Downregulation of Immunodetectable Connexin43 and Decreased Gap Junction Size in the Pathogenesis of Chronic Hibernation in the Human Left Ventricle

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Background—The regional wall motion impairment and predisposition to arrhythmias in human ventricular hibernation may plausibly result from abnormal intercellular propagation of the depolarizing wave front. This study investigated the hypothesis that altered patterns of expression of connexin43, the principal gap junctional protein responsible for passive conduction of the cardiac action potential, contribute to the pathogenesis of hibernation.

Methods and Results—Patients with poor ventricular function and severe coronary artery disease underwent thallium scanning and MRI to predict regions of normally perfused, reversibly ischemic, or hibernating myocardium. Twenty-one patients went on to coronary artery bypass graft surgery, during which biopsies representative of each of the above classes were taken. Hibernation was confirmed by improvement in segmental wall motion at reassessment 6 months after surgery. Connexin43 was studied by quantitative immunoconfocal laser scanning microscopy and PC image software. Analysis of en face projection views of intercalated disks revealed a significant reduction in relative connexin43 content per unit area in reversibly ischemic (76.7±34.6%, P<.001) and hibernating (67.4±24.3%, P<.001) tissue compared with normal (100±30.3%); ANOVA P<.001. The hibernating regions were further characterized by loss of the larger gap junctions normally seen at the disk periphery, reflected by a significant reduction in mean junctional plaque size in the hibernating tissues (69.5±20.8%) compared with reversibly ischemic (87.4±31.2%, P=.012) and normal (100±31.5%, P<.001) segments; ANOVA P<.001.

Conclusions—These results indicate progressive reduction and disruption of connexin43 gap junctions in reversible ischemia and hibernation. Abnormal impulse propagation resulting from such changes may contribute to the electromechanical dysfunction associated with hibernation. (Circulation. 1998;97:651-660.)

Key Words: junctions, gap ■ myocardial contraction ■ hibernation

The term “hibernating myocardium” describes a state of persistently impaired myocardial function at rest due to severely reduced coronary blood flow that is reversible after successful revascularization.1,2 Previous studies on pathophysiological mechanisms in hibernating human tissue have focused on the identification of pathognomonic myocardial structural changes. Histological and electron microscopic examination of biopsy material obtained from hibernating regions reveal a variable degree of fibrous tissue replacement, with a proportion of the myocytes showing depleted contractile material, numerous and small mitochondria, and irregular nuclear envelopes.3,4 Although early studies suggested that such altered myocytes characterized the hibernating state3 and could explain the delay in recovery in contractile function after successful revascularization,5 more recent reports have described them in equal frequency in both hibernating and nonhibernating ventricular segments.6,6 Similar cellular features have also been described in ventricular specimens from patients with dilated, nonischemic cardiomyopathy7 as well as in rat cardiac myocytes after a short period of unloading.8 It can therefore be hypothesized that these are secondary features of cells contracting against a low afterload and are not characteristic of hibernation.

See p 630

Two PET-based studies have shown that the prognosis in medically treated patients with ischemic heart disease and impaired ventricular function is poorer when there is additional evidence of hibernating myocardium, but is significantly improved by successful revascularization.9,10 The majority of deaths in these studies were sudden, possibly implicating
arrhythmias as being a more common feature in the presence of myocardial hibernation.\textsuperscript{9} This combination of segmental wall motion abnormalities and clinically important ventricular arrhythmias could plausibly result from severe disruption in conduction of the depolarizing wave front, leading to asynchronous contraction of individual myocytes and predisposing to reentry arrhythmias.\textsuperscript{11} Coordination of the electrical, and conduction of the depolarizing wave front, leading to asyn-

wall motion abnormalities and clinically important ventricular stress procedure was performed by infusion of adenosine 140 min after surgery. Images were analyzed by two independent observers for the thallium studies and by two others for the MRI. Single photon emission CT images were graded for thallium uptake on a 5-point scale (4=normal, 3=slightly reduced, 2=mod-

erately reduced, 1=severely reduced, 0=absent). Wall motion on cine-MRI was also graded on a 5-point scale (3=normal, 2=mild hypokinesia, 1=severe hypokinesia, 0=akinesia, −1=dysskinesia). Coronary territories were assigned as anterior, septum and apex to the left anterior descending coronary artery, lateral to the circumflex artery, and inferior to either the right or circumflex artery depending on dominance. A nine-segment left ventricular model (basal and apical parts of the septum, anterior, lateral, and inferior walls, together with the apex) was used for segmental classification as follows: The normally perfused classification was defined as preoperative MRI wall motion grade ≥2 and thallium uptake grade 4. Reversibly ischemic was preoperative MRI wall motion grade ≥2 and improvement in thallium uptake by at least one grade on stress/redistribution images. These segments did not show any improvement in contractile characteristics after successful revascularization. Hibernating was de-

fined as preoperative MRI wall motion grade ≤1 and improvement in contraction of at least one grade recorded with postoperative MRI. (Late rest/redistribution thallium uptake grade ≥2 and/or improve-

ment by at least one wall motion grade with low-dose dobutamine infusion was used to predict hibernation before surgery, directing biopsy sites). Infarcted myocardium showed preoperative MRI wall motion grade ≤1 and late rest/redistribution thallium uptake ≤1, with no recovery of wall motion after surgery.

Twenty-one patients with more than two of nine ventricular regions provisionally classed as hibernating (likely to improve in function with revascularization) proceeded to CABG. Per-operative transmural biopsies representing each of the four classes were taken from each patient with a metal cork-borer of 2-mm ID; the surgeon was directed by a map of the nine-segment left ventricular model described. This study was approved by the local Ethics Committee, and all patients gave written consent using a specifically designed consent form.

**Processing of Specimens**

All biopsies were immediately fixed in freshly prepared Zamboni’s fixative on removal in the operating theater.\textsuperscript{10} Biopsy orientation was recorded before further division into epicardial and endocardial samples. For histology and confocal microscopy, tissue was fixed for 2 to 6 hours, washed overnight, and embedded in paraffin. Sections were routinely stained with hematoxylin-eosin, periodic acid-Schiff reagent, and phosphotungstic acid–hematoxylin. For electron microscopy and high-resolution light microscopy, tissue divided from the original biopsy was fixed in 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer at pH 7.3, postfixed in 2% osmium tetroxide, stained en bloc in saturated uranyl acetate in 50% ethanol, and embedded in epoxy resin. Semithin sections for light microscopy were stained with toluidine blue. Ultrathin sections for electron microscopy were stained with uranyl acetate and lead citrate before examination.

**Immunofluorescence Microscopy**

Sections (10 \( \mu \text{m} \)) on polylysine-coated slides were dewaxed with xylene and rehydrated. Microwave treatments in different buffers\textsuperscript{26} and 0.1% trypsin\textsuperscript{27} for retrieval of antigenic sites masked by fixation were compared. Optimal antigen detection was found with 7 minutes of microwave oven treatment in 0.01% sodium bicarbonate at pH 7.4. Sections were blocked with 0.1 mmol/L 1-lysine in PBS containing 0.1% triton X-100 for 45 minutes before overnight incubation with mouse monoclonal anti-connexin43 antibody (Chemicon Interna-

tional Ltd) at dilution 1:1000 in 0.5% BSA in PBS at room tempera-

ture. The detection system used was monoclonal sheep anti-mouse biotinylated antibody/streptavidin Texas red (Amersham Life Scienc-
es), both at 1:250 dilution in PBS containing 0.5% BSA for 1 hour. The sections were washed for 20 minutes and mounted in 50% glycerol containing para-phenylenediamine as an antibleaching agent. Sections from 20 blocks were processed simultaneously in each immuno labeling run; controls in which the primary antibody was omitted were routinely run in parallel. In addition to connexin43, trials were conducted to test for expression of two more connexin types, connexin40 and connexin45. Affinity-purified polyclonal anti-
sers against synthetic peptides (residues 254 to 268 of rat connexin40
proximate junctions, the binary image was further edited to separate if such artifactual merging occurred between non-contiguous. If such artifactual merging occurred between non-contiguous junctions, the binary image was further edited to separate

**Techniques for Quantification**

Quantification of connexin43 content was performed blind on tissue content was performed blind on tissue samples. Immunofluorescence and confocal microscopy were used to ensure that clearly separated juxtaposed junctions. The total remaining "on" pixels were then automatically counted to yield the total number of gap junctions within the disk, the sizes of these individual junctions, and the total area of the disk itself. The mean gap junction size in square micrometers (total area of junctions/number of junctions in disk) and the total area of junctions/total area of disk were calculated. Because there was variability in the absolute label between immunofluorescence runs, results were expressed as a percentage relative to the normally perfused tissue acting as an internal normal control for each patient. Data obtained were finally pooled into the three clinical groups (normally perfused, reversibly ischemic, and hibernating myocardium) to obtain a distribution plot of results for each group and for subsequent statistical analysis using ANOVA. Secondary analysis was performed between the three groups by use of Bonferroni's correction to Student's t test. Data are expressed as mean±SD; secondary t test significance is expressed as P.  

**Results**

**Patients**

Twenty-one patients were selected for the study: 91% male; median age, 61 years (range, 40 to 71 years); mean NYHA grade, 2.8; mean ejection fraction, 23%. Of the 21 patients initially biopsied, full follow-up investigations were completed in 15, allowing final classification of tissue. Four patients did not survive to follow-up, and the remaining 2 withdrew from the study. The demographic and clinical details of the 15 patients are shown in Table 1.

**General Histology and Ultrastructure**

Ventricular biopsies from all four clinical groups showed variable degrees of fibrosis, occurring both in the interstitial spaces and as separate islands of connective tissue representing local infarction. Tissue classified as infarcted showed extensive fibrous tissue replacement. The myocytes themselves varied from a normal appearance at light microscopy to pathological cells severely depleted of myofibrillar proteins (Fig 2). These cells showed evidence of glycogen accumulation by periodic acid–Schiff staining of histological sections (not shown). On transmission electron microscopy, the pathological cells revealed consistent features of reduced myofibrillar content, glycogen accumulation, numerous and small mitochondria, and irregular nuclear envelopes (Fig 3).

**Distribution of Connexin Label**

Low-power views of connexin43 immunolabeling revealed a normal distribution of label in the tissue away from zones of scarring, the fluorescence being confined to typical intercalated disks (Fig 4). In infarcted tissue, there was disruption of this normal pattern, as reported previously, with degenerative myocytes partially embedded in scar tissue having gap junctions distributed widely over their surface membranes. Reversibly ischemic, hibernating, and normally perfused (acting as internal normal control tissue) biopsies were examined in greater detail at higher magnification in tissue distant from such disrupted zones to determine connexin43 distribution in individual intercalated disks. Disks seen en face in transversely sectioned areas of tissue showed that in the control tissues, there was a normal distribution of gap junction label, with small central gap junctions surrounded by extensive larger spots of label in the disk periphery (Fig 5A). Disks from reversibly ischemic biopsies had a similar pattern of connexin43 labeling sequence; residues 354 to 367 of human connexin45) raised in rabbit were used for this purpose. The procedure was similar to that for connexin43, except that 0.1% trypsin was used for antigen rescue. Connexin40 antibody was diluted to 1:1000 and connexin45 to 1:50; secondary detection was with anti-rabbit Cy3 conjugate (Chemicon International Ltd). Immunostained tissue was coded and examined blind with conventional epifluorescence and subsequently confocal microscopy with a Leica TCS 4D equipped with an argon/krypton laser with the appropriate filter blocks for the fluorescent signal. All images were recorded within 48 hours of labeling with single-channel scanning.  

**Figure 1. Diagrammatic representation of image acquisition by immunofluorescence microscopy, which allows high-resolution, low-background optical sections with 0.5-μm depth of field to be recorded. Multiple images taken as a series at set intervals through thickness of specimen are required to scan all spots of label in a single disk, from which a single composite projection image of entire disk can be reconstructed.**

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but demonstrated a decrease in the amount of label compared with the control disks in the same staining run (Fig 5B). The hibernating disks also showed a diminution of label, although in this case there was a striking loss of the larger peripheral gap junctions (Fig 5C). Endocardial and epicardial areas of clearly oriented full-thickness biopsies were compared to investigate any differences that might reflect the recognized variation in perfusion and degree of scarring present in these two areas, but none were apparent. There were no immunodetectable changes in other connexin isoforms in the samples examined; connexin40, present in arteriolar endothelium, was not detected in myocytes, and connexin45, which is expressed only in very low amounts in the heart, was found not to be detectable in fixed specimens with the antibody used. Label was absent in the controls in which the primary antibody was omitted. Tissue autofluorescence was low after microwave treatment, and highly fluorescent lipofuscin granules ubiquitous in human tissue could easily be distinguished from specific label.

Transmission electron microscopy of representative intercalated disks from each tissue class revealed preservation of the basic ultrastructure of the disk; desmosomes and fasciae adherentes occupied the transverse portions of the disks, with gap junctions situated in the zones parallel to the long axis of the cells (Fig 6). This structure was maintained regardless of the degree of loss of contractile material in a given myocyte. There was no evidence of gap junction internalization at the disk margin or of isolated junctions in nondisk membrane. These findings were consistent with the described pattern of connexin43 immunolabeling being confined to the intercalated disks in areas distant from infarcts.

Quantification

**Disk Gap Junctional Density**

Differences in the extent of labeling were observed between individual immunolabeling runs, with values of percentage gap junction area per unit area of disk in the control biopsies varying between 10.4% and 13.0% (Table 2). Overall, however, there were consistent patterns in the measurements.
between the three groups. When expressed as relative values, mean gap junction density per unit area of disk was significantly reduced compared with control (100±30.3%) in both reversibly ischemic (76.7±34.6%; P<.001) and hibernating (67.4±24.3%; P<.001; ANOVA P<.001) tissues. These last values were not significantly different from one another on secondary analysis (P=.069) (Fig 7). The findings confirmed the visual impressions described above.

Size of Immunolabeled Gap Junctions
The mean gap junction size of control tissue was 0.21±0.06 μm² (Table 2), yielding a mean gap junctional diameter of 0.53 μm (assuming a circular structure to a single gap junction). If mean junctional size of the diseased tissues was expressed as percentage relative to control, there was a significant reduction in size to 87.4±31.2% (P<.001) in reversibly ischemic tissue, and a further significant reduction in hiberna-

Figure 3. Electron micrograph showing characteristic appearances of “pathological” myocytes. Note depletion of contractile material, with myofibrils confined to periphery of cells (arrowhead), empty regions containing glycogen (g), minimitochondria (m), and irregular nuclei (n). Cells of this morphology were found throughout poorly functioning ventricles, regardless of segmental classification. Example from hibernating region. Bar=10 μm.

Figure 4. Low-power views of connexin43-labeled myocardium (longitudinal section) showing normal distribution of label confined to intercalated disks in hibernating (left) and reversibly ischemic (right) segments. Strong fluorescence from lipofuscin (arrowhead) is easily distinguished from punctate labeling of gap junctions (arrow). Bar=25 μm (A and B).
tion to 69.5±20.8% (P’=0.12; ANOVA<.001). This last value reflects the loss of peripheral large junctions observed on examination of the disks from the hibernating tissue, with maintenance of the frequency distributions of area measurements of the smaller-size individual junctions from the different tissue classes (Fig 8).

Discussion

Evidence implicating alterations in connexin43 gap junctions in arrhythmogenesis in different models of human heart disease has steadily accumulated.20–22,26,29,30 The novel findings of the present study are that (1) a reduction in connexin43 gap junctions occurs in hibernating myocardium beyond that seen in reversible ischemia, and (2) a specific feature characterizing hibernating myocardium is a loss of the population of large gap junctions at the disk periphery. These changes seen by laser scanning immunoconfocal microscopy were subsequently confirmed by quantification with PC image analysis. The use of quantitative immunofluorescence for measurement of gap junction size has been previously validated with a polyclonal anti-connexin43 antibody in rat left ventricular tissue by comparison of measurements from immunoconfocal (0.53 μm) and freeze-fracture (0.57 μm) electron microscopy.28 In the present study, quantification of images obtained with a different primary antibody yielded a mean gap junctional diameter of 0.53 μm in control tissue, in accordance with the published data, indicating that the technique reflects true structural measurements. We elected to study connexin43 content in individual intercalated disks. Previous work has shown a lack of significant changes in intercalated disk number per myocyte in ischemic human myocardium,22 indicating that the density of gap junctions in the existing disks closely reflects the tissue content of connexin43 in the ventricle as a whole.

In a study of this type, extrapolations of the nature of regional tissue structure are made from assessment of only a small amount of tissue taken as a biopsy. Because pathological changes resulting from ischemic heart disease are known to be inhomogeneous, there will be an unavoidable sampling error from the examination of such biopsy material. In the present study, such errors were minimized in three ways. First, we were able to examine relatively large samples by use of a metal cork-borer, which provides 2-mm-diameter transmural cylindrical biopsies, significantly larger than a specimen from a needle biopsy. Second, concurrent tissue from an individual patient was taken, whenever possible, from hibernating, reversibly ischemic, and normally perfused segments. This allowed direct comparison of diseased tissue with internal controls of “normal” tissue collected in an identical fashion. Third, all tissue examination was performed with the observer

Figure 5. High-power en face view of control (A), reversibly ischemic (B), and hibernating (C) intercalated disks. Normal pattern of large peripheral and small central gap junctions are seen in control tissue. This distribution is maintained in biopsies from reversibly ischemic regions, with an impression of decrease in overall intensity of labeling of disk. Hibernating intercalated disks show loss of normal pattern of labeling, with absence of large peripheral junctions making disk borders harder to define. Bar=25 μm (A, B, and C).
blinded to the tissue origin of each biopsy. The consistent patterns in immunodetectable connexin43 demonstrated under these conditions indicate that the data reflect true differences between the control and disease groups.

Earlier studies emphasized myofibril depletion as a hallmark of myocytes in hibernation. However, separate assessment of tissue histology and ultrastructure from the present patient series has shown no significant differences in frequency of myofibril-depleted myocytes between the groups of biopsies, including the control tissues, suggesting that alternative pathophysiological mechanisms are required to account for the wall motion abnormalities characteristic of hibernation. Alterations to gap junctions affecting intercellular coupling are one such candidate mechanism. The present study, in accordance with previous work, revealed that in reversibly ischemic myocardium, areas distant from a zone of infarction show a loss in connexin43 gap junction content but with maintenance of their overall tissue distribution. A key finding is that progressive decrease in the density of connexin43 gap junctions and loss of the large gap junctions at the disk periphery occurred in hibernating segments. No significant changes in other connexin isoforms were apparent in working myocytes in our tissue.

A relationship between abundance of gap junctions and intercellular conduction is suggested by studies of the functional role of the gap junction in cardiac muscle, the absence of conduction in vitro between cells without gap junctions, and the findings of sparse junctions in areas of heart with low conductance velocities. A relationship between gap junctional area and conductance has also been demonstrated in newly forming connections between isolated myocytes in vitro, as well as by the finding of concomitant increases in conduction velocity and connexin expression in dibutyryl cAMP–treated cultured cardiac myocytes. Although the precise in vivo relationship between absolute number of gap junctions and conduction velocity is not known, significant cardiac conduction defects are reported in heterozygotes for a connexin43 null mutation with a 50% reduction in gap junction content. Our findings of 23% and 33% reduction of disk gap junction density in reversibly ischemic and hibernating segments, respectively, may therefore plausibly cause significant disruption of intercellular propagation of depolarization. The specific loss of the peripheral large gap junctions in the disks of hibernating myocardium is potentially critical.

Recent findings suggest that tetrodotoxin-insensitive voltage-dependent sodium channels, essential for the rapid early upstroke of the cardiac action potential, have been localized to the lateral sarcolemma and may be preferentially concentrated around the intercalated disks. The peripheral intercalated disk conductance, determined by these large junctional

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**Table 2. Results of Quantification**

<table>
<thead>
<tr>
<th>Gap Junction Content of Disk, % (Runs 1 and 2)</th>
<th>Mean Relative Gap Junction Content of Disk, %</th>
<th>Gap Junction Size, ( \mu \text{m}^2 ) (Runs 1 and 2)</th>
<th>Mean Relative Gap Junction Size, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal perfusion</td>
<td>10.4±3.4</td>
<td>100±30.3*</td>
<td>0.21±0.06</td>
</tr>
<tr>
<td>Reversible ischemia</td>
<td>7.6±4.0</td>
<td>76.7±34.6*</td>
<td>0.17±0.06</td>
</tr>
<tr>
<td>Hibernation</td>
<td>7.1±2.6</td>
<td>67.4±24.3*</td>
<td>0.13±0.04</td>
</tr>
</tbody>
</table>

Absolute and mean relative values of intercalated disk gap junction content and gap junction size (mean±SD).

*ANOVA, \( P<.001 \); †ANOVA, \( P<.001 \).
plaques, may be of particular importance because of this spatial relationship. On theoretical grounds, intercellular conduction depends on the reciprocal relationship between intercalated disk resistance, determined by the absolute number of connexin channels, and cell size. Although cell volume was not directly assessed in our study, intercalated disk area, which reflects the cross-sectional area of the corresponding myocyte, provides an indirect measure of cell size. The finding of progressively decreasing density of labeling in the disks in reversible ischemia and hibernation partly reflects larger disk sizes in these groups. Disruption in passive intercellular communication\(^2\) from such changes may, in turn, predispose to the increasing frequency of arrhythmia and sudden death associated with reversible ischemia\(^4\) and hibernation.\(^9\) Furthermore, regional contractile dysfunction in hibernating areas may be partly accounted for by local incoordination in contraction of individual myocytes secondary to fragmentation of the depolarizing wave front.

The mechanisms by which reduced intercellular coupling predisposes to arrhythmia may involve the interaction of the characteristics of the action potential of individual myocytes and the degree of intercellular coupling. Human and canine myocytes from endocardial, epicardial, and the deep subepicardial (M-cell layer) regions of normal and diseased ventricular myocardium have been shown to have different action potential durations, suggested to be due to a variable transient outward K\(^+\) current \(I_{\text{to}}\).\(^{41-45}\) The coupling of these cells to their neighbors in vivo masks such differences by modulating action potential duration of each cell to reach a homogeneous depolarization.\(^34\) A moderate reduction in such communication by disruption of gap junctions could result in an unmasking of these variable action potential characteristics and hence to slowing and inhomogeneities in the conduction of the depolarization wave.\(^1\) This may, in turn, be manifest at the clinical level by the establishment of potential reentrant circuits and hence ventricular arrhythmias.\(^1\)

A common feature of hibernating myocardium is the ability to respond to inotropic stimulation with dobutamine. It is becoming increasingly clear, however, that certain myocardial segments are resistant to dobutamine stimulation but eventually show recovery of function after revascularization and hence are defined as hibernating.\(^4\) A variable severity of gap junctional disruption could provide a mechanism for this phenomenon, with the dobutamine-resistant tissue being the more severely affected.

Our data are consistent with the hypothesis that a reduction in gap junction coupling is involved in the pathophysiology of
hibernation, possibly by unmasking local inhomogeneities in individual cell action potential durations, hence disrupting wave-front propagation, slowing conduction, and leading to loss of the local coordination in myocyte contraction. The changes at the cellular level would be manifested by nonspecific changes at the surface ECG. The disruption in intercellular conduction and investigation of excitation–contraction coupling needs further documentation by cellular electrophysiological studies. A more complete understanding of the role of gap junctions in hibernation would be the first step for subsequent studies of these channels as targets for therapeutic modulation.

Acknowledgments

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10. Caprioli et al. Kaprielian et al. 659

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