Abstract—We tested the hypothesis that an acute critical limitation in coronary flow reserve could rapidly recapitulate the physiological, molecular, and morphological phenotype of hibernating myocardium. Chronically instrumented swine were subjected to a partial occlusion to produce acute stunning, followed by reperfusion through a critical stenosis. Stenosis severity was adjusted serially so that hyperemic flow was severely reduced yet always higher than the preocclusion resting level. After 24 hours, resting left anterior descending coronary artery (LAD) wall thickening had decreased from 36.3±4.0% to 25.5±3.7% (P<0.05), whereas resting flow had remained normal (67±6 versus 67±8 mL/min, respectively). Although peak hyperemic flow exceeded the prestenotic value, resting flow (45±10 mL/min) and LAD wall thickening (17.0±5.0%) progressively decreased after 2 weeks, when physiological features of hibernating myocardium had developed. Regional reductions in sarcoplasmic reticulum proteins were present in hibernating myocardium but absent in stunned myocardium evaluated after 24 hours. Histological analysis showed an increase in connective tissue along with myolysis (myofibrillar loss per myocyte >10%) and increased glycogen typical of hibernating myocardium in the LAD region (33±3% of myocytes from animals with hibernating myocardium versus 15±4% of myocytes from sham-instrumented animals, P<0.05). Surprisingly, the frequency of myolysis was similar in normally perfused remote regions from animals with hibernating myocardium (32±7%). We conclude that the regional physiological and molecular characteristics of hibernating myocardium develop rapidly after a critical limitation in flow reserve. In contrast, the global nature of myolysis and increased glycogen content dissociate them from the intrinsic adaptations to ischemia. These may be related to chronic elevations in preload but appear unlikely to contribute to chronic contractile dysfunction. (Circ Res. 2002;91:970-977.)

Key Words: hibernating myocardium ■ stunned myocardium ■ myocardial ischemia ■ myofibrillar loss

Regions of the heart supplied by chronic stenoses can develop chronic left ventricular (LV) dysfunction in the absence of infarction. Not all segments supplied by a stenosis develop viable dysfunctional myocardium, and there appears to be a critical relation to the physiological severity of a coronary stenosis (ie, coronary flow reserve or the ability to increase flow during vasodilation), which determines whether intrinsic adaptations to regional ischemia develop.1 Furthermore, there is great physiological and molecular diversity in the adaptive responses that are produced in response to reversible ischemia. At one extreme is hibernating myocardium, characterized by chronic regional reductions in resting flow and function.2-6 These physiological changes occur in the absence of acute metabolic evidence of ischemia or pathological evidence of infarction. When stenosis severity is less marked, chronically stunned myocardium with normal resting flow develops.7,8 At the molecular level, chronic stunning can be distinguished from hibernating myocardium by the relative lack of reductions in sarcoplasmic reticulum (SR) Ca2+-ATPase and phospholamban, which are characteristic of hibernating myocardium.9,10 In addition, hibernating myocardium appears to have an increase in the number of myocytes having increased glycogen stores and expressing structural proteins characteristic of dedifferentiation.11,12

Although serial studies of juvenile swine with a chronic stenosis have demonstrated a systematic progression from chronically stunned to hibernating myocardium,4,8 the extent to which these changes are unique to the growing swine model or are dependent on the physiological severity versus chronicity of a coronary stenosis remains unclear. We hypothesized that the severity of stenosis is the key physiological variable that drives the myocyte adaptation to ischemia, and we also hypothesized that the progression to hibernating myocardium is independent of growth and can occur in
mature swine. To critically test these hypotheses, we determined whether we could reproduce the physiological, molecular, and cellular characteristics of chronic hibernating myocardium by acutely restricting vasodilated perfusion. We used a hydraulic stenosis that could be maintained over a period of weeks in chronically instrumented swine. Swine were evaluated at frequent intervals to confirm that the stenosis critically limited peak hyperemic flow. Importantly, we avoided any circumstance in which the stenosis might cause prolonged acute ischemia, as reported in previous models of “short-term hibernation.”13-18 The results demonstrate that the physiological and molecular changes typical of hibernating myocardium can be induced within days after an acute critical stenosis. Although the myolytic phenotype also develops rapidly, the morphological changes occur globally and are dissociated from the regional adaptive responses in hibernating myocardium.

Materials and Methods
A total of 23 swine were chronically instrumented with catheters and hydraulic occluders, as previously described19 and detailed in an online Materials and Methods section (available in the data supplement at http://www.circresaha.org). In brief, we performed a left thoracotomy and inserted catheters into the aorta and left atrium. A micromanometer was placed into the LV apex, and a volumetric flow probe was placed on the proximal left anterior descending coronary artery (LAD). Ultrasonic crystals were inserted to measure wall thickening. Two hydraulic occluders were placed on the LAD. The primary occluder was used to produce a critical coronary stenosis, which was maintained throughout the study. A secondary occluder was used to produce brief LAD occlusions to confirm the attenuation of reactive hyperemia. The chest was closed, the pneumothorax was evacuated, and analgesics were administered until the animals recovered.

Study Protocol
Studies were conducted with the animals under propofol sedation. After assessment of baseline variables, acute stunning was produced by a 15-minute partial occlusion of the LAD using the primary occluder. Subsequently, hearts were reperfused through a critical stenosis that abolished the reactive hyperemic response to a 20-second occlusion yet allowed peak LAD flow to increase above the preocclusion value. Microspheres were injected to assess the transmural distribution of flow at rest, during a transient total LAD occlusion (to assess collateral flow), and after adenosine vasodilation. In 9 animals, we removed hearts for tissue sampling after 24 hours. The remaining studies were continued for ~2 weeks. In the latter group, serial measurements of flow, function, and hemodynamics were assessed at 1- to 3-day intervals. When necessary, the primary occluder was readjusted to achieve a critical coronary stenosis.

Myocardial Sampling
At the end of the protocols, animals were anesthetized, and flash-frozen subendocardial samples were obtained from the LAD and normal regions for protein and RNA isolation. We assessed regional changes in the expression of SR Ca2+-ATPase, phospholamban, and calsequestrin.9,19 Samples were also obtained to assess microsphere perfusion in 3 transmural layers of the LV.

Histological Analysis
Connective tissue was assessed by point counting, and infarction was excluded by triphenyltetrazolium chloride (TTC) staining.5,19 In animals evaluated for 2 weeks, small subendocardial samples were processed for electron microscopy (EM) and light microscopy and analyzed in a blinded fashion (M.B.).20 At least 200 cells per sample were analyzed. Because the space, formerly occupied by sarcomeres, was occupied by glycogen, quantification of myolysis was performed by planimetrically evaluating the percentage of PAS-positive material per cell. If sarcomere depletion was >10%, cells were classified as being affected by myolysis.20 EM was used to confirm the structural alterations, such as sarcomere depletion and glycogen accumulation, demonstrated on light microscopy.

To evaluate the transmural distribution of myolysis, we also quantified myofibrillar volume loss by use of blinded analysis (G.S.) and a point-counting grid. Trichrome-stained sections were used to identify myocytes, and grid intersections that were not stained in the perinuclear area (myolysis) were expressed as a percentage of the intersections that were overlying myofibrils. Myofibrillar loss was analyzed in subendocardial and subepicardial regions of sham-instrumented animals (sham animals) and animals instrumented for 24 hours and 2 weeks (24-hour and 2-week animals, respectively).

Statistical Analysis
Hemodynamics and wall thickness measurements were recorded (Gould model TA11) and digitized (sampling rate, 500 Hz). Values reported are the mean±SEM. Time-dependent changes were evaluated by ANOVA, with post hoc paired t tests comparing data at each time point with the corresponding baseline. Perfusion in stenotic LAD regions was also compared with that in remote left circumflex (LC) myocardium. A value of P<0.05 was considered significant.

Results
Animals were in good health at the time of study. Of the 23 animals initially instrumented, 7 were excluded from analysis by experimental design. In 3 animals, we were unable to maintain a stable occlusion, and the lack of a significant reduction in flow reserve prevented the development of depressed LAD function. In 4 others, there was TTC evidence of infarction that averaged 5.5±1.0% of LV mass.

Serial Flow, Function, and Hemodynamics
Figure 1 illustrates representative analog recordings from an animal undergoing serial evaluation for the 2-week period. Hemodynamics, flow, and function at selected time points are summarized in the Table. A 15-minute partial occlusion resulted in acute myocardial stunning, with LAD wall thickening decreasing from 36.3±4.0% to 24.0±3.3% (P<0.01), whereas remote-zone LC function remained normal (29.3±4.0% versus 30.9±4.1%). Although flow was somewhat lower than baseline, peak hyperemic flow after a total occlusion exceeded the initial baseline, confirming that the stenosis was not flow limiting. When the heart was reperfused through a critical stenosis, function did not return to normal. After 24 hours, LAD wall thickening averaged 25.5±3.7% (P<0.05 versus baseline), but LAD flow was normal; these findings are consistent with sustained myocardial stunning. There were no significant differences in hemodynamics, and there was no infarction as assessed by TTC.

Serial measurements of flow and function in animals completing the 2-week study protocol are summarized in Figure 2, and hemodynamic measurements are summarized in the Table. The physiological severity of the stenosis varied little, and initial peak reactive hyperemic flow increased to less than twice the preocclusion baseline flow at all time points. There was a progressive reduction in LAD wall thickening, which was initially associated with normal resting perfusion, consistent with myocardial stunning. After ~1 week, resting flow decreased and remained so for the duration
of the study. In animals studied after 2 weeks, flow was 45±10 mL/min (P<0.05 versus preocclusion), and wall thickening averaged 17.0±5.0% (P<0.05). The reduction in flow developed despite the fact that hyperemic flow always exceeded the preocclusion value at rest. These findings indicate that an acute critical stenosis caused a rapid progression from stunned to hibernating myocardium within 1 week. Reductions in function preceded reductions in resting flow and occurred in the presence of residual vasodilator reserve.

To confirm that the reductions in flow were indicative of reductions in tissue perfusion, we assessed regional flow with microspheres. Figure 3 summarizes serial resting flow measurements in animals evaluated for 2 weeks. Before occlusion, resting flow was similar in the LAD and normal LC regions. Although function was reduced after 24 hours, microsphere flow was similar in LAD and remote regions, which is consistent with stunning. After 2 weeks, there was a reduction in resting LAD flow to 1.03±0.08 versus 1.39±0.09 mL·min⁻¹·g⁻¹ in normal regions (P<0.05). Flow was reduced in each myocardial layer (P<0.05) and was consistent with a progression from stunned to hibernating myocardium.

Figure 4 summarizes transmural variations in adenosine flow in the presence of a LAD stenosis. After application of a critical stenosis with the primary occluder (Figure 4, top), adenosine flow was similar to the initial baseline value (1.34±0.19 versus 1.14±0.07 mL·min⁻¹·g⁻¹, respectively; P=NS), with no significant increase in any transmural layer. After 24 hours (Figure 4, middle), the physiological severity of the stenosis had decreased, but full-thickness vasodilated flow still did not significantly increase above resting flow (2.46±0.83 [adenosine] versus 1.28±0.12 [baseline] mL·min⁻¹·g⁻¹ at rest, P=NS). Subendocardial flow continued to be critically reduced (1.79±0.65 versus 1.28±0.18 mL·min⁻¹·g⁻¹). After 2 weeks, adenosine flow was similar to that after 24 hours (2.25±0.46 [full thickness] versus 1.62±0.43 [subendocardium] mL·min⁻¹·g⁻¹). Flow measurements during a total LAD occlusion at 24 hours (0.10±0.06 mL·min⁻¹·g⁻¹) and 2 weeks (0.25±0.17 mL·min⁻¹·g⁻¹, P=NS versus 24 hours) were severely reduced, indicating little change in coronary collateral flow over time.

**Protein and RNA Changes**

Figure 5 summarizes SR protein changes in hearts from swine with hibernating myocardium. Subendocardial samples from the LAD region demonstrated reductions in phospholamban (2.12±0.61 [LAD region] versus 3.52±0.62 [normal region]...
densitometric units, \( P=0.06 \) and SR Ca\(^{2+}\)-ATPase (7.30\(\pm\)0.49 [LAD region] versus 9.81\(\pm\)0.31 [normal region] densitometric units, \( P<0.05 \)). In contrast, there were no regional changes in calsequestrin protein, indicating that the reductions in the SR Ca\(^{2+}\) uptake proteins were not related to a nonspecific loss of SR. There were no systematic changes in any SR protein levels in samples taken after 24 hours. Nevertheless, Northern analysis after 24 hours demonstrated reductions in mRNA levels for phospholamban (0.17\(\pm\)0.03 [LAD region] versus 0.23\(\pm\)0.03 [normal region] densitometric units, \( P<0.05 \)) and SR Ca\(^{2+}\)-ATPase (0.94\(\pm\)0.11 [LAD region] versus 1.13\(\pm\)0.07 [normal region] densitometric units, \( P<0.05 \)), with no changes in calsequestrin mRNA. Collectively, these findings suggest that spontaneous ischemia arising distal to a critical stenosis initiates a transcriptional cascade that results in a regional downregulation in the expression of selected SR proteins during the progression to a physiological phenotype of hibernating myocardium.

### Histological Analysis

Point counting demonstrated a regional increase in connective tissue similar to that in humans with hibernating myocardium. After 24 hours, connective tissue was 6.0\(\pm\)1.5\% in the LAD region versus 2.7\(\pm\)0.4\% in normal regions (\( P=0.06 \)). In the group with hibernating myocardium, connective tissue averaged 10.6\(\pm\)4.7\% in the LAD region versus 7.3\(\pm\)5.4\% in normal regions.

Figure 6 shows PAS/toluidine blue–stained sections from LAD and normal regions along with corresponding EM from swine with hibernating myocardium versus sham control swine. PAS-positive areas (increased glycogen) corresponded to regions of myofibrillar loss (myolysis) by EM. Interestingly, myolysis was present in hibernating LAD regions and in normally perfused remote regions of swine that were evaluated 2 weeks after instrumentation. These changes stood in marked contrast to the lack of myolysis or PAS positivity in sham hearts. Myolytic myocytes averaged 33\(\pm\)3\% in hibernating LAD regions versus 15\(\pm\)4\% in sham control.
regions ($P<0.05$). The frequency of myolysis was identical to that in normally perfused nondysfunctional regions of swine with hibernating myocardium, which averaged 32% ($P=NS$ versus LAD of 2-week animals).

Figure 7 summarizes myofibrillar volume loss on a transmural basis for 24-hour, 2-week, and sham animals. Subendocardial myofibrillar loss was similar in LAD and remote regions of 2-week animals (9.4±1.5% versus 8.1±0.5%, respectively; $P=NS$) but was markedly higher than that in sham control animals (2.0±0.2% versus 1.9±0.2%, respectively; both $P<0.05$ versus 2-week animals). Myofibrillar loss was also increased in subepicardial layers of 2-week animals but was not different between regions or corresponding subendocardial values. Myofibrillar loss had already begun after 24 hours but was intermediate between values of 2-week and sham animals. Thus, these data dissociate myofibrillar loss from the regional physiological and protein changes characteristic of hibernating myocardium.

**Discussion**

There are several important new findings from the present study. First, hibernating myocardium develops within days after a sudden critical impairment in coronary flow reserve. Reductions in contractile function with normal resting perfusion precede the development of reductions in resting flow and are consistent with a rapid progression from stunned to hibernating myocardium. Second, like the regional physiological changes, regional alterations in SR protein expression also develop in an accelerated fashion and go hand in hand with the progression from stunned to hibernating myocardium. In sharp contrast, the cellular morphological changes of myolysis and increased glycogen that have been ascribed to hibernating myocytes from biopsies of dysfunctional regions in humans actually occur globally. Myofibrillar volume loss is similar in subendocardial and subepicardial layers. Although it is already present after 24 hours, it increases globally during the progression to hibernating myocardium. The dissociation of the myolytic cellular phenotype from the regional physiological and molecular changes indicates that these morphological alterations are unrelated to the adaptive responses characteristic of hibernating myocardium.

**Relation to Previous Studies of Chronic Hibernating Myocardium**

The present study demonstrates that the time frame over which adaptive responses characteristic of hibernating myocardium develop can be quite rapid and is primarily related to the physiological significance of a coronary stenosis rather than time. This contrasts with the slow progression of these adaptations in juvenile swine instrumented with a fixed LAD stenosis. In the latter model, the cross-sectional area of the proximal LAD is fixed and progressively limits maximal...
flow. As LV mass increases with growth, there is a gradual but progressive reduction in maximal flow per gram of tissue that reaches a critical level after ≈3 months and remains stable thereafter.21 Although this model also results in a progression from stunned to hibernating myocardium that is accompanied by regional reductions in selected SR proteins,10 the adaptive responses develop over a much more protracted time frame. Because domestic swine grow rapidly during the first 3 months, the physiological adaptations could potentially be unique to the juvenile animal. The present study excludes this possibility and indicates that hibernating myocardium can develop over a time frame as short as 1 week. Thus, the temporal progression from stunned to hibernating myocardium is most closely related to the physiological severity of flow impairment.4,8,9,21 We have also accelerated the development of hibernating myocardium by limiting source collateral flow with an LC stenosis in a 2-vessel chronic stenosis model of hibernating myocardium.6 Although speculative, flow reserve is likely an integrative measure of the propensity of a region of the heart to develop spontaneous ischemia, which increases as the ability of the myocardium to increase perfusion above resting values becomes more limited.

The critical nature of coronary flow reserve reduction required to produce hibernating myocardium likely explains why not all studies have demonstrated a progression to hibernating myocardium distal to a chronic stenosis. For example, circumflex ameroid models (rapidly progressing stenoses) in dogs usually have normal resting function, with a well-developed collateral circulation, yet hibernating myocardium can develop when source collateral flow is limited.2,22 Bolukoglu et al23 produced a near-critical stenosis in swine, but flow reserve was not continuously monitored, and hyperemic flow at the end of the study was not lower than control. Although regional function was depressed in this model, resting perfusion was normal, consistent with chronic

myocardial stunning.24 Contractile function in the absence of infarction in swine ameroid occluder models is equally as variable, with most studies reporting normal resting function25–27 and 1 study reporting evidence of chronic myocardial stunning.7 The variability of function in circumflex ameroid models probably reflects several factors. First, there is considerable variability in the time course of ameroid occlusion, with some studies even demonstrating a lack of total occlusion.7 Second, when vasodilated flow was measured, there was only a modest difference in maximal perfusion between collateral-dependent and normal regions,28,29 which probably reflects the increase in perfusion from extracardiac collaterals. Although this limits infarction, it does not lead to the degree of spontaneous ischemia necessary to produce chronic myocardial dysfunction. Thus, variability in coronary flow reserve is likely the primary cause of different intrinsic adaptive responses of the myocardium to ischemia.

Relation to Previous Studies of Short-Term Hibernation

Previous investigators have attempted to use animal models of prolonged acute ischemia to produce viable dysfunctional myocardium, a phenomenon that has been termed short-term hibernation.30 Matsuzaki et al31 showed that the myocardium could tolerate several hours of moderate ischemia without the development of infarction. Other laboratories have demonstrated that this ability to match flow and function at a reduced level prevents further high-energy phosphate depletion with a resolution of lactate release.14,15,18,31 The original concept of hibernating myocardium proposed that such a precarious low-flow state could be maintained indefinitely without leading to irreversible injury.32 However, animal studies have demonstrated that irreversible injury ultimately develops in these models. For example, Chen et al32 found TTC evidence of infarction in the majority of animals subjected to short-term hibernation for a period that exceeded 24 hours, with rates of subendocardial myocyte apoptosis approaching 10%. Kudej et al17 demonstrated myocyte necrosis after >90 minutes of moderate ischemia in conscious swine. After 24 hours, patchy necrosis involved up to 40% of the subendocardium. Schulz et al33 found necrosis to be infrequent after 12 hours but common after 24 hours of moderate ischemia in anesthetized swine. Thus, although the ability of the heart to tolerate moderate ischemia is better than the ability of the heart to tolerate total coronary occlusion, it is still time-limited.

Our findings differ from studies evaluating short-term hibernation in several important respects. First, we adjusted the physiological significance of the stenosis to allow flow during vasodilation to exceed the preocclusion resting flow. This strategy avoided uncertainty in interpreting whether reductions in resting flow and function were simply consequences of acute ischemia. In this regard, the present study clearly shows that dysfunction with normal resting flow (myocardial stunning) preceded the development of hibernating myocardium and confirms a similar progression demonstrated in other animal models.2,8,22
Reductions in SR Proteins in Viable Dysfunctional Myocardium

Our results demonstrate that a critical coronary stenosis initiates transcriptional changes that downregulate the expression of SR Ca$^{2+}$-ATPase and phospholamban within 24 hours. The regional reductions in mRNA contrast with the lack of change in expression that we$^{19}$ and others$^{34–37}$ have demonstrated after single episodes of reversible ischemia. However, they are remarkably similar to the progression that we have reported in growing swine with a fixed LAD stenosis leading to hibernating myocardium.$^{9,10}$ Reductions in mRNA and protein levels for SR Ca$^{2+}$-ATPase and phospholamban began in chronically stunned myocardium after 2 months and decreased further with the progression to hibernating myocardium after 3 months. A similar acceleration in this phenotypic pattern was also seen in swine instrumented with 2-vessel stenoses.$^{6}$ The consistency of these observations in 3 different models of a chronic stenosis supports the notion that regional reductions in SR Ca$^{2+}$-ATPase and phospholamban are at least partly responsible for altered contraction in hibernating myocardium. Although speculative, these changes may lead to an altered calcium responsiveness similar to that in acutely ischemic myocardium.$^{38}$

Dissociation of Myolysis and Regional Adaptations in Hibernating Myocardium

Our results indicate that myolysis develops rapidly after a critical limitation in flow reserve and is consistent with the acute myofibrillar disassembly and increased glycogen reported after short-term hibernation by Sherman et al.$^{36}$ Like acute myofibrillar disassembly, myofibrillar loss and increased glycogen content occurred globally. Quantitative analysis demonstrated similar values in hibernating as well as normally perfused remote regions of dysfunctional hearts. Differences in regional function were not the result of myofibrillar loss because both subendocardial and subepicardial values were similar in dysfunctional and normal remote regions. Thus, the pathological changes described in humans with hibernating myocardium do not appear to reflect the chronicity of LV dysfunction nor do they appear to arise as a direct consequence of ischemia.

A limitation of most clinical studies has been that the biopsy material has been obtained from dysfunctional regions and that an internal control sample from remote myocardium has not been analyzed. Interestingly, Gunning et al.$^{19}$ have recently reported global myolysis in humans with viable dysfunctional myocardium, depressed ejection fraction, and symptomatic heart failure. The fact that myolysis occurs globally in ischemic cardiomyopathy raises the possibility that these morphological changes reflect a response to chronic elevations in preload or stretch. In this regard, we found that LV end-diastolic pressure progressively increased from 16 to 24 mm Hg at the end of the study (Table). This possibility is also supported by the similarity of the changes in LV myocytes to those in atrial myocytes from models of atrial fibrillation.$^{40}$ Interestingly, conversion of the rhythm failed to reverse the myolytic changes in atrial muscle and was associated with chronic increases in atrial filling pressure. Thus, although these cells may revert to a fetal pheno-typic of development under global increases in LV pressure, repetitive regional ischemia requires a sustained period of structural and functional remodeling to develop.

References


Dissociation of Regional Adaptations to Ischemia and Global Myolysis in an Accelerated Swine Model of Chronic Hibernating Myocardium

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Dissociation of Regional Adaptations to Ischemia and Global Myolysis in an Accelerated Swine Model of Chronic Hibernating Myocardium

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**Supplementary On Line Methods**

All procedures were performed in accordance with the institutional guidelines for the humane use of animals in research. Twenty-three pigs (57.6 ± 2.5 kg) were fasted overnight and premedicated with a tiletamine (100 mg/ml)/xylazine (100 mg/ml) mixture (0.037 ml/kg i.m.). After intubation a surgical plane of anesthesia was maintained with an isoflurane (1-3%)/oxygen (balance) mixture. Using sterile technique, the heart was exposed through a left thoracotomy in the 3\(^{rd}\) or 4\(^{th}\) intercostal space. The experimental preparation is summarized in Figure 1. Pressures were measured via angiocatheters secured to Teflon pledgets placed into the left internal mammary artery and the left atrium. In some animals, an angiocatheter was also placed into the pulmonary artery for administration of drugs. Left ventricular pressure was measured via a high fidelity micromanometer (Konigsberg P6.5 transducer) inserted into the left ventricular apex. The proximal left anterior descending (LAD) coronary artery was dissected free from its surrounding tissue and instrumented with two hydraulic occluders. An ultrasonic volumetric flow probe, (3-4 mm diameter, Transonic Systems Inc., Ithaca, NY) was positioned between the two occluders to measure phasic and mean LAD blood flow (Figure 1). Regional wall thickening measurements were obtained using ultrasonic transit time measurement and a single epicardial crystal system (Wall Tracker, Crystal Biotech Inc., Hopkinton, MA) implanted on the left ventricular free wall. This technique is a modification of the original Doppler displacement system described by Hartley et al.\(^1\) with the exception that there is a feedback system that adjusts the sample volume in response to the integrated velocity of echoes passing through the sample volume\(^2\). Once determined, range gate depth was kept constant throughout the study. Crystals were placed in the anteroapical free wall to measure ischemic zone wall thickening and in the posterobasal free wall
supplied by the left circumflex artery to measure normal zone wall thickening. All instrumentation was routed through the chest wall at the 6th-7th intercostal space. The chest was closed and the pneumothorax was evacuated. Analgesia was provided using an intercostal nerve block (0.5% Marcaine) and butorphanol (0.025 mg/kg/i.m.prn). Prophylactic antibiotics (gentamicin, 60 mg i.v. and cephalothin 500 mg i.v.) were given prior to surgery and repeated once immediately after closing the chest. All externally accessible instrumentation was secured within a jacket placed on each pig. Catheters were flushed at regular intervals (Heparin, 1000 units/ml) and pigs were given aspirin (325 mg p.o.). Studies were conducted in the closed-chest state after allowing the animals to recover for at least 1 week.

**Experimental Protocols**

**Initial Study Day** - At the time of study, pigs were sedated with a tiletamine/xylazine mixture (0.037 ml/kg i.m.). Sedation was then maintained for the remainder of the study using propofol (10 mg/ml, 25-45 ml/hr i.v.). We initially assessed resting microsphere flow along with function and hemodynamics. Subsequently, pigs were subjected to a single episode of reversible ischemia produced by a 15-minute partial occlusion of the LAD where wall thickening was reduced to approximately 60% of the resting values. At the end of the occlusion, we partially released the primary occluder and restricted reactive hyperemic flow to produce a critical coronary stenosis as previously described\(^3\). The heart was reperfused for 15-minutes with the critical stenosis in place after which a second set of microspheres was injected. Using the secondary occluder, we confirmed a critical attenuation of the reactive hyperemic response after a 20-second total LAD occlusion. If necessary, the primary occluder was readjusted until peak reactive hyperemic flow was between 1 and 1.5 times the initial baseline flow level. Once a stable level of stenosis was obtained, regional flow during vasodilation was evaluated during an adenosine infusion (0.45 ±
0.06 mg/kg/min) while phenylephrine (100-200 µg/min i.v.) was co-infused to maintain systolic pressure near resting values. The doses of adenosine and phenylephrine employed in each pig were determined during a study conducted at least 72 hours before each protocol began. At this time we confirmed that the adenosine dose abolished the reactive hyperemic response in the absence of a coronary stenosis. At the end of the study, propofol was stopped, after which the pigs rapidly awoke and were returned to their cage.

24-hour group - In the first experimental series, pigs were subjected to a critical coronary stenosis for 24-hours after which a terminal study was performed under propofol sedation. After 24-hours, we assessed hemodynamics and function along with microsphere flow at rest, during adenosine vasodilation (with phenylephrine infused to maintain arterial blood pressure), and during a 90-second total LAD occlusion to assess collateral flow. The pigs were then deeply anesthetized with isoflurane and euthanized with a left atrial bolus of KCl. The heart was excised for sectioning. The left ventricle was weighed and cut into contiguous concentric rings for microsphere flow measurements. Central core samples from LAD and normal regions were subdivided into subendocardial, mid-myocardial, and subepicardial layers. Additional samples were obtained for RNA, protein, and histological analyses. Samples for RNA and protein analyses were flash frozen in liquid nitrogen and stored at -80°C until analyzed. Finally, thin concentric rings were stained with triphenyltetrazolium chloride (TTC, 1 gm TTC/100 ml phosphate buffer) to exclude the possibility of myocardial necrosis. Rings were scanned, digitized, and any area of TTC negativity quantified (Sigma Scan, Jandel Scientific, San Rafael, CA).

2-week group - The initial 24-hours of the study was conducted as outlined in the previous section. Because of the limited number of microspheres available, only resting flow was assessed at 24 hours in this group. After we completed the measurements at the 24-hour time point, the
primary LAD occluder was, if necessary, readjusted to reduce hyperemic flow to be between 1 and 1.5 times the initial resting flow level. The animals were brought back to the laboratory at 1-3 day intervals and serial measurements of resting flow (ultrasonic flow probe), function, and hemodynamics assessed under propofol sedation. At each study, we reassessed the physiological severity of the chronic LAD stenosis by inflating the secondary occluder for 20-seconds. If there was an increase in peak hyperemic flow, we readjusted the primary occluder until a critical level of stenosis (dilated flow 1 to 1.5 times the initial resting flow level on day 1) was reestablished.

After approximately 2-weeks (14.7 ± 0.3 days) pigs were brought back for a terminal physiological study. The LAD stenosis was not readjusted and hemodynamics, function and microsphere perfusion were assessed at rest, following adenosine vasodilation and after a 90-second total LAD occlusion to assess collateral flow as outlined for the studies completed after 24-hours. At the end of the physiological measurements, the animals were deeply anesthetized with isoflurane and euthanized with KCl.

**Role of acute ischemia in the temporal development of regional contractile dysfunction** – Two additional animals were studied to determine the importance of the initial 15-minute partial coronary occlusion on the development of left ventricular function. We placed a critical stenosis to limit the reactive hyperemic response without producing a period of transient ischemia and performed serial measurements of resting function.

**Myocardial Flow Measurements**

Regional myocardial blood flow was determined by injecting fluorescent microspheres suspended in saline with thimerosal (0.02%) and Tween 80 (0.02%) as previously described\(^4\,^5\). Briefly, microsphere suspensions were sonicated and vortex-agitated immediately before injection. Approximately 2 million microspheres (15 μm diameter), labeled with one of up to seven different
fluorescent dyes (red, blue, green, orange, crimson, blue-green, yellow-green; Triton Technology, San Diego, CA) were administered as a left atrial bolus flushed with saline. An arterial reference sample (via the internal mammary artery catheter) was started prior to microsphere injection at a rate of 6.0 ml/min and continued for 90 seconds.

Flow was assessed in an apical concentric ring divided into contiguous wedges. Each wedge was subdivided into three transmural layers of approximately equal thickness. The microspheres employ fluorescent dyes that absorb light and produce fluorescent signals at selected wavelengths. To quantify the amount of fluorescence emitted by each microsphere, myocardial samples were weighed, digested in 4M KOH, and poured through a Millipore filter (pore size, 10 µm). The filter along with trapped residues were allowed to dry in an Eppendorf tube into which a volume of 1 ml of Di(ethylene glycol) ethyl ether acetate 98% (CAS # 112-15-2) was subsequently added. The tube was then vortexed to elute the fluorescent dye from the filter. Following centrifugation for several minutes, the fluorescent samples were each read at selected pairs of emission and excitation wavelengths ranging from 350-700 nm incorporating the principle intensity peak for each pure fluorescent dye, using a luminescence spectrometer (model LS50B, Perkin Elmer, Buckinghamshire, England). The same procedure was used to analyze the myocardial reference blood samples obtained during the experiment. Using the intensity and flow rate of the arterial reference sample and myocardial intensity per unit tissue weight, regional myocardial perfusion was calculated as follows:

\[ Q_{\text{sample}} = \frac{\text{Int}_{\text{sample}} \times Q_{\text{reference}}}{\text{Int}_{\text{reference}}} \]

where:

\[ Q_{\text{sample}} = \text{the flow (ml/min/g) in the tissue sample} \]

\[ \text{Int}_{\text{sample}} = \text{the intensity of a given dye eluted from the tissue sample} \]
\[ \text{Int}_{\text{reference}} = \text{the intensity of a given dye eluted from the blood reference} \]

\[ \text{Q}_{\text{reference}} = \text{the reference blood sample withdrawal rate (ml/min)}. \]

**Point Counting of Connective Tissue**

Samples from the LAD perfused region and the normally perfused region were taken for histological analysis. The samples were immersed in Z-fix (Anatech Ltd) and later sectioned and stained with Masson or Gomori trichrome stain. Connective tissue staining was quantified with standard point counting techniques using a 121-point grid at a magnification of x200 as previously described\(^6\). Connective tissue area was expressed as a percent of the total of myocyte and connective tissue area.

**Quantitation of Myolysis by Light and Electron Microscopy**

In animals evaluated for 2-weeks, we obtained small subendocardial samples for electron and light microscopy which were analyzed in a blinded fashion (M.B.)\(^7\). These were compared to subendocardial samples from size-matched sham control pigs (n=7, weight 65.6±2.9 kg in shams vs. 65.5±5.8 kg in 2-week hibernating animals). These samples were fixed for at least 24 hours in 3% glutaraldehyde, buffered to pH-7.4 with 90 mM KH\(_2\)PO\(_4\), washed in the same buffer and post-fixed for 1-hour in 2% osmium tetroxide, buffered to pH-7.2 with veronal acetate. Fixed tissue blocs were dehydrated in a graded series of ethanol and embedded in epoxy resin. Two-micron thick sections were cut and stained for light microscopic examination. The staining method used, periodic acid Schiff (PAS) followed by toluidine blue, permits independent visualization of glycogen and the contractile elements of cardiomyocytes respectively. At least 200 cells per sample were analyzed and only those cells in which the nucleus was visible in the plane of observation were included in the analysis. Cardiomyocytes were planimetrically scored for the
degree of myolysis (sarcomere loss). Since the space, formerly occupied by sarcomeres, was filled up by glycogen, quantification of the myolysis was done by planimetrically evaluating the percentage of PAS-positive material per cell. If the sarcomere depletion accounted for more than 10%, cells were classified as affected by myolysis. The 10% cutoff is identical to the one used in previous human studies of myolysis in hibernating myocardium\textsuperscript{7,8}. Ultrathin sections were stained with uranium acetate and lead citrate before examination in the electron microscope (Philips CM 100). This method was used to qualify and confirm the structural alterations, such as sarcomere depletion and glycogen accumulation, demonstrated on light microscopy.

**Quantitation of Regional and Transmural Myofibrillar Loss**

While myolysis has been conventionally quantified from glutaraldehyde fixed sections and biopsies in humans, it is dependent upon identifying relative increases glycogen content, which is depleted in formalin fixed samples. Accurate quantitation of myofibrillar volume loss is limited by the small sample size available and the fact that only subendocardial samples were obtained. We therefore developed a complementary approach to assess relative myofibrillar volume loss in viable dysfunctional myocardium that could be quantified from formalin fixed samples. Since increases in glycogen correspond to a loss of myofibrils using trichrome or hematoxylin eosin stains, we used a point counting technique to assess the relative percentage of the myocyte that was devoid of myofibrils in a fashion similar to that which we have described to quantify connective tissue\textsuperscript{6}. We evaluated transverse myocytes from full-thickness LAD and normal histological sections that were fixed in formalin and stained with Masson’s trichrome. The myocyte intersections on a 121 point grid were quantified as the grid was progressively moved across the myocardial wall at a magnification of 200x. Intersections of the grid with connective tissue and vascular structures were excluded. Intersections with unstained perinuclear areas
within myocytes (reflecting myofibrillar loss) and those that intersected myofibrils were quantified in more than 40 fields from each section (average area 9.9 mm$^2$). Myofibrillar loss was expressed as a percent by dividing unstained perinuclear intersections by the number of intersections overlying myofibrils times 100. Data were analyzed separately for the inner and outer half of the myocardial wall to evaluate transmural variations in myolysis. Both LAD and remote tissue was analyzed in each heart. We compared values among sham, 24-hour and 2-week animals to evaluate the temporal progression of myofibrillar loss.

**RNA Isolation and Northern Analysis**

Total myocardial RNA was isolated from flash frozen subendocardial samples by guanidinium thiocyanate/phenol-chloroform extraction$^9$. Pairs of RNA samples from the ischemic and normal regions of the same heart were handled in parallel. Aliquots of 20 µg of total RNA were electrophoresed on 0.8% agarose formaldehyde gels. The RNA was vacuum blotted to a nylon membrane (Zeta-Probe GT, BioRad) and fixed to it by ultraviolet light. Hybridization was performed for approximately 16 hours with selected cDNA probes labeled with ($\alpha$-$^{32}$P) dCTP. The hybridized membranes were washed under appropriate conditions of stringency and then exposed as outlined below.

Probes were synthesized as previously described$^{10}$. Briefly, reverse transcription of porcine mRNA yielded the porcine first strand complementary DNA. Amplification of the cDNA strand by PCR with primers for the specific nucleotide sequence coding for each protein generated the corresponding double stranded cDNA probe. The cDNA was inserted into a vector (PCR-TRAP kit from GenHunter or a TA Cloning kit from Invitrogen) and transformed into *Escherichia coli*. The hybridization probes for the SRCa$^{2+}$ ATPase, calsequestrin, phospholamban, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were labeled by PCR in the presence of ($\alpha$-
\( ^{32}\text{P} \) dCTP (30 cycles at 94°C, 30 seconds; 50°C, 1 minute; 72°C, 2 minutes) as previously described \(^{10-14}\).

The hybridization signals (beta emissions) were quantified on a Phosphorimager (Molecular Dynamics, Inc., Sunnyvale, CA). For each heart, the relative change in mRNA was expressed by dividing the normalized volume (area of the band times the average optical density) from the ischemic region by the normalized volume from the control region. To control for small variations in RNA loading, the volume of each band was divided by the volume of its corresponding GAPDH signal. The membranes were then stripped in boiling 0.1X standard saline citrate and 0.5% sodium dodecyl sulfate for 40 minutes before subsequent rehybridization with another probe. No more than three cDNA probes were hybridized to a given membrane.

**Protein Isolation and Western Blotting**

Protein was isolated from flash-frozen subendocardial samples using an extraction buffer containing (in mmol/L) Tris (pH 7.4) 20, NaCl 50, EDTA (pH 8) 1, \( \beta \)-mercaptoethanol 5, sodium vanadate 0.2, and phenylmethylsulfonyl fluoride 0.2, as well as 1 \( \mu \)g/mL pepstatin, 0.5 \( \mu \)g/mL leupeptin, and 10% SDS. Total protein (50-200 \( \mu \)g/lane) was electrophoresed and separated on a 12% SDS-polyacrylamide gel and transferred to the PVDF membrane Immobilon-P (Millipore, Corp., Bedford, MA). Membranes were blocked by soaking them in 5% nonfat dry milk in PBS, followed by 5 rinses in PBS. This was followed by a 1-hour incubation period overnight with a mouse monoclonal antibody specific for canine SR Ca\(^{2+}\) ATPase (MA3-919, Affinity Bioreagents, Golden, CO) at a dilution of 1:1500 in TBS-T with 4% milk, followed by incubation with goat anti-mouse IgG (1:10,000 in TBS-T) as the secondary antibody. After thorough washing, bands were visualized with a TMB membrane peroxidase substrate (Kirkegaard and Perry, Gaithersburg,
Background-subtracted signals were quantified on a laser densitometer (Bio-Rad, Melville, NY).

Membranes were also incubated overnight with an anti-canine calsequestrin rabbit polyclonal antibody (06-382, Upstate Biotechnology, Lake Placid, NY), and an anti-canine phospholamban mouse monoclonal antibody (05-205, Upstate Biotechnology). The membranes were blocked in 5% nonfat dry milk in TBS (0.1% Tween-20, (TBS-T)). Membranes were rinsed and incubated overnight with the appropriate primary and secondary antibodies for 1 hour each. For calsequestrin, anti-calsequestrin (1:2000 in TBS-T with 1% milk) and protein A (1:10,000 in TBS-T) were used. Finally, for phospholamban, anti-phospholamban (1:1000 in TBS-T with 1% milk) and goat anti-mouse IgG (1:10,000 in TBS-T in 1% milk) were used. All results were visualized and quantified using the same procedure as stated above.

Data Analysis

Tracings of all hemodynamic parameters and wall thickness measurements were recorded on a Gould model TA11 (Gould Instrument Systems, Inc., Valley View, OH) recorder and simultaneously displayed on a Gateway 2000 computer (sampling rate, 500 Hz) using the HEM analysis program (Notocord Systems, Croissy sur Seine, France). Hemodynamic measurements averaged over 30 seconds were calculated from the digitized data.

All data are expressed as the mean ± SEM. Differences between the experimental and the control regions of the ischemic animals were compared using paired t-tests for two sample means. Significant differences were present when p values less than 0.05 were present.
Supplementary On-line Results

Role of acute ischemia in the temporal development of regional contractile dysfunction – As indicated in Table 1, function after 24-hours was significantly reduced when a 15-minute partial occlusion was performed prior to applying a critical stenosis. On average there was a 29±6 % reduction from initial values. In comparison, regional function in two animals subjected to a critical stenosis without the brief period of ischemia remained normal after 24 hours (+3.3 % change from control values). There was still no change in resting function after 72 hours despite the presence of a critical stenosis (+5.3 % change from control values). In one animal, measurements were carried out for 1-week at which time function became significantly depressed with normal resting flow (37 % reduction from control values). Thus, a period of acute stunning accelerated the development of viable dysfunctional myocardium.
Figure 1 – Schematic of experimental preparation. Ultrasonic crystals were placed in LAD and LC (circumflex) regions to measure function. Aortic and left atrial catheters were placed for microsphere flow measurements and LV pressure was measured with a micromanometer. The proximal LAD was instrumented with a Transonic ultrasonic volumetric flowprobe and two hydraulic occluders. The primary occluder (proximal) was used to produce a chronic critical coronary stenosis. The secondary occluder (distal) was used to produce a 20-second occlusion to assess stenosis severity by evaluating the reactive hyperemic flow response.
References


