Persistent Stunning Induces Myocardial Hibernation and Protection
Flow/Function and Metabolic Mechanisms

Song-Jung Kim, Athanasios Peppas, Suk-Keun Hong, Guiping Yang, Yanhong Huang, Gissela Diaz, Junichi Sadoshima, Dorothy E. Vatner, Stephen F. Vatner

Abstract—To test the hypothesis that persistent myocardial stunning can lead to hibernating myocardium, 13 pigs were chronically instrumented, and persistent stunning was induced regionally by 6 repetitive episodes of 90-minute coronary stenosis (CS) (30% reduction in baseline coronary blood flow [CBF]) followed by full reperfusion every 12 hours. During the 1st CS, CBF fell from 43 ± 2 to 31 ± 2 mL/min, and anterior wall thickening (AWT) fell by 54 ± 8%, but posterior WT did not change. AWT never recovered fully and remained depressed by 31 ± 7% before the 6th CS, reflecting persistent myocardial stunning, but baseline CBF was not changed. Surprisingly, during the 6th CS, AWT did not fall further despite a similar reduction in CBF during CS, as occurred with the 1st episode. Regional MV˙O 2 fell similarly during the 1st and 6th CS. During the 1st CS, plasma glucose uptake increased, whereas free fatty acid (FFA) uptake was reduced. Before the 6th CS, glucose uptake remained elevated, whereas FFA uptake remained reduced. Histology revealed enhanced glycogen deposition, which could be explained by decreased glycogen synthase kinase (GSK)-3β protein levels and activity. These results indicate that persistent stunning, even in the absence of chronic ischemia, can recapitulate the phenotype of myocardial hibernation. This results in a shift in the flow/function relationship where a 30% decrease in CBF is no longer accompanied by a fall in myocardial function, which could be explained, in part, by a shift in substrate utilization. These hemodynamic/metabolic adjustments could facilitate survival of hibernating myocardium. (Circ Res. 2003;92:1233-1239.)

Key Words: hibernating myocardium • myocardial stunning • metabolism
• glycogen synthase kinase-3β • ischemia

Myocardial stunning is defined as the impaired but reversible reduction of contractile function after a brief ischemic episode, where the flow/function relationship is altered.1,2 The related concept of myocardial hibernation is based primarily on clinical observations3–6 and has been thought to involve a self-protective downregulation in myocardial function and metabolism to match the reduced O2 supply, rather than a change in the flow/function relationship. Studies in patients with hibernating myocardium where blood flow was measured with positron emission tomography (PET)7–9 and a study in conscious pigs with progressive coronary stenosis induced by an ameroid constrictor for one month10 all found maintained myocardial blood flow in the face of chronically and severely reduced regional myocardial function, reminiscent of myocardial stunning. The results from these studies raised the possibility that persistent stunning might be a mechanism involved in mediating hibernating myocardium, alternative to the mechanism of downregulated myocardial blood flow and O2 consumption. If this is found to be true, then alternative protective mechanisms must be sought to understand how hibernating myocardium can survive in the face of persistent ischemia.

In order to examine whether persistent myocardial stunning can lead to hibernating myocardium, we induced repetitive episodes of myocardial stunning by reducing coronary blood flow by ≈30% every 12 hours in conscious pigs. The first goal of the study was to determine whether repetitive episodes of myocardial stunning result in the phenotype of hibernating myocardium, eg, exhibiting reduced stable regional myocardial function and glycogen deposition. The second goal was to determine potential mechanisms by which hibernating myocardium tolerates ischemia. To address this goal, we examined the myocardial blood flow/function relationship in the presence and absence of persistent stunning. We also determined whether persistent stunning results in a shift in substrate utilization from free fatty acids (FFAs) to glucose. The third goal of the study was to determine whether the enhanced glucose uptake leads to glycogen deposition and
the molecular mechanism mediating glycogen accumulation in hibernating myocardium.

Materials and Methods

Animals used in this study were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, revised 1996).

In Vivo Studies

Thirteen female domestic swine (22 to 25 kg; Animal Biotechnology Industry, Inc, Danboro, Pa) were sedated with ketamine (10 to 20 mg/kg, IM) and xylazine (2.2 mg/kg, IM). General anesthesia was maintained with isoflurane (0.5 to 2.0 vol%). The animals then were instrumented chronically to measure global and regional myocardial function as described previously,11,12 ie, with left ventricular (LV) pressure gauges to measure LV pressure, ultrasonic crystals to measure regional wall thickening, and catheters to measure aortic and left atrial pressures. In addition, a catheter was implanted into the anterior interventricular vein to collect venous samples for the determination of regional myocardial oxygen consumption (MV\textsubscript{O2}) and plasma levels of glucose, lactate, and FFAs. The swine model was chosen because swine lack preformed collateral vessels and its own control.

After 1 week of postoperative recovery, myocardial persistent stunning was induced regionally by 6 repetitive 90-minute episodes of left anterior descending coronary stenosis (CS) (30% reduction in baseline coronary flow) followed by full reperfusion every 12 hours, because the hearts are large enough to measure both ischemic and nonischemic zone function, thereby permitting each animal to serve as its own control.

Regional MV\textsubscript{O2} and Substrate Utilization

Arterial (from aortic catheter) and venous samples were obtained to assess regional MV\textsubscript{O2} and substrate utilizations in the ischemic region. MV\textsubscript{O2} was calculated using the Fick equation as the product of the CBF and arteriovenous oxygen difference measured with a Radiometer ABL 725. Plasma concentration of glucose and lactate was assessed using a Radiometer ABL 725. Plasma FFAs were also measured using the NEFA C test kit from Wako Diagnostics, which utilized an in vitro enzymatic colorimetric method.

Blood Flow Measurement

Regional myocardial blood flow was measured using colored microspheres (BioPAL) at baseline and during coronary stenosis at the 1st and 6th episodes of myocardial stunning to determine whether the altered flow/function relationship is due to collateralization in persistently stunned myocardium.

Histopathology

Samples for histology were obtained from the persistent stunned and nonstunned myocardium. The samples from each animal were immersed in 10% phosphate-buffered formalin, embedded in paraffin, cut into 6-μm sections, and stained with hematoxylin and eosin and periodic acid-Schiff (PAS). The PAS stained for glycogen. Glycogen accumulation was quantified by scoring the ischemic and persistently stunned region from 0 to 4 in 7 pigs.

Morphological evaluation was also assessed in 3 pigs by triphenyl tetrazolium chloride (TTC). The patchy necrosis in the ischemic region was quantified in 3 pigs for the entire area at risk.

Western Blot Analysis

Myocardial tissue was obtained from repeated stunned and nonstunned regions and from sham controls for immunoblot analysis. Samples were homogenized in 160 mmol/L Tris pH 8.0 and 6 mol/L urea, with protease, phosphatase, and kinase inhibitors. Equal amounts of protein were dissolved in 2% SDS, 62.5 mmol/L Tris-HCl, pH 6.5, 10% glycerol, 0.05% bromophenol blue, 6 mol urea, and 100 mmol/L DTT, and then separated by 12.5% SDS-PAGE using the Biorad Mini-gel system. Equal loading of samples was confirmed by densitometry of actin-troponin I (Tnl) of Coomassie stained gels. Gels were transferred to nitrocellulose using a wet transfer apparatus (Biorad) with 20% methanol, 25 mmol/L Tris, and 19 mmol/L glycine buffer. The primary anti-Tnl Mab 81-7 (Spectral Diagnostics, Toronto, Canada) was used. Primary antibodies were detected using anti-mouse IgG conjugated to alkaline phosphatase (Jackson Immuno Research Labs) and CDP-Star chemiluminescence reagent (NEN-Mandel). All Western blot exposures were in the linear range of detection, and the intensities of the resulting bands were quantified by densitometry (Corel Photo). Blots for glycogen synthase kinase (GSK)-3β were incubated for 30 minutes at room temperature with a 1:2500 dilution of rat Ab (BD Transduction Laboratories) in TBS containing 0.1% Tween-20 and 2.5% nonfat milk.

Determination of GSK-3β Activity

The activity of GSK-3β was measured by the immune complex kinase assay as described previously.13 Briefly, the samples (n=4) from the stunned and nonstunned regions were homogenized with lysis buffer (20 mmol/L Tris [pH 7.5], 25 mmol/L β-glycerophosphate, 100 mmol/L NaCl, 1 mmol/L Na\textsubscript{3}VO\textsubscript{4}, 2 mmol/L EGTA, 2.5 μL protease inhibitor cocktail [Sigma]). The samples were subjected to immunoprecipitation with or without anti-mouse GSK-3β antibody (BD Biosciences) overnight at 4°C, then incubated with protein G-Sepharose (Calbiochem) for 2 hours.

After washing the immune complex, the kinase activity was assayed using 1 μg of GS peptide-2 (Upstate Biotechnology) as a substrate in a reaction buffer containing 25 mmol/L β-glycerophosphate, 40 mmol/L HEPES (pH 7.2), 10 mmol/L MgCl\textsubscript{2}, 50 μmol/L ATP, and 5 μCi of [γ\textsuperscript{32}P]ATP (6000 Ci/mmol). After 30 minutes of incubation at 30°C, the reaction was ended by adding 2 mol/L HCl. Radiolabeled peptide (20 μL) was spotted onto Whatman P81 filter papers. Samples were washed 3 times for 5 minutes each in a large volume of 180 mmol/L of phosphoric acid and then once for 3 minutes in 99% ethanol. After drying, the samples were counted using a scintillation counter.

Statistical Analysis

All data are reported as mean±SE. Hemodynamic comparison during stenosis and among the episodes was examined by ANOVA. Comparisons of the data between the 1st and 6th episodes were examined by Student’s paired t test. Significant differences were taken at P<0.05.

Results

Hemodynamics and Flow/Function Relationship

The Table summarizes mean values for systemic hemodynamics (mean aortic pressure, LV systolic and end-diastolic pressure, LV dP/dt\textsubscript{max}, heart rate) at baseline and during CS of the 1st and 6th episodes. At baseline, systemic hemodynamic variables were not changed throughout the protocol.

Figure 1 shows representative hemodynamic recordings at baseline and during CS at the 1st and 6th episodes. During the 1st episode, 30% reduction of CBF (from 43±2 to 31±2 mL/min) decreased (P<0.05), anterior wall thickening (AWT) by 54±8% (from 2.59±0.20 to 1.13±0.16 mm), but posterior WT, which served as a control, did not change (data not shown). Then, AWT never fully recovered and remained depressed before the 6th CS (1.71±0.09 mm), reflecting persistent myocardial stunning, but baseline CBF (44±2 mL/min) was not different from the baseline before 1st CS. However, during the 6th episode, AWT did not fall further despite a 30% reduction of CBF. Figure 2 summarizes the CBF (a) and AWT (b) in response to 90-minute CS and 60-minute CAR during the 1st, 3rd, and 6th episodes.
Interestingly, during the 6th episode AWT did not change despite the same amount of flow reduction. Peak reactive hyperemia (137±10 mL/min) during initial reperfusion after the 6th episode was higher (P<0.05) than after the 1st episode (110±8 mL/min). Figure 2c shows the flow/function relationships during the 1st and 6th episodes. During the 1st episode, reduction of flow decreased contractile function, ie, flow/function match, whereas during the 6th episode of CS, contractile function was not reduced despite the same degree of CS, indicating a flow/function mismatch. The change in flow/function relationship was progressive and was altered even during the 3rd episode, where the 30% reduction in blood flow failed to decrease function as much as occurred during the first episode (Figure 2b). The increase in reactive hyperemia was also progressive, reaching statistical significance after the 6th episode.

To determine whether collateralization from multiple episodes of CS contributes to the preservation of AWT during the 6th episode, subendocardial blood flow was determined at baseline and during the 1st and 6th episodes of CS. Subendocardial blood flow was similar at baseline (1st, 1.38±0.28 versus 6th, 1.42±0.24 mL/min per g). During CS, subendocardial blood flow fell more than total coronary blood flow, as expected, and fell similarly during the 1st and 6th episodes of CS (1st, 0.75±0.07 versus 6th, 0.79±0.09 mL/min per g) (Figure 3a). Figure 3b demonstrates that AWT showed akinesis after the left anterior descending artery (LAD) was occluded completely for 5 minutes after the 6th episode of CS. Both results suggest that the altered flow/function during the 6th episode of CS was not collateral-dependent.

Regional MV˙O2 and Substrate Utilization
To determine whether the reduced O2 demand contributes to the altered flow/function during the 6th episode of CS, regional MV˙O2 in the ischemic region was determined at baseline and during the 1st and 6th episodes of CS. As expected, the 1st episode of CS reduced (P<0.05) MV˙O2 from 4.03±0.48 to 3.37±0.47 mL/min per 100 g. During the 6th episode of CS, MV˙O2 was reduced similarly, from 3.93±0.37 to 3.21±0.45 mL/min per 100 g.

Figure 4 summarizes myocardial substrate extraction, ie, glucose, FFAs, and lactate at baseline and during the 1st and 6th episodes.
During the 1st episode, CS increased (P<0.01) plasma glucose extraction from 0.30±0.08 to 0.81±0.05 mmol/L. In contrast, plasma lactate and FFA extraction were reduced (P<0.01) from 0.45±0.11 to 0.07±0.07 mmol/L and from 2.72±0.42 to -0.07±0.48 mmol/L, respectively. Before the 6th episode, baseline glucose extraction (0.65±0.1 mmol/L) was enhanced significantly (P<0.05) compared with baseline values from the 1st episode, and it remained at this level during CS (0.73±0.06 mmol/L; P=NS). Baseline plasma lactate and FFA extraction before the 6th episode were reduced (P<0.01) compared with the values at the baseline before the 1st episode, and they also remained at the pre-CS level during CS.

**GSK-3β Protein and Activity**

To determine whether the enhanced glucose extraction leads to an increase in glycogen synthesis, we evaluated the level of GSK-3β protein in the tissues from the nonstunned and stunned myocardium after 6 episodes of stunning. After the 6th episode, the levels of GSK-3β were significantly down-regulated in the myocardium obtained from the persistently stunned region, compared with the nonstunned region (Figure 5a). There was no difference between the sham control and nonstunned region after 6 episodes of stunning. Similarly, the GSK-3β activity (n=4) was also significantly reduced (P<0.05) in the stunned regions, compared with the nonstunned regions (Figure 5b).

**Pathology**

Figure 6a shows a representative slice of myocardium from the central area at risk after the 6th episode of CS, demonstrating no evidence of necrosis as assessed by dual perfusion and TTC. However, the tissue samples from persistently stunned myocardium revealed patchy necrosis preferentially in the subendocardium (Figures 6b and 6c). Quantification indicated that the total area of patchy necrosis comprised 3.4±0.9% of the entire area at risk in 3 pigs. This explains why the TTC technique did not demonstrate infarction. Figure 7 shows that after the 6th episode of CS, the stunned region of the myocardium was stained positively for glycogen deposition (Figures 7b and 7c), but not in the nonstunned region (Figure 7a), ie, one pathognomonic feature of hibernating myocardium.9,14,15 As demonstrated in Figures 7b and 7c, the degree of glycogen deposition was enhanced in the ischemic region. Semiquantitative analysis demonstrated a level of 0 for the nonischemic region and 3.3±0.5 in the

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subendocardium and 1.1±0.3 in the subepicardium for the persistently stunned region in 7 pigs.

Discussion

It remains controversial whether repeated episodes of myocardial stunning can induce hibernating myocardium. Equally important is what mechanisms protect hibernating myocardium from acute ischemia. To answer these questions, we induced myocardial stunning with repetitive coronary stenosis (≈30% reduction of baseline blood flow for 90 minutes followed by full reperfusion) every 12 hours to maintain myocardial stunning. We found that (1) persistent stunning induced a sustained reduction in contractile function without a change in resting blood flow, (2) during the 6th CS, the flow/function relationship was altered, ie, despite the same degree of CS, regional function fell no further, (3) the myocardium in the chronically stunned region revealed pathognomonic features of hibernating myocardium, eg, glycogen deposition, (4) persistent stunning shifted substrate utilization from FFAs to glucose, suggesting one mechanism by which hibernating myocardium is protected against acute ischemia, and (5) GSK-3β was downregulated, suggesting not only a mechanism for the enhanced glycogen deposition in the hibernating myocardium, but also invoking other cardioprotective mechanisms that may aid in survival of hibernating myocardium.

Flow/Function Relationship During Persistent Myocardial Stunning

The closely coupled relationship between coronary blood flow and contractile function is a well-described principle in cardiovascular physiology.16 This perfusion-contraction matching almost always is observed in acute myocardial ischemia.1,16 Similarly, several studies also demonstrated a maintained flow/function relationship in the presence of reduced myocardial blood flow induced by coronary stenosis, so called short-term hibernation.11,17–20 However, no study has reported the flow/function relationship during persistent stunning. In the present study, persistent myocardial stunning was maintained in response to the 6 episodes of repetitive coronary stenosis and reperfusion. Interestingly, before the 6th episode of CS, regional myocardial function was reduced (myocardial stunning), and baseline myocardial blood flow was maintained. Surprisingly, during the 6th episode of CS, despite a 30% decrease in blood flow, regional myocardial function did not fall further, indicating that there was a major shift in the flow/function relationship. Collateralization could have been induced during the initial episodes of CS and accordingly could explain the maintenance of myocardial function during the 6th episode of CS. However, if that were the mechanism, then myocardial blood flow measured with microspheres would have demonstrated less reduction in subendocardial blood flow during the 6th episode. This was not observed. In addition, there was no improvement in function in the ischemic zone during total occlusion of the LAD, suggesting that collaterals were not a contributing factor. These results are consistent with previous studies demonstrating that pigs, similar to humans, lack preformed collaterals.21,22 Another possible mechanism we examined was the downregulation of regional myocardial oxygen consumption, which was proposed initially to explain the mech-

Figure 5. GSK-3β Western blots (a) and activity (b) in samples from control (c), nonstunned (NS), and stunned (S) myocardium. After the 6th episode, the levels of GSK-3β protein and activity were reduced in repeatedly stunned myocardium, compared with the nonstunned myocardium. *P<0.05 vs NS.

Figure 6. TTC-stained myocardium failed to demonstrate significant necrosis (a), but patchy areas of micronecrosis (arrow) were revealed in the subendocardium stained with hematoxylin and eosin (b and c) after 6 episodes of coronary stenosis and reperfusion. RV indicates right ventricle; SEP, septum.
anism of hibernating myocardium. This was not the case, because, during the 6th episode, regional MVO$_2$ fell similarly to the decrease observed during the 1st episode. These results indicate that neither collateralization nor altered regional MVO$_2$ is responsible for the altered flow/function relationship after persistent stunning. However, this altered flow/function relationship could be crucial in permitting hibernating myocardium to tolerate persistent and acute ischemia.

**Shift in Substrate Utilization From FFAs to Glucose**

Although FFAs are a primary substrate in the myocardium in the resting state, the increase in glucose uptake is one of the most prominent changes in myocardial metabolism during myocardial ischemia and reperfusion.$^{20,23–28}$ Previous studies demonstrated that myocardial ischemia induced by single or multiple episodes of ischemia resulted in increased glucose uptake.$^{20,27,28}$ The mechanism for the enhanced glucose uptake was associated with translocation of the GLUT4 protein to the plasma membrane.$^{28–30}$ In the present study, we also found that persistent stunning induced by repetitive stunning shifted substrate utilization from FFAs to glucose. Furthermore, the enhanced glucose and reduced FFA extraction persisted in the baseline state before the 6th episode, ie, in the absence of persistent ischemia. Therefore, switching substrates from FFAs to glucose may be another mechanism that preserves LV function during subsequent episodes of acute myocardial ischemia.

Recently, the functional role of GSK-3β, which was named for its ability to phosphorylate and thereby inactivate glycogen synthase, has been investigated intensively for its multifaceted role in intracellular signaling mechanisms, including development, metabolism, gene transcription, protein translation, and apoptosis.$^{31,32}$ In the baseline condition, GSK-3β is active and inhibits synthesis of glycogen,$^{33}$ whereas insulin stimulates glycogen synthesis by inactivating GSK-3β.$^{34}$ When this protein is reduced, glycogen synthesis is enhanced. Histological changes including glycogen deposits have been found to correlate with glucose extraction by using PET in hibernating myocardium.$^{3,34}$ We observed that the level and activity of GSK-3β protein in the chronically stunned region was significantly reduced after 6 episodes of CS. Therefore, this may be an important mechanism mediating glycogen deposition in hibernating myocardium. Recent studies have also demonstrated that pharmacological inhibition of GSK-3β improved recovery of posts ischemic function and reduced infarct size.$^{35}$ and dominant-negative GSK-3β transfected cells reduced apoptosis and enhanced cell survival,$^{36}$ indicating that inhibition of GSK-3β is also cardioprotective. Therefore, it is conceivable that the reduced GSK-3β protein plays a key role in not only promoting glycogen deposits, but also may contribute to cell survival in chronically stunned myocardium. Indeed, Depre et al$^{37}$ demonstrated that myocardial ischemia triggered a genetic program of cell survival in the stunned myocardium after one episode of CS in conscious pigs. This finding coupled with the current data suggests that persistent stunning elicits a program of genes and proteins that protect hibernating myocardium from further episodes of acute ischemia.

In summary, these results indicate that persistent stunning can induce the phenotype of myocardial hibernation, including an increase in glycogen deposition. The persistent stunning is accompanied by a shift in substrate utilization from FFAs to glucose and a decrease in GSK-3β protein and activity. The altered myocardial flow/function relationship, whereby regional function can be maintained in the face of a 30% reduction of blood flow in combination with a shift in metabolic pathways, may explain why hibernating myocardium can survive in the chronically ischemic heart. The downregulation of GSK-3β could contribute not only to the hemodynamic/metabolic adjustment but also to cardioprotection in persistently stunned myocardium. A note of caution in extrapolating these results to patients with hibernating myocardium is necessary. In the clinical setting, hibernating myocardium develops in the presence of chronic myocardial ischemia. In the present investigation, we demonstrated that the hibernating myocardium phenotype could develop in the absence of chronic ischemia, but only with persistent stunning. This key difference can help understand the mechanisms underlying the pathogenesis of hibernating myocardium, even though it cannot mimic entirely hibernating myocardium as it exists in the setting of chronic ischemia. For example, a recent study by Thomas et al$^{38}$ concluded that conditions resembling hibernating myocardium developed in pigs after chronic stenosis, which induced a limitation in flow reserve. Our study demonstrated that persistent stunning can elicit similar results in the absence of chronic ischemia or limited flow reserve. What both studies have in common is that in both cases chronic stunning will develop the phenotype of hibernating myocardium, because chronic coronary stenosis must elicit repetitive bouts of intensified ischemia secondary to adjustments in activity of the animal during the day, which, in turn, elicits repetitive episodes of stunning. Thus, the phenotype of hibernating myocardium can evolve from either chronic coronary...
stenosis or repetitive, transient periods of stenosis and its relief, resulting in persistent myocardial stunning. In addition, the current investigation suggests that the shifts in the flow/function relationship and metabolism, along with the potential protective role of downregulating GSK-3β, may help explain how hibernating myocardium can resist the deleterious effects of further ischemic episodes.

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