Temporal and Spatial Variations in Structural Protein Expression During the Progression From Stunned to Hibernating Myocardium

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Background—Dysfunctional and normally perfused remote regions show equal myolysis and glycogen accumulation in pig hibernating myocardium. We tested the hypothesis that these arose secondary to elevations in preload rather than ischemia.

Methods and Results—Expression of structural protein (desmin, desmoplakin, titin, cardiotin, α-smooth muscle actin, lamin-A/C, and lamin-B2) in viable dysfunctional myocardium was analyzed by immunohistochemistry. We performed blinded analysis of paired dysfunctional left anterior descending coronary artery and normal remote subendocardial samples from stunned (24 hours; n=6), and hibernating (2 weeks; n=6) myocardium versus sham controls pigs (n=7). Within 24 hours, cardiac myocytes globally reexpressed α-smooth muscle actin. In stunned myocardium, cardiotin was globally reduced, whereas reductions in desmin were restricted to the dysfunctional region. Alterations progressed with the transition to hibernating myocardium, in which desmin, cardiotin, and titin were globally reduced. A qualitatively similar reorganization of cytoskeletal proteins occurred 3 hours after transient elevation of left ventricular end-diastolic pressure to 33±3 mm Hg.

Conclusions—Qualitative cardiomyocyte remodeling similar to that in humans with chronic hibernation occurs rapidly after a critical coronary stenosis is applied, as well as after transient elevations in left ventricular end-diastolic pressure in the absence of ischemia. Thus, reorganization of cytoskeletal proteins in patients with viable dysfunctional myocardium appears to reflect chronic and/or cyclical elevations in preload associated with episodes of spontaneous regional ischemia. (Circulation. 2004;110:3313-3321.)

Key Words: stunning, myocardial hibernation proteins structure ischemia

Hibernating myocardium is accompanied by structural changes reminiscent of cardiomyocyte dedifferentiation.1-4 It has been hypothesized that these alterations represent an intrinsic adaptive response to a metabolic imbalance5,6 because of the absence of morphological and cytochemical characteristics of acute ischemia.6,7 In swine, the transition from chronically stunned to hibernating myocardium is preceded by progressive alterations in the degree of myolysis and regional reductions in the expression of sarcoplasmic reticulum proteins.8,9 Surprisingly, the degree of myolysis and glycogen accumulation in the ischemic regions was identical to that in normally perfused remote regions.8 In addition, the frequency of myolysis by histology was much greater than myofibrillar loss by point counting. The finding of remote-zone myolysis in the absence of a stenosis challenges the notion that the cellular changes attributed to hibernating myocardium arise as an adaptation to ischemia.

We used the porcine model to gain more insight into the temporal progression and spatial variations in structural protein changes in hibernating myocardium. We assessed histological reorganization of several structural proteins, previously identified to be altered in biopsies from humans with hibernating myocardium, in paired subendocardial samples from dysfunctional and normally perfused remote myocardium. Our results show that the expression of these proteins changes continuously during the progression from stunned to hibernating myocardium. The structural changes, previously attributed to repetitive regional ischemia, occur globally and are largely independent of regional reductions in coronary flow reserve. Acute elevation of preload induced similar structural changes, indicating that mechanical factors rather than local ischemia initiate the cardiomyocyte histological phenotype characteristic of viable chronically dysfunctional myocardium.
Methods

Animal Models
The accelerated porcine model of viable dysfunctional myocardium has been described previously in detail.9 Chronically instrumented swine underwent partial occlusion of the left anterior descending coronary artery (LAD) to induce acute stunning, after which perfusion was restored through a critical stenosis. After 24 hours (n=6), physiological features of stunned myocardium (dysfunction with normal resting flow) were present. When the stenosis was maintained for 2 weeks (n=6), hibernating myocardium with regionally reduced resting flow developed in the absence of infarction. Controls consisted of sham-instrumented animals (n=7).

We assessed the effects of preload elevation in closed-chest sedated pigs (n=6, Telazol/xylazine, 0.037 mL/kg IM, and propofol, 5 to 10 mg · kg⁻¹ · h⁻¹). A 5F micromanometer was advanced percutaneously into the left ventricle (LV). The 6F introducer was used to withdraw blood for fluorescent microspheres by use of the reference sampling technique.8 A 7F pigtail catheter was inserted into the left atrium via the carotid artery. After equilibration, hemodynamics and flow were measured under resting conditions. We increased mean left atrial pressure to 20 mm Hg for 1 hour by increasing afterload with phenylephrine (300 μg/min IV). Flow measurements were repeated at rest and after adenosine (79 μg/kg/min IV) at the elevated preload to exclude subendocardial ischemia. After a 3-hour recovery, the animals were euthanized and the heart was sampled.

Immunohistochemistry
Immunohistochemistry was performed as previously described.10 Primary antibodies were targeted against desmosplakin (11-5-F11), desmin (RD30112), titin (9D10113), cardiokin (R2G114), lamin-A/C (R27115), lamin-B2 (LN4315), and α-smooth muscle actin (α-SM actin) (a-SM-117). Frozen sections 5 μm thick were transferred onto slides and air-dried. For desmosplakin, desmin, titin, and cardiokin antibodies, sections were incubated for 5 minutes in 0.5% Triton X-100 in PBS. For lamin-A/C and lamin-B2, sections were incubated in methanol (5 seconds) and 3×5 seconds in acetone (−20°C). After a 3×5-minute wash (in PBS), the sections were incubated for 45 minutes with the primary antibodies. Next, sections were washed 3×10 minutes (PBS), and a fluorescein isothiocyanate (FITC)-labeled subclass-specific secondary antibody was applied for 45 minutes. For double-labeling, the first immunostaining step was repeated with a second antibody of another immunoglobulin subclass, and the second step was repeated with a Texas Red–labeled subclass-specific secondary antibody. Finally, sections were placed in distilled water for 5 minutes, fixed in methanol (except cardiokin) for 5 minutes, air-dried, and mounted in Mowiol containing 4′,6-Diamidino-2-phenylindole (DAPI) for nuclear staining. For negative controls, the primary antibody was not applied.

For staining of α-SM actin, sections were pretreated with 3% phosphate-buffered glutaraldehyde containing 1.4% sucrose for 20 minutes, followed by a 15-minute incubation in 0.5% Triton X-100 and 10 minutes in PBS. Sections were treated with 1 mg/mL NaBH₄ in PBS for 15 minutes, washed (PBS), and then preincubated in 0.1% BSA (PBS) for 30 minutes. The primary antibodies were applied overnight (4°C), and after a 3×10-minute washing (PBS), sections were incubated for 60 minutes with an alkaline phosphatase–conjugated secondary antibody. Finally, sections were washed 3×10 minutes, and phosphatase activity was revealed by use of the alkaline phosphatase substrate kit III (Vector Laboratories). After 5 minutes in distilled water, the sections were dried and mounted in Perpx.

Light Microscopic Evaluation
All sections were evaluated blindly by 2 observers (V.T., M-H.L.). Desmin staining was also assessed at the intercalated disks, as well as the organization of desmin in the cytoplasm (cross-striations versus disorganized patterns). Cytoplasmic organization of titin was evaluated at the level of cross-striations, with specific attention paid to the presence or absence of “doublet” cross-striations. Cardiokin was analyzed by staining intensity and the length of the cardiotin-positive arrays. Lamin staining was determined in myocyte nuclei with respect to coexpression of lamin-A/C with lamin-B2.

Western Blotting
Flash-frozen subendocardial tissue was isolated as previously described.18 Equal amounts of protein (10 to 30 μg) were electrophoresed on 10%
SDS-PAGE gels, transferred to PVDF membranes (Millipore), and blocked in 5% BSA, TBS, and 0.1% Tween-20 for 1 hour at room temperature. Membranes were incubated with antibodies to desmin (1:1000), cardiotin (1:100), and α-SM actin (1:10,000) in blocking buffer (4°C overnight). They were washed in TBS/0.1% Tween-20 and incubated at room temperature (1 hour) with peroxidase-labeled goat anti-mouse antibody to IgG (KPL, 1:5000). After washing, desmin and cardiotin were detected by use of Super Signal Pico chemiluminescent substrate, whereas α-SM actin was detected by use of Super Signal Femto (Pierce), and digital images were quantified (Quantity One, Bio-Rad). To ensure that the intensity values were within a linear range, a standard curve was generated for each experiment. The curve was derived from a normal sample that was serially diluted from 50 to 0 μg and was run concurrently with the experimental samples. Blots were exposed simultaneously, and chemiluminescent images were captured every 30 seconds up to 5 minutes. Optimal exposure time was determined from the standard curve by selecting a time point that yielded the maximum intensity and linearity for the amount of protein assayed with respect to the experimental samples.

**Statistical Analysis**

Data are displayed as mean±SEM. Significant differences between the groups were analyzed by means of the Wilcoxon-Mann-Whitney test.
rank-sum test. The hemodynamic data were assessed by 1-way ANOVA and post hoc paired t tests using the Bonferroni correction for multiple comparisons. Probability values of \( P < 0.05 \) were considered to be statistically significant.

**Results**

Hemodynamics, flow, and wall thickening in the dysfunctional pigs has been described in detail. Briefly, LAD wall thickening averaged 36.3\( \pm \)4%, and flow averaged 67\( \pm \)6 mL/min. After 24 hours of critical stenosis, LAD wall thickening decreased (25.5\( \pm \)3.7%, \( P < 0.05 \) versus control), with normal resting perfusion (67\( \pm \)8 mL/min). Within 2 weeks, hibernating myocardium developed, with reductions in LAD flow (45\( \pm \)10 mL/min, \( P < 0.05 \)) and function (LAD wall thickening, 17\( \pm \)5%, \( P < 0.05 \)). Remote-zone flow and function remained unchanged. Preload was elevated in swine with viable dysfunctional myocardium (LV end-diastolic pressure, 16.4\( \pm \)0.9 under control to 18.4\( \pm \)2.0 in stunned and 24\( \pm \)2.0 mm Hg in hibernating myocardium). There was no triphenyltetrazolium chloride evidence of necrosis in any of the groups.

**Reorganization of Structural Proteins During the Progression From Stunned to Hibernating Myocardium**

Results of immunohistochemical scoring for each of the stains are summarized in Table 1. Desmin and desmoplakin colocalize to desmosomal plaques of cardiomyocytes and align to the Z disk. Its expression and distribution are developmentally regulated. In shams, desmin and desmoplakin colocalized to the intercalated disks, with desmin showing a regular striated staining pattern aligned to the Z disk (Figure 1). In contrast, spatial reorganization of desmin occurred within 24 hours after placing a critical stenosis. Reorganization of desmin in the cytoplasm and loss of colocalization with desmoplakin at the intercalated disks initially began in the dysfunctional region. With the progression to hibernating myocardium, both remote and dysfunctional regions displayed structural reorganization in the cytoplasm and loss of desmin expression at the intercalated disks.

Titin showed a regular cross-striated distribution pattern in sham hearts (Figure 2). Because the antibody was targeted against an I-band epitope, a double-banded staining pattern was visible (Figure 2, inset). Titin expression and organization were not altered in stunned myocardium. After hibernating myocardium developed, the double-banded pattern disappeared, whereas the regular cross-striations were still present. Changes were similar in dysfunctional and normally perfused remote regions.

Like that of titin, expression of cardiotin was restricted to cardiomyocytes. In shams, cardiotin was present in arrays (Figure 3). In stunned myocardium, cardiotin was altered, as reflected by a loss of staining intensity and shortening of the array length. Again, the changes were visible throughout the LV and similar in dysfunctional and normal remote regions.

**Reexpression of the Fetal Smooth Muscle Actin Isoform in Stunned and Hibernating Myocardium**

Because alterations in desmin, titin, and cardiotin are negative indicators of cardiomyocyte adaptation, we also analyzed the expression of \( \alpha \)-SM actin (Figure 4). In shams, \( \alpha \)-SM actin was found only in vascular smooth muscle cells. Within 24 hours after a critical stenosis had been placed, \( \alpha \)-SM actin staining was present in cardiomyocytes. The reexpression was heterogeneous, affecting single cells or clusters of
cardiomyocytes. Like other structural proteins, α-SM actin was increased in dysfunctional and remote regions. After the development of hibernating myocardium, α-SM actin was still present but less pronounced because of the presence of large vacuoles out of which the staining was easily washed away.

**Figure 4.** Immunohistochemical analysis of α-SM cell actin expression. Example of typical α-SM actin staining (dark blue staining) in remote region and LAD region of a sham animal (top) and animals with stunned (middle) or hibernating myocardium (bottom). In sham animals, α-SM actin was restricted to coronary arteries (arrowheads, tops). After 24 hours, α-SM actin was expressed in cardiac myocytes from both dysfunctional and normal remote regions of stunned myocardium (arrows). With progression to hibernating myocardium, α-SM actin expression persisted in cardiac myocytes, but overall, it was less pronounced than that after 24 hours of critical stenosis (asterisk).

**Figure 5.** Immunohistochemical analysis of lamin-A/C and lamin-B2 expression. Top, Typical staining pattern for lamin-A/C and lamin-B2 in remote region (right) and LAD region (left). Middle, staining is shown after 24 hours of ischemia. Bottom represents 2 weeks of ischemia. Arrowheads indicate nuclei that stain positive for both lamins, whereas arrows indicate nuclei that stain positive for lamin-B2 and are negative for lamin-A/C. There was no difference in lamin staining in either region of stunned or hibernating myocardium compared with control animals (magnification ×200).

**Distribution of Nuclear Lamins During the Progression From Stunned to Hibernating Myocardium**

The lamin distribution patterns, which indicate nuclear remodeling when altered, are depicted in Figure 5. As in human hearts, all myocyte nuclei stained positive for lamin-B2.
Lamin-A/C expression was more heterogeneous. Cardiomyocytes that were uniformly lamin-B2–positive did not always react with the lamin-A/C antibody. This dissociation was found even within a row of nuclei from a single cardiomyocyte. No differences were found in lamin-A/C expression between sham, stunned, and hibernating myocardium.

Western Analysis of Structural Proteins During the Progression From Stunned to Hibernating Myocardium

Western blotting was performed to quantify the expression of proteins with the most prominent reorganization (Figure 6). There were no differences in protein levels between LAD and remote regions at any time point. After 2 weeks, cardiotoxin tended to decrease and α-SM actin increased, but the modest differences between groups were not significant. Thus, although pronounced redistribution of structural proteins was evident by blinded immunohistochemical analysis, protein levels in whole tissue were not quantitatively altered among groups or between dysfunctional and remote myocardium.

Cytoskeletal Reorganization After Transient Preload Elevation Resembles Stunning-Induced Remodeling

Because cardiomyocyte remodeling occurred globally and preload was elevated in hearts with dysfunctional myocardium, we tested whether some of the structural changes could occur after transient elevations in LV preload. Hemodynamics are summarized in Table 2. Phenylephrine elevated left atrial pressure from 10±2 to 22±5 mm Hg (P<0.05) and increased LV end-diastolic pressure from 16±1 to 33±3 mm Hg (P<0.005). Myocardial flow demonstrated no evidence of ischemia at any time point (0.93±0.08 mL · min⁻¹ · g⁻¹ in control and 1.07±0.11 mL · min⁻¹ · g⁻¹ in elevated preload) and no impairment of flow reserve during preload elevation (adenosine flow, 4.83±0.45 mL · min⁻¹ · g⁻¹, P<0.01 versus control). Structural protein remodeling 3 hours after a 1-hour elevation of preload are summarized in Table 1 and Figure 7. α-SM actin expression was not analyzed because no measurable increase in protein production was expected within the 3-hour time frame. There was disorganized desmin staining in the cytoplasm and loss of staining at intercalated disks. Cardiotoxin arrays shortened, and overall reactivity decreased. There were no changes in intensity or distribution of titin, lamin-A/C, lamin-B2, and desmoplakin. Collectively, the changes resembled cardiomyocyte remodeling in chronically stunned myocardium.

Discussion

There are several new and important findings from our study. First, swine with viable dysfunctional myocardium exhibited alterations in structural proteins that, with the exception of lamin-A/C, are similar to those in humans with chronic hibernating myocardium. Second, these changes begin within 24 hours and become more pronounced as physiological changes of chronic hibernating myocardium develop. Struc-

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*P<0.05 vs baseline.
tural changes are present in stenotic as well as normally perfused regions of the same heart, and similar changes were produced within 3 hours after a transient elevation in LV end-diastolic pressure. Thus, the structural changes originally believed to reflect an adaptive response to chronic repetitive ischemia actually arise from elevations in preload that affect the cardiomyocyte phenotype globally.

Myocardial hibernation in humans and animal models results in characteristic ultrastructural changes in cardiomyocytes, consisting of myolysis and subsequent glycogen accumulation in the myolytic areas.1,4,8,20 Whereas these changes have been believed to reflect an adaptive response of the heart to ischemia, similar observations have been made in cardiac tissue after atrial fibrillation and mitral valve disease.21,22 In addition to these ultrastructural changes, hibernating myocardium is accompanied by changes in the distribution of structural proteins. These alterations have been hypothesized to be an adaptive response to ischemic stress through cardiomyocyte dedifferentiation.1,23 Previous interpretations in humans have, with 1 exception,24 been limited by the absence of control samples from remote nondysfunctional myocardium. Thomas et al8 found myolysis and glycogen accumulation to be independent of regional flow differences in swine with hibernating myocardium, indicating that ultrastructural adaptations were dissociated from regional ischemia. Similar observations were reported by Gunning et al24 in patients with hibernating myocardium in which myofibrillar loss in dysfunctional and normally perfused regions was similar.

Whether the dissociation between structural remodeling and hibernation is also true for the expression and distribution of structural proteins is not known, because previous studies on structural protein expression always compared the hibernating regions with normal nonischemic donor hearts. To the best of our knowledge, this is the first study in which the structural protein expression in the chronically dysfunctional region was compared with the normal nonischemic region of

Figure 7. Immunohistochemical analysis of protein expression after acute preload elevation. Expression of desmin at intercalated disks is reduced after preload elevation (top, arrowheads). Middle figures show decreased cardiotin expression and shortening of cardiotin arrays. Titin expression was unaltered. Inset, Detail of double-band cross-striations (arrowheads; ×800). Bottom shows no differences in lamin staining. Arrowheads indicate nuclei staining positive for lamin-B2 and lamin-A/C.
the same heart. Most of the changes that we observed in dysfunctional myocardium were similar to previous studies on structural remodeling in human cardiac disease. Loss of cardiac actin, disorganized expression of desmin in the cytoplasm, loss of desmin at the intercalated disks, loss of titin double striations, and reexpression of α-SM actin have all been described in humans with chronic hibernating myocardium.1,10 Surprisingly, the extent of alterations in structural protein expression was similar in both regions of the cardium.1,10 Interestingly, the most important finding of the present study was not the confirmation of cardiomyocyte adaptation in pigs with viable dysfunctional myocardium but rather the rapidity with which the remodeling takes place and the fact that it is present in both viable dysfunctional (stunned and hibernating) myocardium and the remote normally perfused regions of the same heart. In addition, cardiomyocyte remodeling could be observed within 3 hours after transiently elevated preload in the absence of ischemia. This dissociation between ischemic stress and cardiomyocyte remodeling is consistent with global myofibrillar loss in the porcine model.8 This has also been reported in humans and animal models by other laboratories. For example, Gunning et al24 observed global myolytic changes in viable dysfunctional myocardium that occurred in the presence of globally reduced LV function. In a dog model of repetitive stunning, Sherman et al28 reported myofibrillar disassembly in areas with reduced and normal flow. Taken together, these observations suggest the involvement of more global stress rather than regional ischemia in the cardiomyocyte remodeling. This is supported by an in vitro study that suggested that many of the structural changes associated with dedifferentiation did not depend on an ischemic environment.27

The inability to confirm quantitative differences in protein by Western analysis could reflect several possibilities. First, there was variability among animals that could have precluded our ability to identify 20% to 30% reductions in protein among groups. Detection of similar changes has been possible when paired samples from the same heart are analyzed, but the global nature of the structural protein changes precluded this type of analysis. Second, there is considerable heterogeneity among the cellular myocyte phenotype in hibernating myocardium; more than half of the cells in human and swine hibernating myocardium appear to be unaffected. Finally, we have previously demonstrated that the number of affected myocytes with histological myolysis (33%) greatly exceeds the myofibrillar volume loss (9.4%) in hibernating myocardium,8 and the latter changes cannot be quantified by Western blotting or Northern analysis for proteins such as β-myosin.10 Thus, the prominent histological changes demonstrated by immunohistochemistry are most consistent with cytoskeletal reorganization in response to stretch or elevated preload rather than indicating a quantitative alteration in the expression of structural proteins.

In summary, our results show that the expression of structural proteins, indicative of cardiomyocyte adaptation, changes progressively during the transition from stunned to hibernation. These changes are global and independent of flow reductions and can be reproduced rapidly after a transient elevation in preload in normal myocardium. Although further studies will be necessary to assess the precise mechanical stimulus initiating these changes, their presence in normally functioning myocardium as well as in stunned and hibernating myocardium makes it unlikely that the phenotypic alterations of myocytes play a role in determining the magnitude or time course of functional recovery after coronary revascularization of viable dysfunctional myocardium.

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References


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