Gap Junction Remodeling and Spironolactone-Dependent Reverse Remodeling in the Hypertrophied Heart

Jiaxiang Qu,* Frank M. Volpicelli,* Luis I. Garcia, Nefti Sandeep, Jie Zhang, Lucrecia Márquez-Rosado, Paul D. Lampe, Glenn I. Fishman

Abstract—Pressure overload is a common pathological insult to the heart and the resulting hypertrophy is an independent risk factor for sudden cardiac death. Gap junction remodeling (GJR) has been described in hypertrophied hearts; however, a detailed understanding of the remodeling process and its effects on impulse propagation is lacking. Moreover, there has been little progress developing therapeutic strategies to diminish GJR. Accordingly, transverse aortic banding (TAC) was performed in mice to determine the effects of progressive pathological hypertrophy on connexin (Cx)43 expression, posttranslational phosphorylation, gap junction assembly, and impulse propagation. Within 2 weeks after TAC, total and phospho-Cx43 abundance was reduced and incorporation of Cx43 into gap junctional plaques was markedly diminished. These molecular changes were associated with progressive slowing of impulse propagation, as determined by optical mapping with voltage-sensitive dyes. Treatment with the aldosterone receptor antagonist spironolactone, which has been shown to diminish sudden arrhythmic death in clinical trials, was examined for its effects on GJR. We found that spironolactone blunted the development of GJR and also potently reversed established GJR, both at the molecular and functional levels, without diminishing the extent of hypertrophy. These data suggest a potential mechanism for some of the salutary electrophysiological and clinical effects of mineralocorticoid antagonists in myopathic hearts. (Circ Res. 2009;104:365-371.)

Key Words: gap junction ■ spironolactone ■ arrhythmias ■ optical mapping ■ mouse ■ connexin

Hemodynamic overload is a commonly encountered pathological cardiovascular stimulus, and the associated hypertrophy has long been considered an independent risk factor for cardiac mortality, including sudden cardiac death.1–6 The effects of hypertrophic stimuli on gap junction expression and function have been studied in a number of experimental systems, including cultured neonatal myocytes, animal models, and human pathological specimens (reviewed elsewhere7,8). In neonatal cardiac myocytes, connexin (Cx)43 gap junction protein expression increases in response to acute administration of hypertrophic agents such as angiotensin II and cAMP.9,10 However, in vivo, both in experimental animals and in humans, prolonged hemodynamic overload is more commonly associated with significant downregulation of Cx43 expression, as well as lateralization of gap junctional protein away from the intercalated disks, ie, with gap junction remodeling (GJR).11–14 Interestingly, in a study of volume overload–induced hypertrophy, a transient upregulation of Cx43 was observed within hours after imposition of the hemodynamic load, followed by significant reduction in myocardial Cx43 content.15 Taken together, these in vitro and in vivo data suggest that GJR may be biphasic, although the second phase, characterized by diminished density of junctional channels, may be more clinically relevant.

In the present study, we used the well-characterized transverse aortic constriction (TAC) model to evaluate GJR and impulse propagation during progressive cardiac hypertrophy in intact hearts. Our results suggest that GJR significantly diminishes conduction velocity, although not sufficiently to support inducible arrhythmias. Altered post-translational phosphorylation of Cx43 was observed within days after imposition of TAC, suggesting that aberrant processing of this gap junctional protein is an essential element in the remodeling process. We found that treatment with the mineralocorticoid antagonist spironolactone blunts the development of pathological GJR and also potently reverses established GJR, both at the molecular and functional levels, without diminishing the extent of hypertrophy.

Materials and Methods

Animal Model

All animal procedures were carried out in accordance with Public Health Service Guidelines for the Care and Use of Laboratory Animals and approved by the New York University School of
Echocardiography

Left ventricular dimensions and function were assessed by echocardiography as previously described using an ATL 5000CV Ultrasound System (Philips Medical, Bothell, Wash).

Fibrosis Index

Formalin-fixed, paraffin embedded sections were stained with Mason’s trichrome and analyzed with Image-Pro Plus 5.0 software (Media Cybernetics, Bethesda, Md) as previously described.

Western Blot Analysis

Total protein lysates and Triton X-100 insoluble pellet fractions were prepared from the apical two-thirds of the ventricle as previously described. Primary antibodies included rabbit polyclonal 18B, which recognizes all forms of Cx43 and is directed toward an epitope from the carboxyl terminus; mouse monoclonal Cx43NT1, which also recognizes all forms of Cx43 but is directed toward an epitope from the amino terminus; rabbit antiS325/328/330-Cx43 and rabbit antiP365-Cx43 antibodies, which recognize specific phosphorylated forms of Cx43. Immunoblotting was verified by probing for vinculin for total lysates or by Ponceau staining of the membrane for Triton X-100 pellets. Signals were visualized and quantified using the Odyssey Imaging System (Li-Cor, Lincoln, Neb).

Immunohistochemistry

Staining was performed on formalin-fixed, paraffin-embedded hearts using either fluorescein isothiocyanate or peroxidase-conjugated secondary antibodies as previously described.

Heart Isolation and Optical Mapping

High-resolution optical mapping experiments were performed as previously described, using either a charge-coupled device camera (Dalsa, CAD-128, Waterloo, Calif) or, in more recent experiments, a newer generation CMOS video camera (Ultima-L; SciMedia Inc.). Studies were performed in the absence of any pharmacological or mechanical motion-reduction techniques. Epicardial conduction velocity (CV) measurements were obtained from the left ventricular surface, with only those pixels residing between 1 and 3 mm from the stimulation site being included in the analyses. Because of the improved robustness of the signal, we focused primarily on measurements of CVmin although CVmax values are reported as well. For studies of pharmacological gap junction uncoupling, after baseline parameters were determined, hearts were perfused with 18-glycyrrhetinic acid (GA) (7.5 μmol/L) for 15 minutes (Sigma-Aldrich), and CV measurements were repeated.

Programmed Electric Stimulation

Ventricular effective refractory periods (VERPs) of isolated-perfused hearts were calculated with a standard S1S2 protocol at a basic cycle length of 100 ms, with the introduction of progressively premature S2 stimuli at 2-ms intervals. The ERP was defined as the longest coupling interval that failed to capture. Following determination of VERP, arrhythmia susceptibility was assessed by provocative testing using single and double extrastimuli, as previously described.

Statistical Analysis

Data are presented as means±SEM unless otherwise indicated. Probability values less than 0.05 were considered statistically significant.

Results

Progressive Hypertrophy With TAC

TAC resulted in an initial phase of cardiac hypertrophy followed by the development of left ventricular dilatation and deterioration in contractile function, as determined by serial echocardiographic studies. The gross appearance of the heart at 2, 4, and 8 weeks after aortic constriction is shown in Figure 1A. Progressive cellular hypertrophy was also observed; by 8 weeks after TAC, there was an increase of 31% in cell length and an increase of 56% in cell width. These data are summarized in Tables 1 and 2.

GJR and Altered Cx43 Phosphorylation Status

To determine whether cardiac hypertrophy was associated with GJR, including changes in posttranslational phosphorylation.

Table 1. Gross and Cellular Hypertrophy in TAC Mice

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Four Weeks</th>
<th>Eight Weeks</th>
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<tbody>
<tr>
<td>Heart weight, mg</td>
<td>139±5 (12)</td>
<td>282±11 (13)*</td>
<td>324±9 (11)†</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>27.9±0.8 (12)</td>
<td>24.1±0.9 (13)</td>
<td>28.5±0.7 (11)</td>
</tr>
<tr>
<td>HW/BW, mg/g</td>
<td>5.00±0.16 (12)</td>
<td>11.81±0.55 (13)*</td>
<td>11.5±0.48 (11)</td>
</tr>
<tr>
<td>Cell length, μm</td>
<td>158±3 (55)</td>
<td>192±4 (55)*</td>
<td>206±5 (55)*</td>
</tr>
<tr>
<td>Cell width, μm</td>
<td>24.7±0.7 (55)</td>
<td>39.2±1.0 (55)*</td>
<td>38.5±1.3 (55)*</td>
</tr>
<tr>
<td>Cell area, μm²</td>
<td>3934±155 (55)</td>
<td>7528±237 (55)</td>
<td>7873±268 (55)</td>
</tr>
</tbody>
</table>

All values are means±SEM. Numbers in parentheses indicate sample size. *P<0.05 compared to sham, †P<0.05 compared to sham and 4 weeks.
antibodies specific for pS365-Cx43 and pS325/328/330-Cx43

Concordant with the Western blot studies, immunostaining with
gap junctional plaques within 1 week after TAC (Figure 1C).

chemical studies, which revealed substantial loss of Cx43
within a week after imposition of TAC.

tion in both pS365, as well as pS325/328/330 forms of Cx43
phospho-specific antibodies demonstrated a substantial diminu-
/junctional Cx43 abundance following TAC, diminishing to
pan-Cx43 antibodies demonstrated a progressive decline in
junctional membrane proteins. Both N-terminal and C-terminal
TAC on Cx43 within the gap junctions, we performed Western

We next determined whether the changes in Cx43 expression
Impulse Propagation in Hypertrophied Hearts

These results were confirmed and extended by immunohisto-
chemical studies, which revealed substantial loss of Cx43
gap junctional plaques within 1 week after TAC (Figure 1C).
Concordant with the Western blot studies, immunostaining with
antibodies specific for pS365-Cx43 and pS325/328/330-Cx43
also demonstrated a substantial loss of these posttranslationally
modified forms of Cx43, as seen in Figure 2.

Impulse Propagation in Hypertrophied Hearts
We next determined whether the changes in Cx43 expression
were accompanied by alterations in impulse propagation,
using optical mapping techniques. We observed progressive
slowing of transverse conduction velocity (CV_{min}) following
the imposition of TAC, as illustrated in Figure 1D and
summarized in Table 3. As early as 1 week after TAC, CV_{min}
had diminished significantly from 0.55±0.02 to 0.47±0.02
m/sec, and by 8 weeks after imposition of the hemodynamic
overload, CV_{min} had fallen to 0.42±0.03 m/sec. To gain some
insight into the extent of residual cell–cell coupling that
existed in hypertrophied hearts, we assessed the effects of the
relatively selective gap junction uncoupler αGA on impulse
propagation in an additional cohort of hearts studied 4 weeks
after imposition of TAC. Treatment of hypertrophied hearts
with αGA depressed CV further, to 0.25±0.01 m/sec. These
data suggest the presence of some degree of residual gap
junction function, despite the extensive GJR that occurs in
response to pressure overload.

Inasmuch as cardiac hypertrophy in many animal models is
associated with action potential prolongation, we also mea-
sured ventricular effective refractory periods. VERPs in
hearts from 4 week TAC animals were significantly pro-
longed compared to sham operated controls (57.4±3.6 ms
(n=9) versus 42.7±2.7 ms (n=9); P=0.005), suggesting
electric remodeling of repolarizing currents and consistent
with previous reports.25

We next assessed whether hypertrophied hearts were more
susceptible to the induction of ventricular arrhythmias. Some-
what surprisingly, none of the 9 hearts isolated from 4 week

<table>
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<th>Table 2. Echocardiographic Analysis of TAC Mice</th>
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<tr>
<td>Baseline</td>
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<tr>
<td>---------</td>
</tr>
<tr>
<td>Sham (21)</td>
</tr>
<tr>
<td>LVAW, mm</td>
</tr>
<tr>
<td>LVAW_m, mm</td>
</tr>
<tr>
<td>LVPW, mm</td>
</tr>
<tr>
<td>LVPW_m, mm</td>
</tr>
<tr>
<td>LVID, mm</td>
</tr>
<tr>
<td>LVID_m, mm</td>
</tr>
<tr>
<td>FS, %</td>
</tr>
</tbody>
</table>

All values are means±SEM. LVAW, LV anterior wall; LVPW, LV posterior wall; LVID, LV internal dimension; d, diastole; s, systole; FS, fractional shortening. *P<0.05 compared to age-matched sham. Numbers in parentheses indicate sample size.

Figure 2. Aberrant phosphorylation of Cx43 in TAC mice. Sections were prepared at the indicated time points and stained with antibodies recognizing all forms of Cx43 (Total Cx43), S365-phosphoCx43 (pS365), or S325/328/330-phosphoCx43 (pS325). Diminution of both phosphorylated forms of Cx43 is evident as early as 1 week post-TAC. Scale bar=50 μm.
TAC mice developed ventricular arrhythmias in response to programmed electric stimulation.

**Inhibition of Pathological GJR With Spironolactone**

Aldosterone antagonists have been shown to reduce sudden cardiac death in clinical trials of myopathic patients. We therefore explored whether treatment with spironolactone might influence the extent of pathological GJR observed in response to pressure overload. Accordingly, we examined additional cohorts of mice subjected to either sham operation or TAC, with half of each group then randomized to receive either a normal chow diet or chow supplemented with spironolactone (50 mg/kg per day) for a period of 4 weeks. Interestingly, there was no effect of TAC or spironolactone on Cx43 mRNA expression (Figure 3A). Moreover, spironolactone did not diminish the overall hypertrophic response, although there was a demonstrable reduction in the extent of cardiac fibrosis (Table 4). However, compared to TAC mice receiving a normal chow diet, those treated with spironolactone showed a significant increase in the abundance of slower mobility, hyperphosphorylated forms of Cx43 (Figure 3B and Table 4), as well as a marked increase in typical gap junctional plaques (Figure 3C). The normalization of gap junctional appearance was also observed using antibodies specific for pS365-Cx43 and pS325/328/330-Cx43 (Figure 4). These molecular changes indicative of reverse remodeling were associated with a significant improvement in conduction velocity, as shown in Figure 3D and summarized in Table 4.

**Reversal of Pathological GJR With Spironolactone**

The previous experiment established that treatment with spironolactone could blunt the development of pathological GJR. We next addressed the clinically more relevant question of whether mineralocorticoid receptor antagonism could reverse the severity of GJR in hearts with established pathology. Therefore, an additional cohort of mice was subjected to TAC. Two weeks later, at which time substantial hypertrophy

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**Table 3. Conduction Parameters in TAC Mice**

<table>
<thead>
<tr>
<th>Time Course Study</th>
<th>GA Study</th>
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<tbody>
<tr>
<td>Sham</td>
<td>Four Weeks Without GA</td>
</tr>
<tr>
<td>One Week</td>
<td>Four Weeks</td>
</tr>
<tr>
<td>CV&lt;sub&gt;min&lt;/sub&gt;, m/sec</td>
<td>0.55±0.02 (9)</td>
</tr>
<tr>
<td>CV&lt;sub&gt;max&lt;/sub&gt;, m/sec</td>
<td>0.84±0.04 (9)</td>
</tr>
<tr>
<td>AR</td>
<td>1.54±0.07 (9)</td>
</tr>
<tr>
<td>VERP, ms</td>
<td>42.7±2.7 (9)</td>
</tr>
</tbody>
</table>

All values are means±SEM. *P<0.05 compared to sham, †P<0.05 compared to 4 weeks without GA. Numbers in parentheses indicate sample size.

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**Figure 3. Inhibition of GJR with spironolactone.** Groups of mice were subjected to either sham operation (S) or TAC (T) and fed either normal chow (–SPI) or chow supplemented with spironolactone (+SPI). Analyses were performed 4 weeks after surgery. A. Northern blot analysis demonstrates no changes in Cx43 mRNA abundance. B. Western blot analysis with a panCx43 antibody demonstrates increased slow-mobility Cx43 in both S+SPI and T+SPI hearts. C. Representative immunofluorescent staining demonstrates loss of Cx43 gap junction plaques in TAC–SPI hearts but substantial improvement in mice treated with spironolactone (TAC+SPI). D. Representative optical maps from each of the 4 groups. E. Representative immunofluorescent staining in reversal experiment demonstrates improvement after 2 weeks of treatment with spironolactone. F. Representative optimal maps from reversal experiment.
Enhanced Cx43 phosphorylation with spironolactone. Sections were prepared from each of the experimental groups 4 weeks after randomization and stained with antibodies recognizing all forms of Cx43 (Total Cx43), S365-phosphoCx43 (pS365), or S325/328/330-phosphoCx43 (pS325). Representative images are shown. Reduced immunoreactive Cx43 is seen in the TAC–SPI hearts with all 3 antibodies. Spironolactone treatment partially restores the appearance of the gap junctional plaques. Scale bar=50 μm.

Discussion

Impulse propagation reflects a complex interplay between structural factors and active and passive membrane properties. In the setting of pathological stressors, structural and electric remodeling occurs, leading to the formation of a substrate that may be susceptible to the initiation and maintenance of ventricular tachyarrhythmias. In this study, we evaluated the effects of pressure overload, a commonly encountered pathological stimulus, on GJR and impulse propagation. The major findings in this study include the following: (1) pressure overload hypertrophy is associated with early and progressive reduction in junctional Cx43; (2) dephosphorylation of Cx43 at serines 365 and 325/328/330 occur contemporaneously with this loss of junctional Cx43; (3) impulse propagation slows significantly during progressive hypertrophy; (4) mineralocorticoid receptor blockade blunts the development of GJR associated with pathological cardiac hypertrophy, both at the molecular and functional levels; and (5) mineralocorticoid receptor blockade potently reverses established GJR in hypertrophied hearts.

Despite evidence of substantial GJR following TAC, the hypertrophied hearts were not especially sensitive to the induction of ventricular arrhythmias. This behavior contrasts with several genetic models of uncoupling in which Cx43 expression is more profoundly reduced. At the extreme end of the spectrum are the Cx43 conditional knockout mice, in which spontaneous arrhythmias are fully penetrant; less fulminant are the heterozygous I130T ODDD mutant mice,22 with Cx43 levels ~10% of normal and easily inducible but without spontaneous arrhythmias. The TAC mice display an even milder phenotype, and despite evidence of substantial GJR, the incremental slowing of CV after infusion with GA suggests the presence of residual gap junction function. In addition, it is conceivable that the choice of strain used in this study reduced the arrhythmic phenotype; C57BL/6 mice are relatively resistant to induction of ventricular tachycardia.26

Our data are consistent with previous studies demonstrating aberrant posttranslational phosphorylation of Cx43 in response to pathological stimuli, including models of nonischemic heart failure in the rabbit and pacing-induced heart failure in the dog.27–30 With respect to the molecular mecha-
functions of GJR, through the use of phospho-specific antibodies, we identified at least 2 regions, serine 365 and a triplet of serines at 325, 328, and 330, within the Cx43 carboxyl terminus that are dephosphorylated as early as 1 week after imposition of hemodynamic overload. Phosphorylation at serine 365 causes a shift in Cx43 mobility to the P1 form and is thought to play a gatekeeper role preventing downregulation via protein kinase C phosphorylation at serine 368. Nonetheless, additional studies will be required to improve CV through an increase in sodium current.

Mineralocorticoid blockade with spironolactone is not likely to improve CV through an increase in sodium current. Nonetheless, additional studies will be required to improve CV through an increase in sodium current.

In summary, our data demonstrate that hemodynamic overload leads to substantial GJR and that spironolactone potently blunts the development of this pathological remodeling, as well as reverses, established GJR. Posttranslational phosphorylation of Cx43 appears to play an integral role in the regulation of gap junction formation as well as pathological remodeling. We suggest that modulation of gap junction regulation may represent a rational antiarrhythmic therapeutic target.

Sources of Funding
This study was supported by NIH grants HL64757, HL82727, and HL81336 (to G.I.F.) and GM55632 (to P.D.L.); a Glorney-Raisbeck fellowship (to L.I.G.); a Sarnoff Cardiovascular Research fellowship (to F.M.V.); and a Howard Hughes Medical Institute medical student fellowship (to N.S.).

Disclosures
None.

References


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Circ Res. 2009;104:365-371; originally published online December 18, 2008;
doi: 10.1161/CIRCRESAHA.108.184044
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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