Activation of p38 MAPK and increased glucose transport in chronic hibernating swine myocardium

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McFalls, Edward O., MingXiao Hou, Robert J. Bache, Aaron Best, David Marx, Joseph Sikora, and Herbert B. Ward. Activation of p38 MAPK and increased glucose transport in chronic hibernating swine myocardium. Am J Physiol Heart Circ Physiol 287: H1328–H1334, 2004. 10.1152/ajpheart.01188.2003.—In preconditioned myocardium, activation of the mitogen-activated protein kinase (MAPK) p38 leads to increased glucose uptake via enhanced GLUT-4 translocation. Glucose uptake is also increased in chronic hibernating myocardium, but the role of p38 MAPK and GLUT-4 translocation has not been studied. Nine swine underwent instrumentation of the proximal left anterior descending coronary artery (LAD) with a small, external constrictor. At 3 mo after instrumentation, myocardial glucose uptake by PET imaging was higher in the LAD than in the remote region under basal, fasted conditions (0.08 ± 0.02 vs. 0.45 ± 0.01 μmol·min⁻¹·g⁻¹, P < 0.05). Composed with the remote region, mean myocardial glucose uptake was higher in the LAD than in the remote region (P < 0.05) and correlated well with the absolute degree of GLUT-4 membrane-bound translocation (r = 0.81, P < 0.01). Relative increase in glycogen (r = 0.70, P < 0.05), and total NOS activity (r = 0.68, P < 0.05). In chronic hibernating myocardial tissue, p38 MAPK activation is increased under basal fasted conditions and correlates well with the increased degree of GLUT-4 translocation, glycogen accumulation, and NOS activity. As in preconditioned myocardium, activation of p38 MAPK may play an important role in the metabolic adaptations that characterize chronic hibernating myocardium.

GLUT-4; glucose uptake; preconditioned myocardium; inducible nitric oxide synthase

Since the seminal observation of ischemic preconditioning in the anesthetized dog model (16), it is widely accepted that the myocardium can favorably adapt so that necrosis can be attenuated after acute prolonged ischemia. Although this initial period of cardioprotection is brief, it is reacquired 24 h after initiation (1), emphasizing the relevance of preconditioning to patients with chronic myocardial ischemia. The signals that are involved with this early and late period of protection are complex. The mitogen-activated protein kinase (MAPK) p38 belongs to one family of stress-responsive enzymes that may play a critical role in signaling in response to chronic myocardial ischemia. In animal models of myocardial ischemic preconditioning, activation of p38 MAPK is important in reducing infarct size within the “first window of protection” (24) and signals transcriptional events within the “late window of protection,” particularly in regard to expression of inducible nitric oxide synthase (iNOS) (3, 35, 36). It also facilitates increased glucose uptake within the preconditioned myocardium by enhancing translocation of the glucose transporter GLUT-4 to the sarcolemma (31).

Whether p38 MAPK activation is also involved in mechanisms that underlie protection in chronically ischemic myocardium is unknown. With the use of dual tracers with PET imaging, hibernating myocardium has been characterized by increased glucose uptake within hypoperfused regions and is predictive of functional reserve after coronary artery revascularization (20, 30). Morphologically, hibernating tissue demonstrates enhanced deposition of glycogen (27, 32) as well as increased iNOS gene expression (9). It is plausible that hibernating and preconditioned myocardium share common signaling pathways, particularly with regard to p38 MAPK. Accordingly, we wished to characterize the activities of regional myocardial p38 MAPK activation in a swine model of chronic regional myocardial ischemia (12) and determine whether the activity of p38 MAPK is related to the degree of GLUT-4 translocation and glycogen storage.

METHODS

This study was performed under the guidance of the Animal Care Committee at the Veterans Affairs Medical Center and conforms with the National Institutes of Health Guide for the Care and Use of Laboratory Animals [DHHS Publication No. (NIH) 85-23, revised 1996, Office of Science and Health Reports, Bethesda, MD 20892].

Animal Preparation

After an overnight fast, domestic pigs (~10 kg) were sedated with xylazine (2 mg/kg im) and Telazol (tiletamine-zolazepam, 4 mg/kg im), ventilated, and anesthetized with isoflurane (1%). The thorax was prepped and draped, and a left thoracotomy was performed in the fifth intercostal space. The left anterior descending coronary artery (LAD) was dissected free, and a C-shaped occluder (3 mm long, 1.4 mm ID) was secured around the vessel and gently closed with suture. The pericardium and chest were closed in layers. Sterile dressings were applied, and a chest tube was placed and removed within 3 days of surgery. Cephazolin (1 g iv) was given before and 12 h after the procedure and repeated daily for 3 days. Pain prophylaxis was provided during the first 3 postoperative days with buprenorphine (Buprenex, 0.5 mg every 12 h im). Animals were maintained on a diet of standard Purina chow.

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Terminal Study and Data Analysis

At 12 wk after instrumentation, fasted animals were brought to the scanner, sedated, and mechanically ventilated. Anesthesia was maintained with a combination of isoflurane (0.8–1.0%) and room air. Before the PET study, two-dimensional echocardiograms were acquired from the right parasternal and apical views to assess regional myocardial function. Regional wall thickening was measured from the anterior (LAD) and posterior (remote) walls and computed as the difference between end-systolic and end-diastolic wall thickness, expressed as a percentage of end-diastolic thickness. End diastole and end systole were defined as the onset of the QRS complex and the frame with the smallest chamber size, respectively. Animals were then positioned on the table so that the heart was in the center of the field of view. An external surface reference was placed on the animals and aligned with a laser to ensure that the position was similar on serial studies. Attenuation of tissue density was determined by a transmission scan using an internal source of radiation. After the transmission scan, $^{18}$Fammonia (15 mCi) was infused intravenously over 20 s, and dynamic scans were obtained over the next 21.5 min. The scanning protocol consisted of one 30-s, twelve 10-s, two 30-s, three 60-s, and one 900-s frame. After 50 min (5 half-lives of $^{18}$Fammonia), $[^{3}$H$] $fluoro-2-deoxy-d-glucose (FDG, 6 mCi) was infused, and dynamic scans were acquired over the next 40 min with a scanning protocol of twelve 10-s, six 30-s, four 60-s, three 120-s, three 300-s, and one 600-s frame. An arterial sample was collected for plasma glucose during the FDG scan.

Multiple circular regions of interest (ROIs) were chosen from transverse planes from the blood flow image and saved for analysis. For analysis of the tissue studies, approximately five ROIs were obtained in the LAD and remote territories and for the arterial input function; an ROI was carefully placed in the center of the left ventricle (LV) to minimize the spillover effect from the myocardium to the LV cavity (17). For estimation of regional myocardial blood flow (MBF), we applied a three-compartment model. MBFs were obtained by a nonlinear least-square fitting to the model equation using the input function and tissue samples acquired in the first 6 min of the dynamic scan (17). For estimation of regional myocardial FDG uptake, Patlak plots were generated from the time-activity curves from the LV cavity and each myocardial tissue ROI. Values were then averaged for LAD and remote regions. The model has been previously described and is based on a three-compartment model (21).

Tissue Analysis

After the PET scan, the animals were killed. The heart was sliced into five sections along the longitudinal axis, and the distal ventricular section was placed in freshly prepared triphenyltetrazolium solution for visualization of significant necrosis. The other sections were divided into transmural LAD and remote tissue samples, placed in liquid nitrogen, and stored at $-80^\circ$C. Sections were also prepared from each region and analyzed by light microscopy by an experienced pathologist. Although triphenyltetrazolium staining showed no evidence of transmural necrosis, significant histological changes were noted in three of the nine pigs, including subendocardial necrosis in one pig and patchy infiltration of fibrosis in two pigs. Separate analysis of the results of the three pigs with the focal histological changes did not differ from the other six pigs; therefore, they were included in the study.

GLUT-4 transport protein. Frozen heart tissue (30–50 mg) was homogenized (4 times for 30 s each) into 1 ml of iced buffer, and a portion of the homogenate was centrifuged at 1,200 g for 30 min. The pellet was incubated in a detergent-based assay buffer (Pierce Biotech, Rockford, IL) and spun at 10,000 g for 5 min. The supernatant was removed and spun at 10,000 g for 5 min. The resulting hydrophobic fraction was used for membrane-bound determinations of GLUT-4 content, and the crude homogenate was used for the total GLUT-4 content. GLUT-4 assays were performed with antiserum (kindly provided by Dr. Howard Haspel), and purity of membrane fractions was confirmed using an anti-Na$^+$-K$^+$-ATPase antibody (Sigma, St. Louis, MO). The immunoblots were quantified from an imaging densitometer (Bio-Rad). For colocalization of GLUT-4 with nuclei by immunohistochemistry, transmural sections from frozen samples were cut on a cryostat microtome, mounted on Superfrost Plus slides, air dried, and stored at $-80^\circ$C. Slides were fixed in methanol at $-20^\circ$C, rehydrated with Tris-buffered saline, and blocked with 1% BSA (fraction V) and 0.5% Tween 20. Slides were incubated for 2 h at 37°C with polyclonal rabbit GLUT-4 antiserum and then washed for 1 h in Tris-buffered saline. Fluorescein-labeled donkey anti-rabbit IgG and propidium iodide were used at dilutions of 1:50 and 1:200, respectively. The slides were mounted and examined using confocal microscopy.

Glycogen. Tissue was dissolved in 0.3 N HCl and hydrolyzed using amyloglucosidase. The glucose residues were measured by an NADP-linked spectrophotometric method using glucose-6-phosphate dehydrogenase and hexokinase.

Immunoprecipitation and kinase assays. The immunoprecipitations were carried out in 500-μl aliquots, and immobilized antiphosphorylated p38, ERK, and JNKs were used as the enzyme source. The activity studies were performed by using the precipitated protein and in vitro phosphorylations of the substrates. Soluble fractions were subjected to SDS-PAGE, and proteins were transferred onto nitrocellulose membranes. Antiphosphorylated antibodies for activating transcription factor-2 (ATF-2), Elk1, c-Jun, AKT, and AMP-activated protein kinase substrates (Cell Signaling, Beverly, MA) were used as the primary antibodies, and peroxidase-labeled anti-rabbit IgG was used as secondary antibody. Bound antibodies were detected with chemiluminescence using the ECL Plus Western blotting kit (Amer sham), and quantification was done with an imaging densitometer. Values were expressed as arbitrary units.

Because ischemia-induced p38 MAPK activity in preconditioned myocardium is increased to a greater degree than p38 MAPK activity (25), we also measured the phosphorylation of ATF-2 substrate after the kinase assays from immunoprecipitates obtained with specific antibodies for p38 MAPKα (Cell Signaling) and p38 MAPKβ (Abgent, San Diego, CA).

NOS activity. Heart tissue was homogenized from LAD and remote regions in three volumes of ice-cold homogenization buffer. The homogenates were centrifuged at 20,000 g for 45 min, and the supernatants were used for measuring NOS activity. The conversion of [L-$^{14}$C]citrulline from [L-$^{14}$C]arginine by NOS was measured in the supernatants from each region and analyzed by light microscopy by an experienced pathologist. Although triphenyltetrazolium staining showed no evidence of transmural necrosis, significant histological changes were noted in three of the nine pigs, including subendocardial necrosis in one pig and patchy infiltration of fibrosis in two pigs. Separate analysis of the results of the three pigs with the focal histological changes did not differ from the other six pigs; therefore, they were included in the study.

Statistics

Values are means ± SE. Differences between LAD and remote regions were tested at $P < 0.05$ with Student’s t-test.

RESULTS

Regional Myocardial Function, Perfusion, and Glucose Uptake

At 3 mo after instrumentation and at the time of the terminal study, a regional wall motion abnormality was observed by two-dimensional echocardiograms in all animals. Systolic wall thickening was 25 ± 3% in the LAD region and 43 ± 4% in
the remote region ($P < 0.05$). PET studies with tracers of blood flow (MBF) and glucose (FDG) were performed at rest during fasted conditions. In the chronically ischemic LAD region, myocardial FDG uptake was increased and blood flow was reduced compared with the corresponding remote territory (Fig. 1). At the time of the PET scan, plasma glucose and lactate were $5.55 \pm 0.61$ and $1.20 \pm 0.10 \mu$mol/l, respectively, and concentration of total free fatty acids was $782 \pm 104 \mu$mol/l. Insulin levels were $<2.0 \mu$U/ml, confirming fasting conditions.

**Myocardial Glucose Transporters and Glycogen**

The membrane-bound and total content of the GLUT-4 transporter was analyzed by Western blots in the LAD and remote regions. Relative to the total GLUT-4 content, the membrane-bound portion of GLUT-4 in the LAD region was $61 \pm 4\%$ and was higher than in the remote region ($45 \pm 6\%, P < 0.05$; Fig. 2, A and C). Analysis of the enzyme Na$^+$/K$^+$-ATPase in the membrane and cytosolic fractions showed that the differences in membrane-bound GLUT-4 could not be explained by differences in membrane separation between LAD and remote regions (Fig. 2B). Immunohistochemistry and colocalization of GLUT-4 with nuclei showed more GLUT-4 protein in the sarcolemma of the LAD region, whereas GLUT-4 was observed in cytoplasmic stores in the remote region, with minimal membrane definition (Fig. 2D). Glycogen content was $28.37 \pm 4.41$ and $19.26 \pm 1.87 \mu$mol/g wet wt in LAD and remote regions, respectively ($P < 0.05$).

**MAPK and GLUT-4 Translocation**

Total p38 MAPK activation, as determined by the ATF-2 substrate assay, was $47 \pm 14\%$ higher in the LAD than in the remote region ($P < 0.05$). When normalized to the remote region, p38 MAPK$\alpha$ activity in the LAD region was $1.03 \pm 0.22$.

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**Fig. 1.** At 3 mo after instrumentation and under basal, fasted conditions, imaging with dual tracers of PET demonstrated reduced myocardial blood flow (MBF) and enhanced [$^{18}$F]fluoro-2-deoxy-D-glucose (FDG) uptake in the chronically ischemic left anterior descending coronary artery (LAD) region. *P < 0.05 vs. remote region.

**Fig. 2.** Representative Western blots (A) and grouped data (C) show increased membrane-bound (Mem) GLUT-4 content in LAD myocardial region relative to total GLUT-4 content compared with remote region. B: analysis of Na$^+$/K$^+$-ATPase in membrane and cytosolic (Cyt) fractions showed that differences in membrane-bound GLUT-4 could not be explained by differences in membrane separation between LAD and remote regions. D: immunohistochemistry and colocalization of GLUT-4 (green) with nuclei (red) used to show that GLUT-4 protein was localized to the sarcolemma in the LAD region, whereas it resided in cytoplasmic stores in the remote region, with minimal membrane definition. Magnification $\times200$. *$P < 0.05$ vs. LAD.
0.04 (not significant), whereas p38 MAPK activity was 1.30 ± 0.08 (P < 0.05), demonstrating that, similar to preconditioned myocardium, ischemia-induced changes in p38 MAPK in hibernating myocardium involved predominantly the β-isozyme (25). ERK activity, as measured by the Elk1 assay, was also higher in the LAD region by 32 ± 6% (P < 0.05), whereas no differences were noted in the activity of the JNK pathway (c-Jun assay; Fig. 3). When p38 MAPK activity in the LAD region was normalized to that of the remote region, a significant correlation existed between the absolute increase in membrane-bound GLUT-4 (r = 0.81, P < 0.01) and the relative increase in glycogen content within the LAD region (r = 0.70, P < 0.05; Fig. 4). There was no correlation between p38 MAPK activation and relative changes in wall thickening, suggesting that p38 MAPK signaling with global LV dysfunction in congestive heart failure may differ from that with the regional dysfunction that is observed in this model of ischemia (23).

Because protein kinase A and B can also increase GLUT-4 translocation, their activities were measured in the LAD region and normalized to the remote territory. Protein kinase A and B were 66 ± 21% and 73 ± 35% higher in the LAD region, respectively (P < 0.05); however, the relative changes in regional activities did not correlate with the absolute degree of GLUT-4 translocation or the relative increase in glycogen in the LAD region.

NOS Activity

In the hibernating LAD region, total NOS and Ca$^{2+}$-independent iNOS activities were at least twofold higher than in the remote region (Fig. 5). Because the NOS activity from the remote region was higher than expected, two additional pigs (shams) were instrumented with dissection of the LAD artery but not placement of the occluder. Total NOS was 0.31 ± 0.09 and 0.70 ± 0.10 activity/mg protein in the LAD and remote regions, respectively, whereas iNOS in the remote region was 0.07 ± 0.01 activity/mg protein. These values in the remote region were lower than those in the remote region of the hibernating hearts, suggesting that some expression of iNOS may have been a result of signaling to myocardial tissue remote from the ischemic LAD region (29).

A significant correlation existed between the relative degree of p38 MAPK activity and the relative degree of total NOS activity (r = 0.68, P < 0.05). These data suggest that, similar to the second window of preconditioning, p38 MAPK activation may play an important role in the expression of NOS within hibernating myocardium.

**DISCUSSION**

The principal findings of this study are that p38 MAPK activation within chronic hibernating swine myocardium is increased relative to the remote territory and that this increase correlates well with the increase in membrane-bound GLUT-4 glucose transporter, regional glycogen levels, and total NOS activity. This is the first study that has shown that signaling within preconditioned and hibernating myocardium may share common pathways with regard to activation of p38 MAPK.

**Myocardial Hibernation In Vivo**

With dual tracers of PET imaging, hibernating myocardium has been characterized by increased glucose uptake within hypoperfused regions and is predictive of functional reserve after coronary artery revascularization (20, 30). We previously
showed that this swine model of chronic myocardial ischemia is associated with increased glucose uptake relative to MBF by PET and preserved transmural ATP and creatine phosphate (12). In the original description of the model, important adaptations were shown to occur in the coronary arterial resistance vessels in the chronically ischemic LAD region (14). At 3 mo after implantation of the coronary artery constrictor, regional function by segment length crystals and basal blood flow after implantation of the coronary artery constrictor, regional vessels in the chronically ischemic LAD region (14). At 3 mo, collateral development within the LAD region makes it possible to produce theta-quantitative (2). Although we did not measure vasodilator reserve in the present study, animals were studied 3 mo after instrumentation, which is consistent with the time that hibernation is known to evolve (5).

**p38 MAPK and Myocardial Protection**

The signals that are involved with the metabolic adaptations that lead to a state of myocardial hibernation are poorly understood (8). The present data suggest that signals that are responsible for early and late preconditioning may also be important in hibernation. The stress-responsive MAPKs consist of a family of highly conserved signaling enzymes that phosphorylate Ser/Thr residues in proteins. p38 MAPK, a subfamily of these enzymes, is activated in response to extracellular stimuli and may play an important role in protein regulation and cell signaling in chronic myocardial ischemia. Activation of p38 can occur in response to free radical generation during low-flow ischemia and hypoxia (10, 11) and may be an important mediator in the infarct-sparing effects of ischemic myocardial preconditioning (34). Thus, when p38 MAPK activation is inhibited before an ischemic preconditioning protocol, the reduction in necrosis during a subsequent sustained period of ischemia and reperfusion is lost (15, 19).

Conversely, when p38 MAPK is activated by administration of anisomycin, a preconditioning effect is induced that mimics the protective effects of ischemic preconditioning (18). Activation of p38 MAPK may play a role during the “late” or “second” window of protection (1), possibly by virtue of transcriptional events. For instance, p38 MAPK is activated 24 h after brief regional myocardial ischemia and reperfusion by a mechanism that involves adenosine A1 receptor stimulation (3). Once activated, p38 MAPK, along with the transcriptional factor NF-κB, can lead to the expression of Ca2+-independent NOS (iNOS) (35). The latter is an important component in the cardioprotection observed in the “late window of ischemic preconditioning” (7).

**Myocardial Ischemia and GLUT Transporters**

During sustained reductions in coronary blood flow, GLUT-1 and GLUT-4 are mobilized from cytoplasmic stores to the sarcolemma in the absence of insulin (28, 33). Although the precise signaling cascade responsible for this translocation and activation of the GLUT transporters to the sarcolemma is still under investigation, AMP-activated protein kinase and α-adrenergic receptors have been shown to play an important role in this process (4, 22). The focus of the present study was on GLUT-4 translocation during chronic hibernation, rather than acute ischemia. GLUT-4 is the predominant glucose transporter that has been shown to increase glucose uptake in preconditioning (31) and is the most abundant transporter in fasted pig myocardium (13). Similar to preconditioning, hibernating myocardium demonstrated increased GLUT-4 translocation that was highly correlated with the degree of p38 MAPK activity. Although phosphorylation of protein kinase A and B was also increased in the hibernating myocardial region, there was no correlation between their relative activities and the degree of GLUT-4 translocation.

**Methodological Considerations**

The hypothesis that a preconditioning effect exists in chronically ischemic, hibernating myocardium has not been tested. Although it might seem reasonable to test whether myocardium within the hibernating regions is protected from necrosis during a prolonged period of severe ischemia, the variable degree of collateral development within the LAD region makes it impossible to impose severe ischemia sufficient to produce infarct. The chronically hibernating myocardial regions share common features with preconditioned myocardium, including increased glucose uptake related to GLUT-4 translocation (31).
and enhanced iNOS expression (9). Whether these factors lead to a protection that is comparable to that observed during late preconditioning is speculative and can only be tested after an infusion of agents that inhibit p38 MAPK activation chronically.

“Short-term hibernation,” induced by a sustained reduction in regional blood flow in swine, is associated with partial recovery of energy and activation of p38 MAPK by a mechanism that does not involve adenosine receptor stimulation or ATP-dependent K+ channel opening (26). Although this suggests that hibernation and preconditioning do not share common protective mechanisms, this does not eliminate the possibility that a protection in chronic hibernation and the late window of preconditioning share common signaling related to transcriptional events.

In conclusion, in this swine model of chronic hibernation, activation of p38 MAPK is increased and correlates well with the degree of increased GLUT-4 translocation, glycogen accumulation, and NOS activity. The findings suggest that, as with preconditioned myocardium, important metabolic changes within hibernating myocardium are signaled by p38 MAPK.

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