Internalization and Dephosphorylation of Connexin43 in Hypertrophied Right Ventrices of Rats With Pulmonary Hypertension

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Background Altered expression and distribution of gap junctions might provide substrates for abnormal conduction and arrhythmogenesis in the heart, but little is known about the regulation of gap junctions under pathological conditions. The organization and phosphorylation state of connexin43 (Cx43) in ventricular hypertrophy will be investigated.

Methods and Results Right ventricular (RV) hypertrophy was induced in rats by treatment with monocrotaline. Subcellular Cx43 distribution was assessed by immunofluorescence and electron microscopy. Immunolabeling of Cx43 was confined to the intercalated disks in the normal ventricular myocytes of control rats, but hypertrophied RV cells from monocrotaline-treated rats showed dispersion of Cx43 immunolabeling over the cell surface and in the cytoplasm; cytoplasmic Cx43 was increased by ~7-fold (n=15). The Cx43 internalization was confirmed by the double staining of monocrotaline-treated RV tissues for Cx43/wheat germ agglutinin (WGA) and Cx43/zonula occludens protein-1 (ZO-1). Electron microscopy of hypertrophied RVs showed an increase in annular gap junctions immunolabeled with Cx43. Immunoblotting revealed a significant increase in non-phosphorylated Cx43 in hypertrophied RVs (by ~5-fold, n=8) without changes in the total amount of Cx43. The accumulation of non-phosphorylated Cx43 in hypertrophied RVs was also recognized by immunofluorescence-microscopy with an isoform-specific antibody.

Conclusion Ventricular hypertrophy is associated with the dephosphorylation of Cx43 and its translocation from the intercalated disks to intracellular pools, suggesting accelerated gap junction degradation. (Circ J 2007; 71: 382 – 389)

Key Words: Connexin43; Gap junction; Hypertrophy; Phosphorylation/dephosphorylation; Remodeling
Methods

Animals

The present study experimental protocol was approved by the Animal Research Committee of the Research Institute of Environmental Medicine, Nagoya University, and all procedures were conducted in accordance with the statutory Japanese regulations.

In rats, a parenteral dose of MCT is known to cause pulmonary hypertension within a few weeks and the pressure overload results in right ventricular (RV) hypertrophy, leading to right-sided congestive heart failure within several weeks. In the present study, MCT dissolved in water was injected (60 mg/kg) intraperitoneally into 5-week-old Wistar rats. Saline-injected rats served as controls. Animals were killed 4 weeks after the injection. RV hypertrophy was evaluated by the heart-to-body weight (HW/BW) ratio and the tissue weight ratio of the RV free wall to the left ventricular (LV) free wall plus the interventricular septum (IVS) (RV/[LV+IVS]). We also estimated cell hypertrophy; single cardiomyocytes were enzymatically isolated from both ventricles and the cell dimensions were measured.

Antibodies

In the present study, 3 different antibodies for Cx43 were used. A mouse monoclonal antibody (Chemicon) and a rabbit polyclonal antibody (Zymed) were used to recognize total Cx43 (both phosphorylated and non-phosphorylated isoforms). A mouse monoclonal antibody (Zymed) was used to recognize selectively the non-phosphorylated isoform of Cx43. The specificity of this antibody has been extensively characterized previously by Nagy et al. To detect zonula occludens protein-1 (ZO-1), a PDZ domain-containing protein localized at the intercalated disks, a rabbit polyclonal anti-ZO-1 antibody (Zymed) was used.

Immunofluorescent Microscopy

Single cardiomyocytes were isolated from both ventricles and fixed with 2% paraformaldehyde (PFA). In addition, cryosections (8 μm thick) of ventricles were prepared from frozen samples fixed with 4% PFA. The samples (single cells and tissue sections) were immunolabeled with the Chemicon mouse monoclonal anti-Cx43 antibody (1:200) by the methods similar to those described previously. Primary antibody-bound Cx43 complexes were detected by fluorescent isothiocyanate (FITC)-conjugated anti-mouse IgG. In some experiments, tissue sections were double immunolabeled with a mixture of the Zymed mouse monoclonal (1:200) and Zymed rabbit polyclonal (1:200) anti-Cx43 antibodies. Primary antibody-bound non-phosphorylated Cx43 complexes were detected by FITC-conjugated anti-mouse IgG, while total Cx43 complexes were detected by using biotinylated anti-rabbit IgG and Texas Red-conjugated streptavidin.

For double labeling of Cx43 and ZO-1 in tissue samples, a mixture of Chemicon mouse monoclonal anti-Cx43 antibody (1:200) and the rabbit anti-ZO-1 antibody (1:200) was used in combination with appropriate fluorescent secondary antibodies. Samples processed without primary antibody served as negative controls.

Wheat germ agglutinin (WGA) binds to N-acetyl-d-glucosamine and sialic acid in the glycocalyx covering the external surface of the cell membrane. To specify the subcellular localization of Cx43, some RV tissue sections, which had been labeled with the Chemicon mouse monoclonal anti-Cx43 antibody, were treated with rhodamine-
Table 1 RV Hypertrophy in MCT-Treated Rats

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<th>Control</th>
<th>MCT</th>
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<tr>
<td>n</td>
<td>34</td>
<td>28</td>
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<tr>
<td>BW (g)</td>
<td>305.3±4.1</td>
<td>245.7±3.7*</td>
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<tr>
<td>HW (g)</td>
<td>1.17±0.03</td>
<td>1.22±0.04</td>
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<tr>
<td>HW/BW (%)</td>
<td>0.39±0.01</td>
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<tr>
<td>RV/(LV+IVS) (%)</td>
<td>24.1±0.6</td>
<td>44.8±0.9*</td>
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RV, right ventricle; MCT, monocrotaline; BW, body weight; HW, heart weight; LV, left ventricle; IVS, interventricular septum. *p<0.01 vs the control.

Fig 2. Quantitative analysis of Connexin43 (Cx43) immunolocalization in right ventricular (RV) myocytes. The Cx43 domain on the lateral sarcolemma was estimated from 2 confocal optical slices sectioned at the top and bottom surface of each myocyte (A). The Cx43 domain in the cytoplasm was estimated from the remaining optical slices and their projection images (B). *p<0.01 vs the control.

coupled WGA (Vector, Burlingame).

Series of confocal optical slices were obtained at 0.5 μm intervals from the bottom to the top surface of isolated myocytes, and a projection image was constructed from these optical slices covering the whole thickness of each myocyte (20–33 images). For quantitative analysis, immunolabeled Cx43 domains were classified into 3 categories: intercalated disk, lateral sarcolemma and cytoplasmic domains, according to their subcellular localization. Clusters or aggregates of multiple punctuate Cx43 immunolabeled domains running across the longitudinal axis either at the cell termini or close to the sites of bifurcations were defined as intercalated disk domains (arrows in Fig 1). Scattered fluorescent domains distant from the intercalated disk regions on the top and bottom surface were recognized as lateral sarcolemma domains (solid arrowheads in Fig 1). The remaining widely dispersed domains distant from the intercalated disks and the lateral sarcolemma on the optical slices other than the top and bottom cell surface were recognized as cytoplasmic domains (open arrowheads in Fig 1). The areas occupied by each group of domains were estimated on confocal optical planes of the top and bottom surface as well as on the total projection image using NIH Image software.

Immunoblotting

The amount of Cx43 was evaluated by the immunoblotting of RV homogenates using Zymed rabbit polyclonal and Zymed mouse monoclonal antibodies. The intensity of Cx43 bands was quantified by densitometry and normalized to that of actin.

Electron Microscopy

For conventional transmission electron microscopy, ventricles were fixed with 2% PFA/2.5% glutaraldehyde and then processed by using a standard method. For immunogold electron microscopy, ventricles fixed with 2% PFA/0.1% glutaraldehyde were frozen in liquid nitrogen and ultra-thin cryosections (80–90 nm thick) were incubated with the Chemicon mouse monoclonal anti-Cx43 antibody and 10 nm-gold anti-mouse complexes. Ultra-thin sections were examined by a JEOL-1210 electron microscope.

Statistical Analysis

Data are expressed as mean±SE and were analyzed by using an unpaired t-test. A value of p<0.05 was considered to be statistically significant.

Results

RV Hypertrophy in MCT-Treated Rats

An intraperitoneal injection of MCT resulted in RV hypertrophy in rats. Table 1 summarizes animal characteristics 4 weeks after the MCT injection. The BW was significantly less in MCT-treated rats than in controls, whereas the HW of MCT-treated rats was similar to controls. The HW/BW ratio and the RV/(LV+IVS) ratio were significantly larger in MCT-treated rats than controls. There were no appreciable physical signs of right-sided congestive heart failure in the MCT-treated rats at this stage. Hypertrophy of RV myocytes of MCT-treated rats was confirmed by an increase in cell dimensions: RV myocytes obtained from MCT-treated rats were significantly wider and considerably longer than controls. The cell dimensions of LV myocytes (both cell width and cell length) from MCT-treated rats were comparable to controls. These observations are consistent with our earlier study.

Distribution of Immunolabeled Cx43 in Hypertrophied Ventricles

Ventricular myocytes isolated from control and MCT-treated rats were immunolabeled with a Chemicon mouse monoclonal anti-Cx43 antibody to study the localization of gap junctions. Representative confocal images are shown in Fig 1; top panels are from a normal RV myocyte of a control rat, whereas bottom panels are from a hypertrophied RV myocyte from a MCT-treated rat. The left 6 images (A through C, E through G) represent single optical slices at the top surface (A, E), midway (B, F) and bottom surface (C, G) in the respective myocytes. Right end panels (D, H) are composite projection images constructed by the superimposition of a series of single optical slices (0.5 μm interval) to cover the full thickness of the respective myocytes (22 slices for the control and 29 for the MCT myocyte). In the control myocyte, Cx43 immunolabeled punctuate domains were almost exclusively confined to the intercalated disks at the cell termini or at the sites of bifurcations of the main cell body (arrows), and were rarely observed on the...
Lateral surface (solid arrowheads) and in the cytoplasm (open arrowheads). Such a distribution pattern of Cx43 was common to all RV and LV myocytes (n=15, and n=10) isolated from 5 control rats. In the hypertrophied RV myocytes, unlike controls, Cx43 immunolabeled spots were not confined to the intercalated disks, but widely distributed on the lateral surface (solid arrowheads) as well as in the cytoplasm (open arrowheads). All the hypertrophied RV myocytes (n=15) from 6 MCT-treated rats showed the similar dispersed pattern of Cx43 distribution. In contrast, non-hypertrophied LV myocytes (n=10) from the same MCT-treated rats showed normal localization of Cx43 immunolabeling at the intercalated disks.

Immunolabeled Cx43 domains were classified into 3 groups, intercalated disk, lateral sarcolemma and cytoplasmic domains, according to their subcellular localization, and the proportion of each group was calculated in RV myocytes isolated from 5 control and 6 MCT-treated rats (Fig 2). The proportion of cytoplasmic Cx43 domains was significantly increased (by ~7-fold on average) in hypertrophied RV myocytes (0.71±0.14, n=15) compared with controls (0.11±0.02, n=15, p<0.01). The proportion of lateral sarcolemma domains was also higher in hypertrophied RV myocytes (0.47±0.04, n=15) than in controls (0.15±0.04, n=15, p<0.01).

To verify the characteristic subcellular localization of Cx43 in the hypertrophied ventricular myocytes, RV tissue sections along the myocardial fiber orientation were double stained for Cx43/WGA and for Cx43/ZO-1. Representative Cx43/WGA images are shown in Figs 3A,B. In the control (Fig 3A), punctuate spots of immunolabeled Cx43 (green) and ZO-1 (red) were localized closely to each other (arrows), giving rise to a mosaic pattern or yellow spots at the intercalated disk region running across the longitudinal axis. In the hypertrophied RV tissue from a MCT-treated rat (Fig 3D), ZO-1 (red) remained at the intercalated disk regions but immunoreactive Cx43 (green) spots were distributed widely; some of them were co-localized with ZO-1 at the intercalated disks (arrows), but others were recognized alone at the lateral cell abutment (solid arrowheads) and in the cytoplasm (open arrowheads).

**Ultrastructure of Internalized Gap Junctions**

Ultrastructural changes in gap junctions of RV tissues were investigated by conventional and immunogold electron microscopy in each of 3 RV tissues from control and MCT-treated rats (Fig 4). In the normal RV myocardium of control rats, large gap junction membranes were present predominantly in a longitudinally oriented (interplicate) segment of the intercalated disks and smaller ones between fasciae adherentes in a transverse (plicate) segment (Fig 4A). These gap junctions were consistently labeled with Cx43 immunogold (Fig 4B). In hypertrophied RVs of MCT-treated rats, in addition to normal gap junctions at the intercalated disks, annular profiles of the gap junction membrane (Fig 4C) were frequently observed in the cytoplasm. These annular gap junctions were also clearly labeled with Cx43 immunogold (Fig 4D), suggesting that these membrane structures may correspond to the immunolabeled Cx43 detected by confocal microscopy in the cytoplasm.
Characterization of Cx43 Phosphorylation State by Immunoblot Analysis

The expression and phosphorylation state of Cx43 were investigated by Western blotting of the RV tissue samples from control and MCT-treated rats. Fig 5A shows representative blots probed with the Zymed rabbit polyclonal anti-Cx43 antibody that detects both the phosphorylated and non-phosphorylated isoforms of Cx43. In blotting of the normal RV myocardium of control rats, the antibody detected major bands between 43 and 46 kDa and a faint minor band at ~41 kDa. This blotting pattern is consistent with previous reports by other investigators. Immunoblotting of the same sample with the Zymed mouse monoclonal antibody, which binds selectively to the non-phosphorylated isoform of Cx43, showed a single faint band at ~41 kDa (Fig 5B).

This suggests that the major bands between 43 and 46 kDa and the minor band at ~41 kDa in immunoblots with the Zymed polyclonal antibody correspond to the phosphorylated and non-phosphorylated isoform(s) of Cx43, respectively. Based on these results, it is concluded that Cx43 proteins in the normal ventricular myocardium are largely phosphorylated. Blotting of RV tissue samples of MCT-treated rats with the Zymed polyclonal antibody showed weaker phosphorylated bands and a more intense non-phosphorylated band compared with controls (Fig 5A). The latter was verified by immunoblotting with the Zymed monoclonal antibody (Fig 5B). Densitometric analysis of immunoblots with the Zymed mouse monoclonal antibody showed a significant decrease in the phosphorylated isoform (*p<0.05 vs the control). MCT, monocrotaline.
are shown in Figs 5C–E, and those with the Zymed monoclonal antibody are shown in Fig 5F.) In hypertrophied RVs of MCT-treated rats, phosphorylated Cx43 was significantly decreased (p<0.05, Fig 5D) and non-phosphorylated Cx43 was significantly increased compared with controls (p<0.05, Figs 5E, F). The total amount of Cx43 protein was unaffected (p=0.983, Fig 5C).

**Distribution of Non-Phosphorylated Cx43 in Hypertrophied Ventricles**

The distribution of phosphorylated and non-phosphorylated Cx43 isoforms was assessed by the double labeling of RV tissue sections with the Zymed rabbit polyclonal and Zymed mouse monoclonal anti-Cx43 antibodies (Fig 6). In the normal RV myocardium, the pattern of Cx43 labeling with the Zymed rabbit polyclonal antibody was similar to that with the Chemicon mouse monoclonal antibody: immunolabeled spots were almost entirely confined to the intercalated disks (Fig 6A). The Zymed mouse monoclonal anti-Cx43 antibody, which binds selectively to the non-phosphorylated isoform, hardly detected Cx43 in the same section (Figs 6B, C), indicating that Cx43 located in the intercalated disks of the normal ventricle was predominantly in the phosphorylated state. In hypertrophied RVs of MCT-treated rats, the pattern of immunolabeling with the Zymed rabbit polyclonal antibody (Fig 6D) was again analogous to that with the Chemicon mouse monoclonal antibody: immunolabeled spots for Cx43 were more dispersed and recognized not only at the intercalated disks (arrows), but also in the cytoplasm (open arrowheads) as well as at the lateral cell abutment (solid arrowheads). The distribution of non-phosphorylated Cx43 assessed with the Zymed mouse monoclonal antibody shows an almost similar pattern to that of the Zymed polyclonal antibody (Fig 6E), indicating that Cx43 proteins at the intercalated disks and those displaced from there were largely dephosphorylated (Fig 6F). The distribution pattern and phosphorylation state of the LV myocardium of MCT-treated rats were essentially the same as those of controls.

**Discussion**

It is known that the organization and distribution of myocardial gap junctions is remarkably altered in various models of cardiac hypertrophy. In the present study, subcellular localization of disorganized Cx43 gap junctions was extensively assessed in a large series of confocal optical slices covering the whole thickness of isolated myocytes from MCT-induced hypertrophied ventricles. The results reveal that the cytoplasmic population of immunoreactive Cx43 far away from the intercalated disks and the lateral sarcolemma, is increased dramatically in hypertrophied ventricular myocytes. This finding is novel and suggests that internalization of Cx43 may be an important feature of gap junction remodeling in cardiac hypertrophy, in addition to the lateralization of Cx43 gap junctions that has been characterized by previous studies.

Frequent observation of cytoplasmic annular gap junction profiles, intracellular vesicular double-membrane structure labeled with Cx43 immunogold particles, in transmission electron-microscopy of the present study may also suggest a substantial increase in the translocation of Cx43 gap junctions from the intercalated disks into a intracellular compartment in hypertrophied ventricles. Annular gap junctions have been recognized as endocytosed fragments of gap junction plaques and early degradation products of gap junction membrane. Intracellular sites and pathways responsible for subsequent gap junction degradation.
have not been fully understood in cardiac cells, but previous molecular studies have suggested that both the lysosome and proteasome participate in cardiac gap junction degradation.\(^{27,28}\)

Gap junctions have a remarkably dynamic structure, because the turnover of connexin proteins is surprisingly rapid (the half-life of Cx43 in cardiac myocytes ranges 1–2 h).\(^{11,27}\) Internalization of Cx43 gap junctions and the accumulation of cytoplasmic pools observed in the present study may reflect an accelerated turnover of gap junction proteins under structural remodeling in response to pressure overload.

The second important finding of the present study is that ventricular hypertrophy was associated with the dephosphorylation of Cx43: in Western blots, Cx43 in normal ventricles of control rats was predominantly phosphorylated, which is consistent with previous reports involving other animal species and humans.\(^{3,17–19,25,27}\) and there was a significant decrease in phosphorylated Cx43 and a concomitant increase in the non-phosphorylated isoform without changes in the total amount of Cx43 in hypertrophied ventricles of MCT-treated rats. Dephosphorylation of Cx43 in hypertrophied ventricles was also demonstrated in immunoblotting with an isoform-specific antibody that selectively binds to dephosphorylated Cx43. Cx43 proteins can be phosphorylated by several serine/threonine kinases, including protein kinase A (PKA), protein kinase C (PKC), protein kinase G, mitogen-activated kinase (MAPK) and casein kinase 1, and dephosphorylated primarily by protein phosphatase 1 and 2A (PP1 and PP2A, respectively).\(^{12–14}\) In addition, it is known that some tyrosine residues of Cx43 are phosphorylated by tyrosine kinases such as v-Src and c-Src.\(^{15–16}\) Increased non-phosphorylated Cx43 could be the result of decreased phosphorylation by kinases and/or increased dephosphorylation by protein phosphatases. The specific Cx43 antibody used in the present study recognizes the non-phosphorylated serine-rich segment of the cytoplasmic C-terminal domain that includes putative phosphorylation sites for PKA, PKC and MAPK.\(^{21}\) but it still remains unknown which kinases and/or phosphatases are responsible for the increased non-phosphorylated Cx43 in hypertrophied ventricles of MCT-treated rats. A recent report demonstrating that a decrease in Cx43 phosphorylation in rabbits with non-ischemic heart failure is associated with increased levels of PP2A colocalization with Cx43, suggesting that local levels, rather than global cellular levels, of enzymes directly interact with Cx43, which might play an important role in the regulation of the Cx43 phosphorylation state.\(^{18}\)

There was no significant difference in the total Cx43 protein expression between hypertrophied and normal ventricles in the present study, and this is consistent with our earlier report.\(^{9}\) However, it is generally believed that Cx43 expression is downregulated in ventricles under various pathological conditions, especially in patients with chronic ischemic heart disease (healed myocardial infarction and chronic hibernation) and end-stage non-ischemic heart failure caused by idiopathic dilated cardiomyopathy and valvular heart disease.\(^{25,18}\) A similar downregulation of ventricular Cx43 has also been observed in some animal models of congestive heart failure.\(^{2,3,18,19}\) The reasons for the discrepancy in Cx43 expression are unknown, but such conflicting data could be explained partly by the difference in the stage of cardiac hypertrophy and heart failure. Molecular studies using cell culture have suggested that compensatory hypertrophic growth in response to pulsatile stretch as well as its chemical mediators, such as angiotensin II and vascular endothelial growth factor (VEGF), is associated with the significant upregulation of Cx43 expression and increased number of gap junctions.\(^{29–32}\) In addition, in-vivo experiments, no change or a slight increase in Cx43 protein and/or mRNA levels has been reported in the early stage of systemic hypertension-induced cardiac hypertrophy in rats with deoxycorticosterone/salt administration, renal artery clipping or abdominal aorta banding.\(^{10,33}\)

In the present study, immunoconfocal microscopy using the isoform-specific Cx43 antibody demonstrated that accumulated non-phosphorylated Cx43 in the hypertrophied ventricles was not restricted in the intercalated disks but distributed widely in the cytoplasm and at the lateral cell abutment. This might suggest that the dephosphorylation of Cx43 could be an initial step in the internalization of Cx43 gap junctions from the surface sarcolemma into the cytoplasm for degradation, although evidence for the causative relationship between the dephosphorylation of Cx43 and internalization of gap junctions are still lacking.

In the same animal model (MCT-induced RV hypertrophy) that was used in the present study, we have previously demonstrated that gap junction remodeling is correlated with a significant decrease in longitudinal conduction velocity.\(^{3}\) Although direct translation of altered gap junction expression and organizational changes in conduction properties may not be possible, the dephosphorylation of Cx43 and translocations of Cx43 gap junctions from the intercalated disks into the cytoplasm and lateral sarcolemma could play a role in intercellular electrical uncoupling and altered coordination of electrical activation in cardiac hypertrophy, which may create structural substrates of re-entrant arrhythmias.

Gap junction remodeling in the hypertrophied heart is a complex process involving the activation of a number of signal transduction pathways initiated by multiple chemical mediators partly through autocrine/paracrine mechanisms.\(^{2}\) It has been reported in several in-vitro and in-vivo experiments that activation of c-Jun-activated N-terminal kinase (JNK) plays an important role in the downregulation of Cx43 in failing hearts.\(^{34}\) Recently, heparin-binding epidermal growth factor has been shown to lead to cell hypertrophy and reduced Cx43 expression in cultured cardiomyocytes in a local autocrine/paracrine manner.\(^{35}\) In contrast, angiotensin II, transforming growth factor-\(\beta\) (TGF-\(\beta\)) and VEGF are reported to be involved in pulsatile stretch-induced upregulation of Cx43 in cell culture.\(^{29,31,32}\) Nevertheless, further experimental studies are required to elucidate the precise molecular mechanisms underlying dephosphorylation and translocation of Cx43 in gap junction remodeling associated with cardiac hypertrophy.

**Conclusion**

Pressure-overload-induced ventricular hypertrophy is associated with the considerable dephosphorylation of Cx43 proteins and translocation of Cx43 gap junctions from the intercalated disks to intracellular pools. Such alterations in myocardial gap junctions could contribute to conduction disturbances and arrhythmogenesis in cardiac hypertrophy.

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Gap Junction Remodeling in Ventricular Hypertrophy

References


