Dobutamine responsiveness, PET mismatch, and lack of necrosis in low-flow ischemia: is this hibernation in the isolated rat heart?

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Southworth, Richard, and Pamela B. Garlick. Dobutamine responsiveness, PET mismatch, and lack of necrosis in low-flow ischemia: is this hibernation in the isolated rat heart? Am J Physiol Heart Circ Physiol 285: H316–H324, 2003. First published March 13, 2003; 10.1152/ajpheart.00906.2002.—The clinical hallmarks of hibernating myocardium include hypotrophy while retaining an inotropic reserve (using dobutamine echocardiography), having normal or increased [18F]fluoro-2-deoxyglucose-6-phosphate ([18F]FDG6P) accumulation associated with decreased coronary flow (flow-metabolism mismatch) by positron emission tomography (PET), and recovering completely postrevascularization. In this study, we investigated an isolated rat heart model of hibernation using experimental equivalents of these clinical techniques. Rat hearts (n = 5 hearts/group) were perfused with Krebs-Henseleit buffer for 40 min at 100% flow and 3 h at 10% flow and reperfused at 100% flow for 30 min (paced at 300 beats/min throughout). Left ventricular developed pressure fell to 30 ± 8% during 10% flow and recovered to 90 ± 7% after reperfusion. In an additional group, this recovery of function was found to be preserved over 2 h of reperfusion. Electron microscopic examination of hearts fixed at the end of the hibernation period demonstrated a lack of ischemic injury and an accumulation of glycogen granules, a phenomenon observed clinically. In a further group, hearts were challenged with dobutamine during the low-flow period. Hearts demonstrated an inotropic reserve at the expense of increased lactate leakage, with no appreciable creatine kinase release. PET studies used the same basic protocol in both dual- and globally perfused hearts (with 250 MBq 18FDG in Krebs buffer ± 0.4 mmol/l oleate). PET data showed flow-metabolism “mismatch,” whether regional or global, [18F]FDG6P accumulation in ischemic tissue was the same as (glucose only) or significantly higher than (glucose/oleate) control tissue (0.023 ± 0.002 vs. 0.111 ± 0.002 normalized counts·s⁻¹·g⁻¹·min⁻¹, P < 0.05) despite receiving 10% of the flow. This isolated rat heart model of acute hibernation exhibits many of the same characteristics demonstrated clinically in hibernating myocardium.

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UP TO 50% OF ALL PATIENTS suffering from coronary heart disease are thought to have hibernating myocardium that would benefit from revascularization (17, 24). Myocardial hibernation was first described in 1989 by Rahimtoola (28) as the “persistently impaired function of viable myocardium in the setting of reduced coronary blood flow.” More recently, however, some patients exhibiting hibernating regions of myocardium have been shown to have near-normal coronary flow. In these patients, it has been suggested that a decreased coronary reserve would lead to successive periods of ischemia and reperfusion, i.e., repetitive stunning (5, 36). However, the presence of normal blood flow during hibernation is not universally accepted (6), and the role of repetitive stunning thus remains the subject of debate (8, 12). Over a decade since the first description of hibernation, little is known about the cellular events involved in its induction and maintenance, and the conditions under which it exists clinically remain controversial. In a 1998 report (18) on the medical and cellular implications of the ischemic syndromes, resulting from a National Heart, Lung, and Blood Institute workshop, Kloner et al. concluded that an experimental model of myocardial hibernation, which was validated by “generally accepted clinical criteria for hibernating myocardium,” was urgently required.

The key feature of hibernating myocardium is that, after revascularization, cardiac contractility returns to normal (or near normal) values (3). Thus the correct diagnosis of hibernation in a patient presenting with left ventricular dysfunction has important prognostic and therapeutic implications. Clinically, hibernating regions of the heart display a number of common features. Functionally, they are characterized as being hypo- or even akinetic while retaining a contractile reserve, as demonstrated by increased contractility in response to low-dose dobutamine during echocardiography (1). Morphologically, they display little evidence of ischemic damage, although in chronically hibernating tissue, there is some evidence of loss of myofibrillar content and accumulation of glycogen (26). Biochemically, they are often characterized by a normal or increased [18F]fluoro-2-deoxyglucose ([18F]FDG) uptake despite a limited blood flow (flow-metabolism “mismatch”). This is demonstrated using positron emission tomography (PET), which is generally considered to be the “gold standard” technique for diagnosing hibernating myocardium (reviewed by Dutka and Camici (9)).

The lack of agreement on the clinical correlates of hibernation highlights the importance of developing and characterizing models of hibernation achieved by
either route. In this study, we investigated a low-flow model of hibernation in the isolated rat heart, employing experimental equivalents of the clinical techniques used for its diagnosis, namely, recovery of function on reperfusion, lack of tissue necrosis, dobutamine responsiveness, and flow-metabolism mismatch by PET.

MATERIALS AND METHODS

Reagents

Perfusion media were supplied by BDH (Dorset, UK) except for oleate (albumin bound), which was supplied by Sigma (Dorset, UK). 18FDG was kindly provided by the Clinical PET Centre, St. Thomas’ Hospital (London, UK).

Heart Preparation

Male Wistar rats (220–260 g, 9 groups, n = 5 rats/group) were anesthetized with Sagatal (100 mg ip), and heparin (200 IU) was injected into the femoral vein. Hearts were excised and immediately immersed in ice-cold Krebs-Henseleit buffer. Hearts were cannulated and placed in a heart chamber designed to fit within the mini-PET scanner as previously described (14). Buffer oxygenation was performed using membrane oxygenators rather than gassing sticks to prevent excessive frothing. After 20 min of constant pressure perfusion, the perfusion mode was switched to constant flow, and 200 MBq 18FDG was introduced into the perfusion buffer reservoir. After a further 40 min, the flow rate was decreased to 10% for 3 h, the hibernation period, and then reperfused under constant pressure for 30 min. PET scans were acquired throughout the protocol. In group VII, this protocol was repeated with glucose only-containing buffer.

During regional low-flow perfusion (groups VIII and IX). During global low-flow perfusion (groups VI and VII). In group V, hearts were instrumented with an intraventricular balloon as previously described, and the hibernation protocol was repeated with bolus of 10⁻⁶ 10⁻⁷, and 10⁻⁸ M dobutamine in 100 μl of water being injected into a side arm immediately above the aortic cannula after 60, 100, and 140 min of low-flow ischemia, respectively. Coronary flow was measured throughout the protocol, and aliquots of perfusate were collected for analysis of lactate and creatine kinase (CK).

Investigation of 18FDG-6-Phosphate Accumulation by PET

During global low-flow perfusion (groups VI and VII). In group VI, the perfusion buffer contained 0.4 mmol/l oleate (bound to bovine serum albumin) in addition to the standard 11 mmol/l glucose. Hearts were cannulated and placed in a heart chamber designed to fit within the mini-PET scanner as previously described (14). Buffer oxygenation was performed using membrane oxygenators rather than gassing sticks to prevent excessive frothing. After 20 min of constant pressure perfusion, the perfusion mode was switched to constant flow, and 200 MBq 18FDG was introduced into the perfusion buffer reservoir. After a further 40 min, the flow rate was decreased to 10% for 3 h, the hibernation period, and then reperfused under constant pressure for 30 min. PET scans were acquired throughout the protocol. In group VII, this protocol was repeated with glucose only-containing buffer.

Functional Study (Groups I and II)

Immediately after cannulation, a polythene intraventricular balloon was inserted into the left ventricle via the left atrium and connected to a pressure transducer and recording apparatus. The balloon was inflated to an end-diastolic pressure of ~6 mmHg, and the left ventricular pressure was recorded. Coronary flow was measured throughout the protocol, and aliquots of perfusate were collected for lactate and creatine kinase analysis. After the stabilization period, hearts were switched to constant flow perfusion for a further 20 min (flow adjusted to obtain the same perfusion pressure as during the stabilization period, monitored using pressure transducers in the afferent buffer lines). The flow rate was then decreased to 10% for 3 h, the “hibernation period,” and the hearts were then reperfused under constant pressure for 30 min in group I. In group II, the stability of recovery of function postreperfusion was investigated by repeating this protocol and extending the reperfusion period from 30 min to 2 h.

Investigation of Tissue Morphology (Groups III and IV)

Hearts (group III) were perfused as described in Functional Study (Groups I and II) in parallel with a timed matched aerobically perfused group (group IV) and perfusion fixed with glutaraldehyde (25% in aqueous solution) infusion for the last 4 min of the hibernation period at a rate equal to 10% of the control coronary flow. The hearts were then removed from the cannula, and a midventricular section of the free left ventricular wall was cut and stored in paraformaldehyde. Each sample was identified with a code known to only one of the authors (R. Southworth) before being sent for electron microscopic analysis.
international units of CK released per minute per gram of heart wet weight.

Electron Microscopy

Tissue samples were embedded in Spurr resin following routine embedding procedures. Several ultrathin sections were prepared using a Reichart OMU4 ultramicrotome and stained with uranyl acetate, followed by Reynolds lead citrate. The sections were then examined and photographed with a JEOL 100CX transmission electron microscope operated at 80 kV. All sections were evaluated (blind) to determine the degree of ischemic injury and the extent of glycogen granule accumulation.

Acquisition and Analysis of PET Scans

Short-axis midventricular PET scans of each heart were obtained using a 5-cm-diameter, single-slice mini-PET scanner, as described previously (14). In the global ischemia studies, $^{18}$FDG-6-phosphate ($^{18}$FDG6P) accumulation across the whole midventricular slice was considered. In the regional ischemia studies, the distribution of the blue dye was used to map two "regions of interest" on the PET images. These were then used to calculate the accumulation of $^{18}$FDG6P into the left and right sides of the heart. All images were corrected for decay and initial activity and analyzed using standard ECAT software; the resulting data were expressed as normalized $^{18}$FDG6P accumulation per unit area of the region of interest.

Statistical Analysis

All data are means ± SE. Data were analyzed using ANOVA, followed by Student’s $t$-test for unpaired data with a Bonferroni correction. A $P$ value of <0.05 was considered statistically significant.

RESULTS

Recovery of Cardiac Function

Cardiac function, lactate release, and CK leakage during the hibernation protocol are shown in Fig. 1. After an initial fall in developed pressure to ~15% of the preischemic value, cardiac function quickly recovered to 30% within 5 min and remained stable throughout the rest of the 3-h ischemic period. Function recovered instantaneously on reperfusion and was not significantly different from preischemic values throughout the 30-min reperfusion period. At no time during the protocol was end-diastolic pressure elevated above preischemic values. Whereas there was no elevation in the rate of CK release during low-flow ischemia, myocardial lactate release approximately doubled, rapidly normalizing on reperfusion. When the reperfusion period was extended from 30 min to 2 h in group II hearts (shown in Fig. 2), functional recovery was shown to be preserved over this prolonged reperfusion period.

Tissue Morphology

Representative electron micrographs of sections from low-flow ischemic hearts at the end of the hibernation period (group III) and time-matched controls (group IV) are shown in Fig. 3. There was no evidence of ischemic damage in any of the sections from any of the hearts (including all of the sections not shown). Across all the samples investigated, there was a marked increase in glycogen at the margins of the mitochondria and myofilaments in hearts subjected to the hibernation protocol compared with the control group; this is shown in the higher-magnification images.
Response to Dobutamine

During the hibernation period, group V hearts demonstrated a strong dobutamine response at all three doses used, responding maximally after $10^{-7}$ M (Fig. 4). Each bolus elicited a marked increase in myocardial lactate release, which closely followed the observed increases in developed pressure. CK release under these stimuli, however, was unaffected. At the end of this protocol, developed pressure recovered to preischemic values, albeit with a slight but nonsignificant elevation in end-diastolic pressure during early reperfusion.

Analysis of Dual-Perfusion PET Data

Figure 5, A and B, shows a representative demarcation of the two coronary vascular beds obtained by perfusing one side of the heart with blue dye at the end of the protocol. This delineation was then used to construct the regions of interest shown in Fig. 5C on a representative PET scan. These regions of interest were then used to calculate the accumulation of $^{18}$FDG6P into the two sides of the heart during the hibernation protocol.

Flow-Metabolism Mismatch by PET

A representative series of PET scans from a regionally ischemic heart (from group VIII) perfused in the presence of $^{18}$FDG, glucose, and oleate is shown in Fig. 6. During aerobic perfusion, very little $^{18}$FDG6P accumulation was evident in either side of the heart. After the onset of regional ischemia, there was a marked increase in $^{18}$FDG6P accumulation in the ischemic side, whereas the rate of accumulation remained low in the control side. Quantitative analysis of the PET data from these regionally ischemic hearts is shown in Fig. 7. In hearts perfused with glucose only (group IX; Fig. 7A), there was no regional difference in phosphorylated glucose tracer accumulation during the aerobic perfusion period. There continued to be no difference in uptake between the two sides of the heart throughout the ischemic period, despite one side of the heart receiving only 10% of the control flow. In hearts perfused with glucose and oleate (group VIII; Fig. 7B), again there was no regional difference in phosphorylated glucose tracer accumulation during the aerobic perfusion period, although the absolute rates observed were lower than with glucose alone. However, during regional ischemia, the accumulation rate increased markedly in the ischemic side, whereas it remained unaffected in the control side.

The same phenomenon was also observed in hearts exposed to global ischemia (groups VI and VII; Fig. 8). $^{18}$FDG6P accumulation progressed at a constant rate throughout the protocol in hearts perfused with glu-
cose only, independent of the flow received, whereas hearts perfused with glucose and additional oleate demonstrated a much lower rate during aerobic perfusion, which increased during ischemia. A comparison of the rates of accumulation of phosphorylated tracer in globally ischemic hearts is shown in Fig. 9. In glucose-only hearts, the rate of accumulation was not statistically different before and during hibernation (3.0 ± 0.4 vs. 2.7 ± 0.3 U/s), whereas in hearts perfused with glucose plus oleate, phosphorylated tracer accumulation increased dramatically during hibernation (1.1 ± 0.2 vs. 2.3 ± 0.2 U/s, P < 0.05).

**DISCUSSION**

In this study, we describe a short-term model of acute hibernation in the isolated perfused rat heart. We showed that the model demonstrates the same basic physiological, morphological, and biochemical characteristics observed clinically in the hibernating heart, using experimental equivalents of the techniques used for its diagnosis in the patient.

Although the role of decreased flow in mediating hibernation has recently been the subject of much debate, we demonstrated that decreasing the flow to 10% induces a dramatic and persistent decrease in contractile function while being sufficient to maintain myocardial viability and allow instantaneous and complete recovery of function on reperfusion. This phenomenon has been previously described as “perfusion-contraction matching,” and it seems likely that the decrease in flow induces the heart to downregulate its contraction, establishing a new balance between energy supply and demand, thus conserving viability. Low-flow ischemic models of acute hibernation via perfusion-contraction matching have been demonstrated in a number of other species, including pigs (31), dogs (22), and rats (30).

In our model, we demonstrate that through such perfusion-contraction matching, rat hearts are capable of enduring low-flow ischemia over a prolonged period of 3 h with no impact on cell viability. Furthermore, we provide evidence, albeit nonquantitative, that under these conditions of low flow, intracellular glycogen accumulation occurs, a striking parallel with observations made from biopsies from hibernating tissue obtained clinically (26).

Recovery of function postocclusion in a model of acute hibernation in open-chest pigs has demonstrated an initial improvement of function on early reperfusion that declined with time, presumably as a late response to either ischemic or reperfusion injury (27). We were therefore concerned that the complete recovery of function we observed after 30 min may not indicate the true viability of our model. We therefore extended the reperfusion period in one group of hearts to 2 h. As can be seen, contractile function was preserved and no late injury, due to either ischemia or reperfusion, was evident.

In isolated perfused rat (34) and rabbit (11) hearts, it was suggested that a 5-min period of zero flow before the 3-h low-flow period was essential to trigger the protective mechanism of hibernation. However, it was subsequently shown in pigs that this protection was abolished by glibenclamide, indicating that this effect...
was due to the coexistence of preconditioning via ATP-dependent K\(^+\) channels rather than being indicative of a new hibernation protocol (32). In the isolated rat heart study, it is difficult to understand why the authors observed ischemic injury during 10% flow without the trigger, when we observed complete recovery using a similar regime. We can only conclude that this is due to differences in the perfusion methodologies used.

With the use of our protocol, the heart maintains its capacity to respond to adrenergic stimulation with dobutamine, another characteristic used to diagnose hibernating segments clinically during stress echocardiography. This effect has been previously demonstrated experimentally in an open-chest pig preparation (31) but has not been demonstrated in an isolated heart to our knowledge. As shown in Fig. 4, when the equilibrium of the hibernating heart is challenged by dobutamine, the subsequent increase in anaerobic metabolism results in increased lactate production and release. With high-dose dobutamine (10\(^{-6}\) M), no further increase in contractility was observed, due to either adrenoceptor saturation or the incapacity of the heart to respond under the prevailing oxygen and nutrient conditions. Even under this apparent stress, the absence of CK in the coronary effluent indicated that no significant tissue damage was induced. In summary, the dobutamine response observed in our model is analogous to that observed clinically in hibernating myocardium.

The final piece of evidence that we provide for the similarity between this model and the clinical situation is the flow-metabolism mismatch demonstrated by PET, generally considered to be the gold standard of hibernation diagnosis clinically (15). With the use of our mini-PET scanner, we (14) previously demonstrated cessation of \(^{18}\)FDG accumulation during a regional zero-flow ischemia and reperfusion regime in the isolated perfused rat heart. With the present hibernation protocol, we demonstrated normal \(^{18}\)FDG accumulation despite only 10% of the flow in both regionally and globally ischemic hearts, with glucose...
as the sole substrate. When oleate was used as an additional substrate, $^{18}$FDG accumulation increased during the low-flow period in both regionally and globally ischemic hearts. Because hibernating cardiac regions are diagnosed clinically by normal or increased $^{18}$FDG accumulation, these data further confirm the validity of our model.

$^{18}$FDG accumulation in the myocardial cell depends on its uptake by glucose transporters GLUT1 and GLUT4 and its phosphorylation by hexokinase [for a review, see Lopaschuk (19)]. We (14) previously demonstrated translocation of GLUT4 to the sarcolemma during total ischemia and reperfusion in the isolated rat heart, and it has also been demonstrated during low-flow ischemia in the canine heart (38). Hexokinase has been shown to translocate to the mitochondria during ischemia (29), increasing its activity by receiving ATP directly from the mitochondria and decreasing its susceptibility to inhibition by glucose-6-phosphate.

It is likely that both these mechanisms are involved in the increased $^{18}$FDG accumulation observed both in the model described in this paper and in hibernating myocardium clinically. Our data demonstrate the capacity of this mechanism to increase the extraction of glucose by over 10-fold in response to low-flow ischemia. Perfusion with fatty acids has been demonstrated to internalize GLUT4 (37), and $\beta$-oxidation of fatty acids will indirectly inhibit hexokinase via increased levels of glucose-6-phosphate (7); these mechanisms would lead to the low $^{18}$FDG accumulation that we observed in the regions of oleate-perfused hearts receiving normal flow. However, during ischemia, low oxygen levels inhibit $\beta$-oxidation, and it is likely that in turn GLUT4 and hexokinase translocation are pro-

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**Fig. 7.** Graph showing the accumulation of $^{18}$FDG-6-phosphate ($^{18}$FDG6P) in the control (●) and ischemic (○) regions of interest in dual-perfused hearts subjected to the regional hibernation protocol. Hearts were perfused with glucose only (A) or glucose plus oleate (B) as the substrates. Data are expressed as means ± SE; n = 5.

**Fig. 8.** Accumulation of $^{18}$FDG6P in a midventricular section through hearts subjected to the global hibernation protocol. Hearts were perfused with glucose (●) or glucose plus oleate (○) as the substrates. Data are expressed as means ± SE; n = 5.

**Fig. 9.** Rate of accumulation of $^{18}$FDG6P in short-axis midventricular sections through hearts during aerobic control perfusion or 10% low-flow ischemia. Hearts were perfused with glucose or glucose plus oleate as the substrates. Data are expressed as means ± SE; n = 5.
moderated, leading to the observed increased uptake of glucose necessary for maintenance of cell viability. The relative importance of low blood flow and repetitive myocardial stunning in the aetiology of hibernation is currently the subject of much debate. Hibernation was initially thought to be a state of decreased contractility resulting from decreased blood flow (perfusion-contraction matching). Canty and Fallavollita (6) noted that 10 of 13 clinical studies on hibernation demonstrated decreased blood flow in the hibernating region by up to 58%. However, it has also been proposed that hibernation may exist in regions of the heart where resting perfusion is normal, but coronary flow reserve is decreased (20, 35); proponents of this theory argue that early blood flow measurement techniques were subject to errors caused by the “partial volume effect” (23) and that such errors could lead to the underestimation of blood flow in the hibernating region (5, 9). Under conditions of decreased coronary flow reserve, each demand placed on a compromised region of the heart potentially subjects that region to a period of ischemia; reperfusion of the region will occur when heart rate and contractility return to normal. Repeated cycles of such ischemia-reperfusion under these conditions may then result in chronic stunning leading to hibernation (36).

Revascularization of hibernating tissue in patients has therefore been suggested to restore the coronary flow reserve rather than the resting coronary flow (26). It has also been suggested that blood flow may become reduced in chronically stunned tissue due to the decreased energy demands of the hypoxic contractile region, i.e., that blood flow is reduced as a consequence of hibernation rather than a cause (10). While this may be the case, there remains no proof that stunning per se is the causative link between chronic stenosis and reduced contractile function either clinically or when experimentally induced. While the perceived importance of repetitive stunning in hibernation gains ground in the clinical setting, considerable experimental evidence contradicts this theory. Although repetitive stunning protocols in in vivo animal models yield cumulative ventricular dysfunction, the increased 18FDG6P accumulation observed clinically has not been demonstrable in all experimental models (8, 16). It has also been shown that the coronary reserve is maintained in a model of hibernation in the pig, whereas perfusion pressure and flow are reduced by an 80% stenosis (21). Furthermore, it has been argued that reperfusion of a chronically stunned region of the heart in the context of an unchanged coronary flow would not necessarily lead to the near instantaneous functional recovery of hibernating tissue observed clinically (12).

It is this uncertainty as to the nature of hibernation in the patient that currently confounds the basic science approach to modeling the phenomenon. If regions of low flow are demonstrable, then some degree of perfusion-contraction matching must be responsible for hibernation; however, the demonstration of regions with normal flow would suggest stunning as a key mechanism. It seems likely that both mechanisms are involved in hibernation, either in combination or possibly independently in two subpopulations of patients. Further research into both mechanisms is therefore necessary.

Limitations of the Study

While the isolated heart preparation provides invaluable information on the biochemistry and function of the heart without interference from autonomic and hormonal influences, it cannot be used to monitor long-term chronic conditions. In the present study, we were therefore limited to examining a model of acute hibernation, using 3 h of low-flow ischemia. We believe that extension of the ischemic period beyond 3 h would say more about the stability of the isolated heart preparation itself than the model of hibernation and would provide little additional relevant information compared with hibernation periods of days or weeks in vivo. Our model therefore clearly lacks a significant factor in the pathophysiology of hibernation: that of time. While we observed acute changes in contractile function, myocardial glucose metabolism, and glycogen accumulation, all characteristic of the clinical situation, other effects associated with hibernation, such as interstitial fibrosis and loss of the contractile apparatus, are chronic events and are thus not demonstrated in our model. While extrapolation of our data to the chronic clinical situation must clearly be performed with caution, we believe that our model will prove invaluable in the examination of events early in the onset of myocardial hibernation.

In conclusion, using experimental equivalents of standard clinical techniques, we demonstrated that the isolated rat heart exposed to low-flow ischemia exhibits many of the characteristics seen in clinical hibernation.

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