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Abstract—Hibernating myocardium refers to chronically dysfunctional myocardium in patients with coronary artery disease in which cardiac viability is maintained and whose function improves after coronary revascularization. It is our hypothesis that long-term adaptive genomic mechanisms subtend the survival capacity of this ischemic myocardium. Therefore, the goal of this study was to determine whether chronic repetitive ischemia elicits a gene program of survival protecting hibernating myocardium against cell death. Accordingly, we measured the expression of survival genes in hibernating myocardium, both in patients surgically treated for hibernation and in a chronic swine model of repetitive ischemia reproducing the features of hibernation. Human hibernating myocardium was characterized by an upregulation of genes and corresponding proteins involved in anti-apoptosis (IAP), growth (VEGF, H11 kinase), and cytoprotection (HSP70, HIF-1α, GLUT1). In the swine model, the same genes and proteins were upregulated after repetitive ischemia, which was accompanied by a concomitant decrease in myocyte apoptosis. These changes characterize viable tissue, because they were not found in irreversibly injured myocardium. Our report demonstrates a novel mechanism by which the activation of an endogenous gene program of cell survival underlies the sustained viability of the hibernating heart. Potentially, promoting such a program offers a novel opportunity to salvage postmitotic tissues in conditions of ischemia. **(*Circ Res*. 2004;95:433-440.)**

Key Words: gene expression ■ coronary artery disease ■ ischemia ■ stunning ■ hibernation

Because the cardiac myocyte has a limited capacity for regeneration, most forms of heart disease, including ischemic heart disease and heart failure, are characterized by a loss of cardiomyocytes.[1–3] Historically, any evidence of prolonged left ventricular dysfunction after myocardial ischemia was thought to be caused by irreversible myocyte damage. This traditional concept was challenged with the discoveries of myocardial stunning (in which ischemia is followed by prolonged but fully reversible dysfunction),[4] ischemic preconditioning (in which a brief ischemic episode protects the myocardium against a subsequent episode of sustained ischemia),[5] and hibernating myocardium (in which chronic dysfunction resulting from repeated ischemia recedes after bypass revascularization).[6,7] Hibernating myocardium is submitted to repetitive bouts of ischemia caused by normally occurring increases in myocardial metabolic demand in the face of significant coronary stenosis and limited coronary reserve, yet it does not develop irreversible damage.[8,9] The diagnosis of this condition is of the highest priority in patients with chronic coronary artery disease, because successful coronary revascularization and restoration of coronary flow reserve in hibernating myocardium are followed by an improvement of contractile performance;[10,11] however, if left untreated, this condition leads to progressive exacerbation of heart failure and death.

Despite the abundant descriptions of altered flow, contraction, and metabolism,[12–17] mechanistic insights into why hibernating myocardium survives during chronic ischemia have been limited. Previous studies at the molecular level have focused mainly on the regulation of genes and proteins, which might explain the reduced cardiac contraction of hibernating myocardium in an animal model or in the human heart,[18–22] because these studies focused on proteins regulating Ca^{2+} flux,[18–20] inflammatory response,[21] or β-adrenergic receptor density.[22] No study specifically addressed the mechanisms related to prolonged survival, except for the measurement of the heat-shock protein HSP70 in an animal model[19] and in patients.[20] It is our hypothesis that long-term genomic

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From the Department of Cell Biology and Molecular Medicine and the Cardiovascular Research Institute (C.D., S.-J.K., Y.H., V.G., D.E.V., S.F.V.), University of Medicine and Dentistry New Jersey, Newark; National Heart and Lung Institute (A.S.J., O.E.R., J.R.P., G.D.D., D.J.P., P.G.C.), Imperial College, London, UK; and Medical Research Council Clinical Sciences Centre (O.E.R., P.G.C.), London, UK.

*Both authors contributed equally to this work.

Correspondence to Stephen F. Vatner, MD, Cardiovascular Research Institute, Department of Cell Biology and Molecular Medicine, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, 185 S Orange Ave, MSB G-609, Newark, NJ 07103. E-mail vatnersf@umdnj.edu

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mechanisms also subtend the survival capacity of this chronically ischemic myocardium. Therefore, the goal of this study was to determine whether the hibernating heart activates the expression of genes promoting cell survival, both in biopsy samples from patients with chronic hibernation and in a swine model, which reproduces the pattern of repetitive ischemia characterizing hibernation in the human heart.[23]

We focused our analysis on transcripts that are known to protect the heart by blocking apoptosis while promoting cytoprotection and cell growth.[24] This cluster of genes includes the inhibitor of apoptosis (IAP), a powerful caspase inhibitor,[25] as well as cytoprotective heat-shock proteins (such as HSP70), which dramatically reduce ischemic cell death both in vitro and in vivo.[26–28] We also described an upregulation of H11 kinase (H11K), a novel signaling molecule promoting both cytoprotection and cell growth in the heart.[29] In addition, the hypoxia-inducible factor-1α (HIF-1α) represents a master switch in the metabolic and functional adaptation to chronic anaerobic conditions[30] by stimulating glucose metabolism (through the stress-responsive glucose transporter [GLUT1]) and angiogenesis (through the vascular endothelial growth factor [VEGF]). Increased reliance on glucose and increased collateral flow are 2 characteristics of the hibernating heart.[9] Because the simultaneous activation of these genes represents a powerful cardioprotective mechanism to limit irreversible cell damage in vivo, we tested whether this pattern of gene induction applies to the heart of patients with evidence of hibernating myocardium.

Materials and Methods

Human Studies

Twelve patients with coronary artery disease and chronic left ventricular (LV) dysfunction without previous clinical myocardial infarction in the area of interest and awaiting bypass surgery were studied. The main eligibility criterion was the demonstration of tissue viability in 1 or more chronically dysfunctional segments subtended by stenotic coronary arteries in patients devoid of acute coronary syndromes. Global and regional LV function was assessed by cine cardiovascular magnetic resonance (CMR), and the presence of viable myocardium was confirmed by $<$50% transmural uptake of gadolinium using CMR.[31] In 6 patients, myocardial viability was confirmed by maintained uptake ($>$0.25 μmol/min per gram of tissue) of ^{18}F-fluorodeoxyglucose (FDG) with positron emission tomography (PET).[32,33] Absolute regional myocardial blood flow was measured with PET using oxygen-15–labeled water.[34] During bypass surgery, 2 full-thickness biopsy samples (1 from the hibernating, 1 from a remote normally contracting area) were taken from the LV of each patient and frozen immediately. A follow-up cine CMR scan was performed 6 months after bypass to confirm the functional improvement of all hibernating segments for which biopsy samples were obtained. Regional LV function was graded according to a 4-point scale (0=normal, 1=moderate hypokinesis, 2=severe hypokinesis, 3=akinesis or dyskinesis). Changes in wall motion $\geq$1 grade at follow-up were considered significant.

Animal Model

A hydraulic occluder was implanted around the base of the left anterior descending (LAD) coronary artery in female domestic swine (22 to 25 kg). After 3 to 5 days of recovery, ischemia was induced in the conscious animal by inflating the coronary occluder to reduce the blood flow in the LAD by 30% to 40% from baseline.[23] The coronary stenosis was maintained for 90 minutes, followed by complete deflation of the occluder, and repeated every 12 hours up to 6 times. One hour after reperfusion, myocardial samples were taken from both the hibernating area and the remote area of the beating heart after 1, 3, or 6 episodes of ischemia. Three instrumented pigs, in which no occlusion was performed, were used as shams. Myocardial infarction was induced in 3 additional pigs by occluding the LAD artery for 72 hours. Samples were taken in the middle of the hibernating area and in the remote myocardium. In each case, tissues were frozen immediately or fixed in formalin. Measurements were performed in samples taken from subendocardium, and the results were subsequently confirmed in subepicardial samples.

Quantitative Reverse-Transcriptase Polymerase Chain Reaction

The mRNA of interest was reverse-transcribed and used for quantitative 2-step polymerase chain reaction (7700 Prizm; Perkin-Elmer/ABI) as described.[35] Data are reported per number of cyclophilin transcripts, used as a normalizer (copies of cyclophilin transcript per nanogram of RNA was $1.8\pm0.1\times10^4$ in remote versus $2.2\pm0.3\times10^4$ in hibernating myocardium; not significant).

Western Blotting

Tissue was homogenized as described.[29] Protein extracts were resolved on 12% SDS-PAGE gels and transferred to membranes. Primary antibodies were added at the recommended dilution and incubated overnight. Detection was performed by chemiluminescence. Equal protein loading was verified by Coomassie staining.

TUNEL Staining

Formalin-fixed tissues were dehydrated, embedded in paraffin, and sectioned. TUNEL staining was performed as described.[36] After washing, slides were mounted in a Vector DAPI medium for fluorescent microscopic observation at $\times$40 objective field (Zeiss).

Statistical Analysis

Results are presented as the mean$\pm$SE for the number of samples indicated in each figure legend. Statistical comparison was performed using the Student *t* test with paired comparison for human samples and using a 2-way ANOVA with Bonferroni correction for swine samples.

An expanded Material and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Results

Patient Characteristics

Clinical characteristics are reported in the Table. All biopsy samples were taken from dysfunctional regions without partial-thickness infarction, ie, with no evidence of late gadolinium uptake at CMR. In the 6 patients in whom FDG-PET was also performed, the uptake of the tracer in hibernating segments where the biopsy samples were obtained showed the presence of maintained viability and was not different from the uptake in remote myocardium (Table). The segment in which biopsy samples were harvested and the number of segments considered as hibernating are indicated for each patient in the Table. Resting myocardial blood flow was 0.91 ± 0.05 mL/min per gram in the dysfunctional area where biopsy samples were obtained and 0.94 ± 0.06 mL/min per gram in the remote segments (not significant). Six months after surgery, cine CMR confirmed functional improvement by at least 1 grade in all the dysfunctional segments where the biopsy samples were taken (Table).

Patient Characteristics

Patient	Gender	Age, y	EF, %	Number of Hibernating Segments	FDG Uptake Hibernating (μmol/min/g)	Hibernating Biopsy	Coronary Stenosis to Hibernating Region	Mean Hibernating Region Wall Motion Score at Baseline	Mean Hibernating Region Wall Motion Score at Follow-up	Remote Biopsy	FDG Uptake Remote (μmol/min/g)	Mean Remote Region Wall Motion Score at Baseline	Mean Remote Region Wall Motion Score at Follow-up
1	M	65	36	8	0.38	Ant	LAD 90%	2	0	Inf	0.34	0	0
2	M	66	54	5	0.50	Ant	LAD 90%	1	0	Post-lat	0.46	0	0
3	M	76	40	8	0.34	Ant	LMS 70%	2	0	Post-lat	0.42	0	0
4	M	69	31	3	0.43	Inf-lat	RCA 99%	1	0	Lat	0.41	0	0
5	M	63	25	6	—	Inf-lat	RCA 90%	2	0	Ant	—	0	0
6	M	55	29	6	—	Ant	LAD 70%	2	1	Inf	—	1	1
7	F	77	53	3	0.41	Ant	LMS 95%	1	0	Inf	0.47	0	1
8	F	82	28	5	—	Ant	LMS 90%	2	0	Lat	—	0	0
9	F	42	31	9	0.64	Ant	LMS 70%	3	0	Inf-lat	0.47	0	0
10	M	63	47	3	—	Inf-lat	LCX 100%	3	2	Ant	—	0	0
11	M	54	42	4	—	Lat	LCX 99%	2	1	Ant	—	0	0
12	M	72	23	8	—	Ant	LAD 90%	2	*	Ant-lat	—	1	*

EF indicates ejection fraction; ant, anterior; ant-lat, antero-lateral; inf-lat, infero-lateral; lat, lateral; inf, inferior; post-lat, postero-lateral; LAD, left anterior descending artery; LMS, left marginal superior artery; RCA, right coronary artery; LCX, left circumflex artery; M, male; F, female.

*No follow-up available.

Regulation of Survival Genes in Human Hibernating Myocardium

Tissue samples were obtained from both the hibernating territory and the normally contracting segments. The specific cluster of genes described above was measured by quantitative polymerase chain reaction, and values were compared by paired analysis between hibernating and remote myocardium. The mRNAs encoding IAP, HSP70, H11K, HIF-1α, VEGF, and GLUT1 were all significantly ($P<0.05$) upregulated in hibernating compared with remote myocardium (Figure 1). In particular, the increase in HIF-1α can activate the expression of genes involved in angiogenesis (VEGF) and glucose metabolism (GLUT1), suggesting that the hemodynamic and metabolic adaptations[9,15] described in myocardial hibernation may be a consequence of the gene adaptation. Transcripts unrelated to survival (cyclophilin, adenylyl cyclase 5, T-type Ca^{2+} channel) did not show significant differences in the hibernating compared with remote area (not shown).

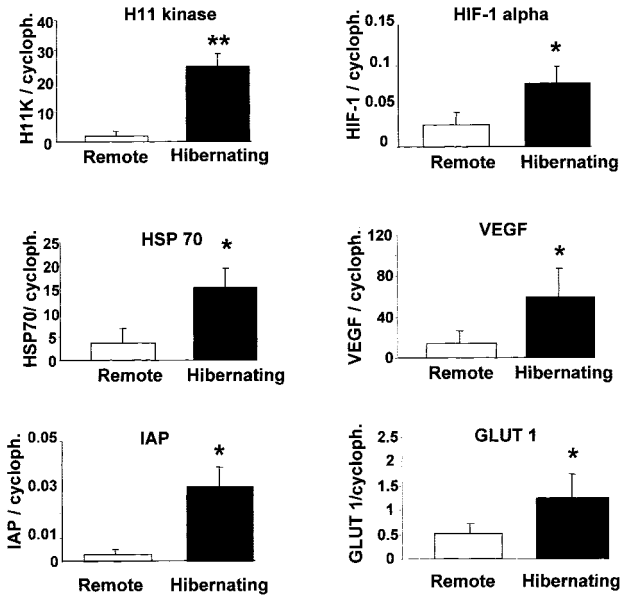

Figure 1. Activation of genes of cell survival in human hibernating myocardium. Quantitative polymerase chain reaction was performed for H11K, HSP70, IAP, HIF-1α, VEGF, and GLUT1 mRNAs from both remote and hibernating myocardium (n=7 per group) in samples taken at the time of bypass surgery. **$P<0.01$; *$P<0.05$ versus remote.

Regulation of Corresponding Proteins in the Human Heart

To test whether the upregulation of these genes was accompanied by an increase in the corresponding proteins, Western blot analysis was performed for H11K, HSP70, and IAP in 5 additional pairs of samples. Figure 2 shows that the protein level of these products was more than doubled in hibernating myocardium compared with the remote area of the same hearts. Therefore, the genomic adaptation is accompanied by a concomitant change in the translation of the regulated genes.

Regulation of Survival Genes in a Swine Model of Repetitive Stunning

Because it has been proposed that the human hibernating myocardium may result from repetitive episodes of ischemia–reperfusion, we determined next whether the recurrence of ischemia plays a role in the expression of this cardioprotective mechanism. Therefore, similar measurements were performed in a swine model in which repeated episodes of ischemia–reperfusion reproduce the pattern of myocardial hibernation described in patients.[23] Myocardial samples were taken from the dysfunctional hibernating area (where regional wall thickening was reduced by −57%, −54%, and −36% after 1, 3, and 6 episode of ischemia, respectively) and from the normal territory from the same hearts. As shown in Figure 3, HSP70, H11K, and IAP transcripts were upregulated after the first episode of ischemia, consistent with our previous observation.[24] We showed before that the expression of these

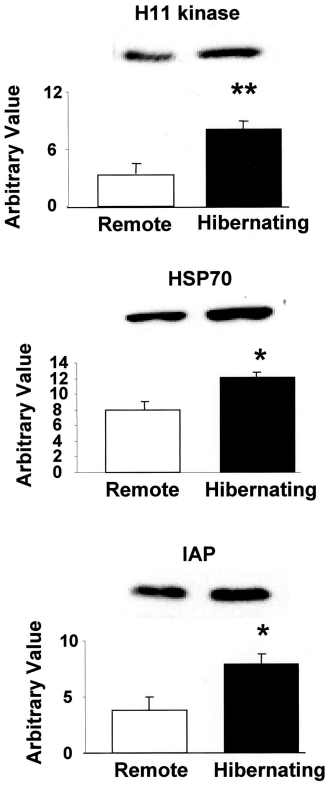

Figure 2. Expression of survival proteins in human hibernating myocardium. Western blotting was performed for H11K, IAP, and HSP70 from both remote and hibernating myocardium (n=5 per group) in samples taken at the time of bypass surgery. $^{**}P<0.01$; $^{*}P<0.05$ versus remote.

genes progressively returns to basal values during the reperfusion after ischemia and is normalized after 12 hours of reperfusion.[24] However, as shown in Figure 3, these genes were re-induced by subsequent ischemic episodes. In contrast, the mRNA encoding HIF-1α was not different from control after the first episode but progressively increased as ischemia was repeated (Figure 3). Such increase was accompanied by a concomitant upregulation of the downstream target VEGF. In this model, the glucose transporter GLUT1, another target of HIF-1α, was not significantly affected (not shown), in contrast to the human samples (Figure 1). The proto-oncogene EGR-1, an immediate/early gene with pro-survival function, was induced after the first episode only, and was not reactivated by further episodes of ischemia/reperfusion. Therefore, a gene program of cell survival including anti-apoptotic, cytoprotective, and growth-promoting genes is induced by ischemia and is sustained chronically as ischemic episodes are repeated. Although some genes already respond to a single episode of ischemia, the full cytoprotective program (including HIF-1α and VEGF) requires a repeated pattern of ischemia–reperfusion.

Regulation of Corresponding Proteins in the Swine Heart

To determine whether this transcriptional activation described in swine myocardium is followed by an increase of the corresponding proteins, myocardial expression of HSP70, H11K, and IAP was measured by Western blot in samples submitted to 1, 3, or 6 cycles of ischemia–reperfusion (Figure 4A), and the expression was compared between remote and ischemic areas (Figure 4B). There was no significant change in the expression of these proteins in the remote zone of the myocardium after 1, 3, or 6 episodes (Figure 4A). As shown in Figure 4B, the expression of these 3 proteins was not increased after a single episode of ischemia in the ischemic territory compared with the nonischemic zone, but increased progressively and significantly ($P<0.05$) by the third episode and remained elevated after 6 episodes. Interestingly, the 3- to 4-fold increase of survival proteins in the swine model is comparable to the amplitude of the changes described in the human hibernating heart (Figure 2). The data in Figure 4 were

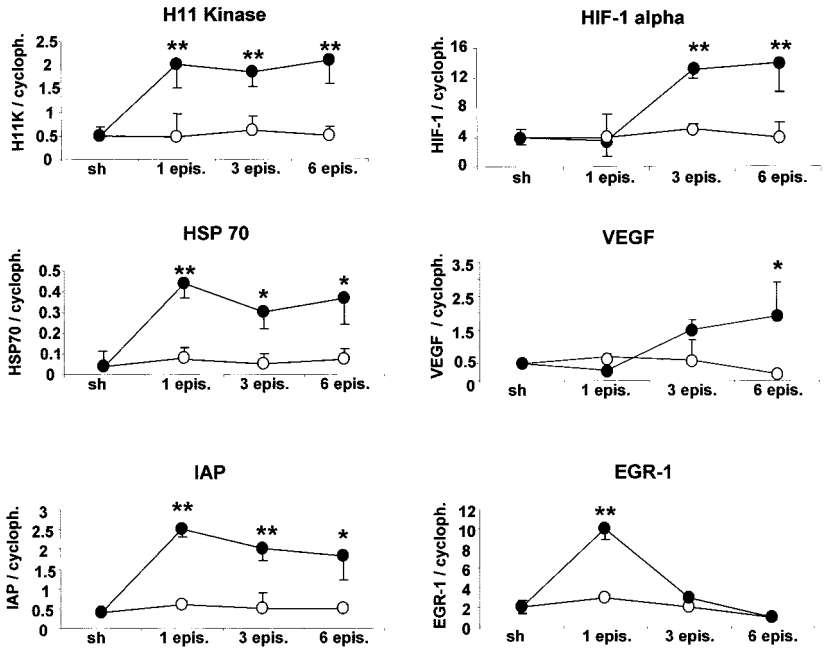

Figure 3. Activation of genes of cell survival in swine hibernating myocardium. Quantitative polymerase chain reaction was performed for H11K, HSP70, IAP, HIF-1α, VEGF, and EGR-1 mRNAs from both ischemic (black circles) and remote (white circles) myocardium after 1, 3, and 6 episodes (epis) of ischemia–reperfusion and compared with sham (sh) controls (n=4 per group). $^{**}P<0.01$; $^{*}P<0.05$ versus remote.

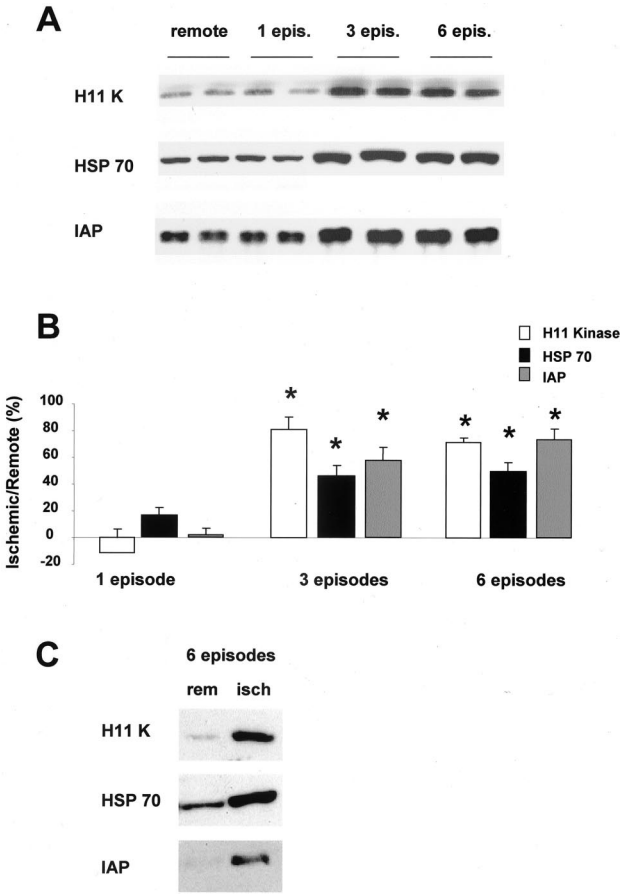

Figure 4. Expression of survival proteins in swine hibernating myocardium. A, Western blotting was performed for H11K, HSP70, and IAP from remote myocardium and from ischemic myocardium after 1, 3, and 6 episodes (epis) of ischemia/reperfusion. Two representative examples are presented for each time point. B, Values are reported as a percentage increase in the ischemic versus remote territory (n=8 per group). C, Example of a Western blot for H11K, HSP70, and IAP in cardiac myocytes isolated from both remote (rem) and ischemic (isch) areas of a swine heart submitted to repetitive stunning. *$P<0.05$ versus corresponding remote territory.

collected in subendocardial samples. We examined the expression of these proteins separately in the subepicardium, where we found directionally similar changes (not shown). To verify that the changes in gene expression occur in cardiac cells, a Western blot for H11K, HSP70, and IAP was performed in isolated cardiac myocytes prepared from the remote and ischemic area of a swine heart submitted to repetitive stunning. As shown in Figure 4C, similar changes were observed in this preparation compared with intact hearts.

The Survival Program Characterizes Viable Myocardium

Because the swine model and human hibernating myocardium are not totally devoid of necrosis,[23,37] we verified next that the changes in gene expression described are specific of viable rather than necrotic tissue. Three pigs were submitted to myocardial infarction induced by 72 hours of total coronary occlusion. Samples were taken from both the infarcted

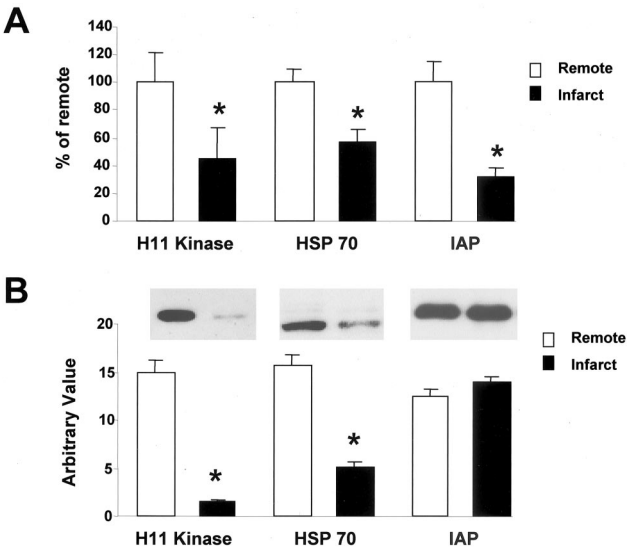

Figure 5. Expression of survival genes and proteins in swine infarcted myocardium. Swine hearts were submitted to myocardial infarction by occluding the LAD artery for 72 hours. A, Quantitative polymerase chain reaction for H11K, HSP 70, and IAP from remote (open bars) and infarcted (closed bars) myocardium. B, Western blotting for H11K, HSP 70, and IAP from remote (white bars) and infarcted (black bars) myocardium (both panels, n=3 per group). *$P<0.05$ versus corresponding remote territory.

area and the normal remote tissue. Both the gene and protein expression of H11K, HSP70, and IAP were measured. As shown in Figure 5, there was a significant decrease of all these parameters (except no change of IAP protein) in infarct tissue compared with normal. Therefore, the survival program cannot be attributed to irreversible injury, but rather characterizes viable myocardium.

Reduction of Apoptosis During Repeated Stunning

We tested the physiological relevance of this progressive upregulation of cytoprotective genes by measuring the rate of cardiac cell apoptosis in the swine myocardium submitted to repeated episodes of ischemia. As shown in Figure 6, apoptotic cell death measured by TUNEL was increased after a single episode of ischemia but markedly increased after 3 episodes. Subsequently, however, the rate of apoptosis significantly ($P<0.05$) decreased, and the number of apoptotic cells detected after 6 episodes was less than one third of the corresponding value after 3 episodes (Figure 6). This protection was accompanied by a significant increase in the anti-apoptotic protein Bcl2 (Figure 6). Interestingly, Bcl2 increased significantly after the sixth episode at the time when apoptosis decreased.

Discussion

Myocardial hibernation represents a condition of chronic regional ventricular dysfunction in patients with coronary artery disease, which is reversible after revascularization. The term "hibernation" referring to this condition was first used by Diamond et al in 1978.[38] Rahimtoola expanded on this concept and proposed that the hibernating heart downgrades its contractile function to reach a new equilibrium with

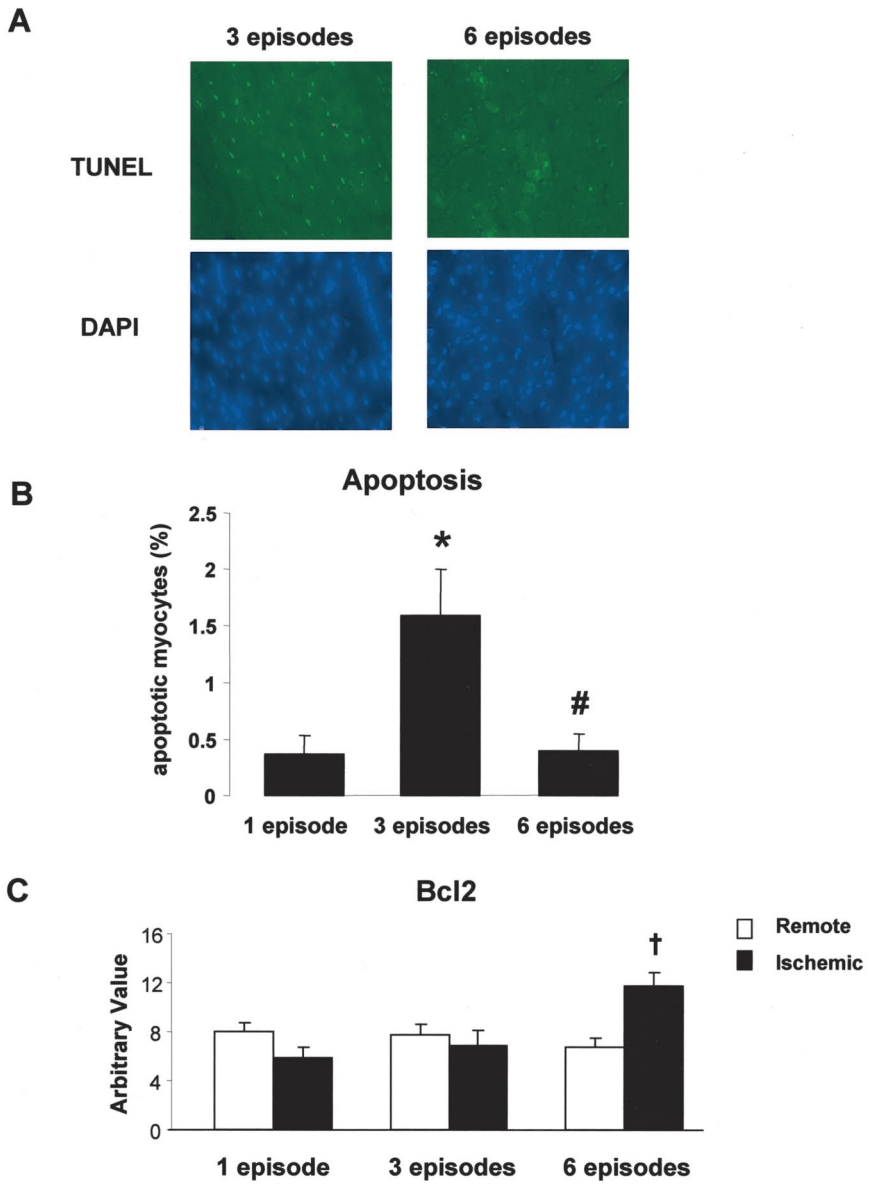

Figure 6. Decreased rate of apoptosis after repeated cycles of ischemia/reperfusion. A, Illustrative example of TUNEL measurement of apoptotic cells and nuclear counterstaining by DAPI in myocardial samples submitted to 3 and 6 episodes of ischemia–reperfusion. B, Averaged percentage of TUNEL-positive myocytes in ischemic myocardium after 1, 3, or 6 episodes of ischemia–reperfusion (n=5 per group; 88 500±6000 nuclei counted per sample). *$P<0.05$ versus 1 episode; #$P<0.05$ versus 3 episodes. C, Increase in Bcl2 protein after 6 episodes of ischemia–reperfusion (n=4 per group). †$P<0.05$ versus remote.

reduced blood flow.[6,39] An alternative hypothesis is that baseline blood flow is maintained in the absence of stress but, because of impaired coronary flow reserve, stress results in an imbalance between myocardial supply and demand. On recovery, myocardial stunning occurs, and when this process is repeated chronically, a chronic depression in regional function, ie, hibernating myocardium, develops.[7,40] Animal models with reversible regional dysfunction demonstrating either a constant reduction of resting blood flow[18,41–43] or repetitive stunning, without a chronic severe reduction in resting blood flow, have been developed.[23,44] In both situations, the mechanisms responsible for the protection of hibernating myocardium remain unclear. Several experimental models reproduce the hallmarks of human hibernating myocardium, including chronic dysfunction, loss of myofibrils, accumulation of glycogen, alteration of Ca^{2+} metabolism, and some extent of cell degeneration.[18,19,23,41,44] The current study, which compares hibernating myocardium in patients and in a swine model of the clinical condition, shows that these similarities can be extended at the genomic level.

Our findings illustrate a novel mechanism underlying hibernating myocardium in the form of an endogenous genomic program of cytoprotection that could subtend cell survival under conditions of prolonged ischemia. The corollary in our animal model shows that a repetitive pattern of ischemia–reperfusion progressively results in a program of cytoprotection and survival. To assess a functional correlate, we measured apoptosis, which was already increased after the first episode and increased further after the third episode. However, after the sixth episode, apoptosis decreased significantly, most likely because of these mechanisms of cardioprotection. Most importantly, we describe the simultaneous activation of an array of genes, all with the potential of protecting the myocardium when overexpressed individually.[25–28,30] Therefore, we propose that such program of cell survival represents a coordinated effort to protect the myo-

cardium by additive and complementary mechanisms, which would limit cell degeneration and irreversible injury in hibernating heart.[37,45] This endogenous mechanism of self-defense is reminiscent of ischemic preconditioning, in which cardioprotection is conferred by the application of a series of short-term ischemic episodes,[46] and which is also followed by a second window of protection resulting from gene regulation.[47,48]

Previous investigations have addressed the regulation of genes and proteins in hibernating myocardium to better understand the molecular basis for myocardial dysfunction in this condition.[18,19,21] As for survival mechanisms, an upregulation of HSP70 has been shown, both in the human myocardium[18] and in a swine model.[19] The study conducted in humans found an increased expression of HSP70 in cardiac tissue from patients with functional reversibility, preserved metabolic activity, and normal resting flow,[20] which corresponds to the criteria of our patient population. Except for HSP70, the survival mechanisms in hibernating heart have been so far reported mainly as a metabolic adaptation. Recently, it was proposed that increased glycolysis is a protective mechanism in the human hibernating myocardium,[45] which is compatible with our observation that HIF-1α, a strong activator of the expression of genes encoding glycolytic enzymes, and the glucose transporter GLUT1 are upregulated. Therefore, the novelty of our study is the demonstration that the survival mechanisms in hibernating myocardium involve more than a metabolic shift and include the coordinated activation at the gene level of complementary mechanisms limiting cell death. The physiological relevance of this mechanism is illustrated by the decreased rate of apoptosis as ischemia is repeated in the swine model (Figure 6). An alternative, but less likely, explanation for the decrease of apoptosis after 3 episodes is that all the cells meant to die are gone at this time. This is unlikely because the upregulation of a survival program in itself shows that the myocardium remains threatened by ischemic damage, and the efficiency of the proteins described here to limit cell death has been demonstrated in several experimental models.

In addition to illustrating a novel mechanism of protection in a postmitotic tissue, the current study also offers insight into the pathophysiological mechanisms of myocardial hibernation. Figure 3 clearly illustrates that the survival genes activated by a single episode of ischemia are subsequently reactivated as ischemia is repeated, which shows a molecular link between the concepts of stunning (temporary dysfunction after a single episode of ischemia)[24] and hibernation (sustained decrease in contractility caused by repetitive ischemia). This progressive adaptation to ischemia has also been shown for proteins involved in Ca^{2+} homeostasis.[49]

Because it is recognized that hibernating myocardium may include some necrotic tissue,[37] it was important to demonstrate that our results reflected changes in dysfunctional, but viable, chronically ischemic myocardium and not in irreversibly injured tissue. We verified this important point, and our conclusions are supported by at least 4 lines of experimental evidence. The strongest evidence is that the genes and proteins of survival found to be elevated in the current study were also measured in infarcted myocardium with directionally opposite results, ie, they were reduced in infarcted myocardium (Figure 5). In addition, we found similar upregulation of genes and proteins in the subepicardium as well as in the subendocardium in the chronically stunned swine heart. As noted in our previous study describing this model, there was very little subendocardial necrosis (3.4% of the area at risk) in this model, and virtually no subepicardial necrosis.[23] Third, the changes in protein expression were found specifically in isolated cardiac myocytes (Figure 4) and therefore cannot be ascribed to an inflammatory reaction or other cell contamination related to the preparation. Finally, the human biopsy samples were taken from areas of myocardium with maintained glucose uptake and presenting no evidence of irreversible damage at CMR. Therefore, the changes in genes and proteins measured in the current study cannot be ascribed to irreversible injury, but rather characterize a genomic adaptation of viable myocardial tissue.

Using the paradigm described here in hibernating myocardium, it is likely that an activation of this gene program in postmitotic tissues could offer a new therapeutic alternative to prevent irreversible cell loss. Our study addressed a specific subset of genes to demonstrate the concept of survival in hibernating myocardium, and it is likely that this concept could be expanded by large-scale genomics techniques. A better understanding of this concept is of clinical importance to expand conditions of maintained cellular viability and functional salvage of the ischemic myocardium. Therefore, deciphering the molecular mechanisms balancing cell growth and apoptosis may help develop strategies to improve the prognosis of both ischemic heart disease and heart failure, which is secondary to coronary artery disease in the majority of cases. It is also conceivable that these novel findings may be useful in determining future tests for the diagnosis of human hibernating myocardium.

Acknowledgments

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METHODS

Human studies. Twelve patients with coronary artery disease and chronic left ventricular (LV) dysfunction without previous clinical myocardial infarction in the area of interest and awaiting bypass surgery were studied. The main eligibility criterion was the demonstration of tissue viability in one or more chronically dysfunctional segments subtended by stenotic coronary arteries in patients devoid of acute coronary syndromes. Global and regional LV function was assessed by cine cardiovascular magnetic resonance (CMR), using steady state free precession cine sequences[1]. Late gadolinium imaging was used to define myocardial scar, using an inversion recovery sequence with FLASH readout >10 minutes after intravenous injection of 0.1mmol/kg gadolinium-DTPA[2]. In all patients, the presence of viable myocardium was confirmed by <50% transmural uptake of gadolinium using CMR, as described by Kim et al[3]. All images were gated to the electrocardiogram. Cine images were acquired in both short-axis and long-axis views before and after injection of 0.1 mmol/kg gadolinium-DTPA. In six patients, myocardial viability was confirmed by maintained uptake (>0.25 μmol/min per g of tissue) of ^{18}F-fluorodeoxyglucose (FDG) with positron emission tomography (PET)[4,5]. Absolute regional myocardial blood flow was measured with PET using oxygen-15 labeled water[6]. At the time of bypass surgery, two full thickness biopsies (one from the hibernating, another from a remote normally contracting area) were taken from the LV of each patient, and frozen immediately. A follow-up cine CMR scan was carried out six months after bypass to confirm the functional improvement of all hibernating segments where biopsies were obtained. Regional LV function (16 segment model) was graded according to a 4 points scale (0= normal, 1= moderate hypokinesis, 2= severe hypokinesis, 3= a- or dyskinesis). Changes in wall motion ≥ 1 grade at follow-up were considered significant. Informed consent was obtained and the protocol was approved by the Royal Brompton and Hammersmith Hospitals. Radiation exposure was licensed by the UK Administration of Radioactive Substances Advisory Committee.

Animal model. Animals used in this study were maintained in accordance with the Guide for the Care and Use of Laboratrory Animals (National research Council, revised 1996). Female domestic swine (22-25 kg) were sedated with ketamine (10 to 20 mg/kg, IM) and xylazine (2.2 mg/kg, IM), and general anesthesia was maintained with isoflurane (0.5 to 2.0 vol%). A hydraulic occluder was implanted around the base of the left anterior descending (LAD) coronary artery. Myocardial blood flow through the LAD was monitored by a Transonic flow probe. Regional wall thickening in ischemic and remote area was measured by ultrasonic crystals. After 3-5 days of recovery, ischemia was induced in the conscious animal by inflating the coronary occluder to reduce the blood flow in the LAD by 30-40% from baseline[7]. The coronary stenosis was maintained for 90 min, followed by complete deflation of the occluder, and repeated every twelve hours up to six times. In this model, the wall thickening in the ischemic territory never fully recovers from the first to the sixth ischemic episode, thereby creating a condition of persistent stunning. One hour after reperfusion, myocardial samples were taken from both the hibernating area and the remote area of the beating heart following one, three or six episodes of ischemia. Three instrumented pigs, in which no occlusion was performed, were used as shams. In three additional pigs, myocardial infarction was induced by occluding the LAD coronary artery for 72 hours. Samples were taken in the middle of the hibernating area and in the remote myocardium. In each case, tissues were frozen immediately or fixed in formalin. Measurements presented in the Figures were performed in samples taken from subendocardium, and the results were subsequently confirmed in subepicardial samples.

Quantitative RT-PCR. About 100 mg of each sample was homogenized in 3 ml of the guanidium thiocyanate-phenol-chloroform solution (Triazol, Gibco Life Technologies). Total RNA was extracted by phenol-chloroform, resuspended in 50 µl DEPC-water, and its concentration was measured spectrophotometrically by the absorbance at 260 nm. The integrity of the RNA pool was checked on a 1%-agarose denaturing gel stained with ethidium bromide. The mRNA of interest was reverse-transcribed and used for quantitative 2-step PCR (7700 Prizm, Perkin-Elmer/ABI) with

specific fluorogenic probes and primers designed from public databases with the Primer Express software. Internal RNA standards were prepared from the PCR-amplified cDNA after ligation of the T7 promoter using the MegaShortScript kit (Ambion, Austin, TX)[8]. Data are reported per number of cyclophilin transcripts, used as a normalizer (copies of cyclophilin transcript per 100 ng of RNA was $1.8 \pm 0.1.10^4$ in remote versus $2.2 \pm 0.3.10^4$ in hibernating myocardium, NS).

Western blotting. Tissue was homogenized in 7M urea, 2M thiourea, 4% CHAPS, 0.5% IPG-ampholytes pH 3-10, 1% Triton X-100, 1% DTT, supplemented with phosphatase and protease inhibitors, and centrifuged at 50,000 g for 30 min at 4°C. Protein extracts were resolved on 12% SDS-PAGE gels and transferred to PVDF membranes. Primary antibodies for H11 kinase (custom-made antibody as reported in ref. 29, dilution 1/1,000), HSP70 (Santa Cruz, dilution 1/500) and IAP (Cell Signaling, dilution 1/500) were incubated overnight. Detection was performed by chemiluminescence (New England Biolabs). Equal protein loading was verified by Coomassie staining.

TUNEL staining. Tissue samples were fixed by immersion in 10% phosphate-buffered formalin dehydrated, embedded in paraffin and sectioned at 6-µm thickness. Sections were treated with 2% H_2O_2 to inactivate endogenous peroxidases, then with 20 µg/ml proteinase K for permeabilization, and washed in PBS. For nick end labeling, DNA fragments were labeled with 2 nM biotin-conjugated dUTP and 0.1 U/µl TdT for 1 hour at 37°C. Incorporation of biotin-16-dUTP was measured by incubating the sections with FITC-ExtrAvidin (Sigma) at room temperature for 30 minutes[9]. After washing, slides were mounted in a Vector DAPI medium for fluorescent microscopic observation at x40-objective field (Zeiss). FITC-stained nuclei were counted and reported as a percentage of the total DAPI-stained nuclei population. The number of nuclei counted in the different groups is reported in the corresponding Figure legend.

Statistical analysis. Results are presented as the mean ± standard error for the number of samples indicated in each figure legend. Statistical comparison was performed using the Student's *t* test with paired comparison for the measurements of human samples, and using a two-way ANOVA with Bonferroni correction for the time-course experiments in the swine model.

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