by the Corin deficiency, and not an observably contributing factor in the cardiac pathology observed here. In order to further study mast cell involvement, one might think of performing whole bone marrow, or *ex vivo* differentiated mast cell reconstitution in irradiated Wsh mice. A notable concern with a study involving whole bone marrow reconstitution is that mast cells would not be exclusively reconstituted, all leukocyte populations would be reintroduced. The reconstitution of *ex vivo* differentiated mast cells would also prove unsatisfactory, as this would not restore mast cell populations to all organs, notably the brain, spinal cord, lymph nodes, and most notably heart [25].

We conclude that Corin is necessary for appropriate myocardial compensation under conditions of increased cardiac afterload and the mutation in the Kit^{W-sh/W-sh} mouse makes it an appropriate model for the study of Corin deficiency, but a less than optimal model for investigating mast cell, and immunological, functions in cardiovascular pathologies that involve ANP and the renin-angiotensin system.

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Abbreviations

ANP	atrial natriuretic peptide
TAC	transverse aortic constriction

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Research Highlights

C57BL/6-KitW-sh/W-sh mice lack the Corin gene > Corin is needed for the processing of atrial natriuretic peptide to its active form > transverse aortic constriction of these mice leads to rapid progressive and substantial left ventricular dilation, hypertrophy, and impaired cardiac function.

Figure 1.

(A) RT-PCR analysis of mRNA for corin expression in WT and corin-deficient (Wsh) mice reported as fold change in TAC-treated mice relative to sham-treated mice. (B) RT-PCR analysis of mRNA for ANP expression in WT and Wsh mice normalized to cyclophilin housekeeping gene (C) RT-PCR analysis of mRNA for ANP expression for TAC-treated WT and Wsh mice using sham-treated mice as internal control (D) Example Western blot of pre and or pro-ANP illustrates a lack of pre and or pro-ANP in sham-treated Wsh mice (n=2) and TAC-treated Wsh mice (n = 3) compared to sham and TAC-treated WT (n = 2,3). GAPDH is used as a loading control.

Figure 2.

A) Heart weight/body weight ratio and **(B)** heart weight/tibia length ratio in WT and Wsh mice 8 wks post TAC ($n = 14,10$) compared to sham-treated mice ($n = 11,8$). **(C)** M-mode images of WT and Wsh mice at 8 wks post-TAC **(D)** Left ventricular internal diameter, diastolic (LVID, d) from baseline to 8 wks in sham-treated and TAC-treated WT and Wsh mice. Values are presented as means $\pm$ SEM. **(E)** Heart function measured by percent ejection fraction (%EF) from baseline to 8 wks in sham-treated and TAC-treated WT and Wsh mice.

Figure 3. (A) WGA (top) and picrosirius (bottom) stained heart sections from sham- and TAC-treated WT and Wsh mice, scale bar = 20 μm, (top) and 50 μm (bottom) (B) Quantification of cardiomyocyte cross sectional area (n=100) from WGA stained sections and percent collagen from picrosirius stained sections. (C) RT-PCR analysis of mRNA for Collagen I expression in sham- and TAC-treated heart tissue relative to the housekeeping gene, cyclophilin. (D) Fold change of collagen I mRNA transcripts for TAC-treated WT and Wsh mice using sham-treated mice as internal control.

Table 1

Peak pressure as measured by Doppler, heart weight, body weight and tibia length in sham and TAC-treated WT and Wsh mice.

	WT-Sham	Wt-TAC	W ^{sh} -Sham	W ^{sh} -TAC
n=	11	14	8	10
Peak Velocity (mm/sec)	935±14	3845±248	993±69	4093±125
Peak Pressure (mm Hg)	3.5±0.1	59.3±7.8	3.9±0.5	67.1±4.1
Heart Weight (mg)	141.1±13.3	195.7±66.3 *	158.8±9.7	294.5±101.7 **
Body Weight (g)	27.2±1.6	26.5±1.4	29.4±2.3	27.8±1.1
Tibia length (mm)	19.3±0.3	18.9±0.3	20.3±0.6	20.6±0.7

*
 $p < 0.05$

**
 $p < 0.01$ vs. sham.