Ischemic preconditioning and heat shock activate Akt via a focal adhesion kinase-mediated pathway in Langendorff-perfused adult rat hearts

Hongguang Wei1 and Richard S. Vander Heide1,2

1Department of Pathology, Wayne State University Medical School, and 2Department of Pathology, John D. Dingell Veterans Affairs Medical Center, Detroit, Michigan

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Wei H, Vander Heide RS. Ischemic preconditioning and heat shock activate Akt via a focal adhesion kinase-mediated pathway in Langendorff-perfused adult rat hearts. Am J Physiol Heart Circ Physiol 298: H152–H157, 2010. First published October 30, 2009; doi:10.1152/ajpheart.00613.2009.—Heat stress (HS)-induced cardioprotection is associated with the activation of focal adhesion kinase (FAK) and protein kinase B (Akt) in neonatal rat ventricular myocytes (NRVMs), suggesting that stress-induced activation of survival pathways may be important in protecting intact hearts from irreversible injury. The purposes of this study were 1) to examine the subcellular signaling pathways activated by HS and ischemic preconditioning (IP) in intact hearts, 2) to determine whether HS and IP activate an integrated survival pathway similar to that activated by HS in cultured NRVMs, and 3) to determine whether HS and IP reduce lethal cell injury in perfused intact hearts. Adult rat hearts perfused in the Langendorff mode were subjected to 25 min of global ischemia and 30 min of reperfusion (I/R) either 24 h after whole animal HS or following a standard IP protocol. Myocardial signaling was analyzed using Western blot analysis, whereas cell death was assayed by measuring lactate dehydrogenase release into the perfusate and confirmed by light microscopy. Similar to NRVMs, HS performed in the whole animal 24 h before I/R increased phosphorylation of FAK at tyrosine-397 and protein kinase B (Akt) and resulted in protection from cell death. Using IP as a myocardial stress caused by whole animal HS activates cytoskeletal-based survival signaling pathways in whole heart tissue and reduces lethal I/R injury and 2) IP activates the same stress-induced survival pathway and the activation correlates with the well-known cardioprotective effect of IP on lethal I/R injury.

cytoskeleton; protection; survival signaling

PROLONGED PERIODS of myocardial ischemia, if not relieved, inexorably result in cell death. In contrast, brief episodes of ischemia (termed “reversible ischemia”) result in a mild injury pattern that eventually reverts to normal upon the restoration of normal arterial blood flow. Despite many years of active research, the exact series of events underlying the transition from reversible to irreversible injury remains elusive. It is known that certain interventions are capable of modulating or delaying the onset of irreversible injury in experimental model systems such as hypothermia (2, 12, 13), calcium channel blockade (16, 22, 34), heat stress (HS) (17, 35), and ischemic preconditioning (IP) (15, 20, 26, 29). However, even in experimental model systems, the mechanism(s) responsible for protection are not fully understood. If any intervention is to have impact in the clinical arena and reduce the mortality from acute myocardial infarction, a better understanding of the mechanism of cardioprotection is critical.

IP provides the most dramatic and consistent protection against cell death, but the subcellular mechanism of protection has remained controversial. Although “classic” IP is induced by a brief episode of reversible ischemia followed by reperfusion, a wide range of pharmacological agents has been described that mimic the protective effect of IP including drugs that activate adenosine, α1-adrenergic, muscarinic, angiotensin, and bradykinin receptors. As a consequence of these studies, it has been suggested that the subcellular signaling pathways used by these receptors may underlie the mechanism of IP (3, 21, 27, 39). Although it is an attractive idea that a common subcellular signaling pathway may underlie cardioprotection, to date there is no unifying hypothesis to explain the diverse number of pharmacological agents capable of mimicking the cardioprotection of classic IP.

Focal adhesion kinase (FAK) is a nonreceptor protein tyrosine kinase that normally exists in nonmuscle cells at cell-matrix junctions known as focal adherens or focal adhesion sites. In our most recent studies we have shown that HS, representing a cause of acute myocardial stress, causes the activation of a cytoskeletal-based survival pathway that includes integrin, FAK, phosphatidylinositol 3-kinase (PI3K), and Akt. Furthermore, the activation of the pathway reduced both oncotic and apoptotic cell death, and the inhibition/interruption of either FAK or PI3K (members of the pathway) resulted in an increased cell death that correlated with a reduced activation of Akt (37). However, these studies were conducted in cultured neonatal rat myocytes rather than intact, adult hearts where stress-induced signaling may be different and/or more complex. In addition, our previous studies used HS followed by 24 h of recovery and therefore did not test for protection immediately after the activation of the pathway. The purpose of the present study was to determine whether myocardial stress (HS and classic IP) activates the same survival pathways in intact adult hearts.

Address for reprint requests and other correspondence: R. S. Vander Heide, Dept. of Pathology, LSU Health Science Ctr., 1901 Perdido St., New Orleans, LA 70112 (e-mail: rvand3@lsuhsc.edu).

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MATERIALS AND METHODS

All experiments reported here conformed to the standards in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996). All animal protocols were approved by the Animal Investigation Committee at Wayne State University.

Perfused hearts. Male Sprague-Dawley rats (200–250 g) were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). After the absence of a tail-flick response was confirmed, the chest was opened and heparin was injected into the inferior vena cava and allowed to circulate for 5 s. The hearts were rapidly removed and plunged into ice-cold buffered Krebs-Henseleit solution. Excess tissue was removed, and the aorta was cannulated and perfused in the Langendorf mode at a constant pressure of about 75 mmHg at 37°C. Control perfusate was a modified Krebs-Henseleit bicarbonate buffer containing (in mmol/l) 118 NaCl, 4.7 KCl, 1.2 KH2PO4, 1.2 MgSO4, 10 glucose, 2.5 CaCl2, and 2.5 NaHCO3, equilibrated with 95% O2-5% CO2. The coronary flow rate was monitored via the effluent and ranged from 10–15 ml/min. Any heart with a flow rate of <10 ml/min was excluded from further manipulation.

Experimental design/protocol. Forty-four adult male Sprague-Dawley rats were used in these studies. Hearts were divided into three main groups: control, HS, and IP. For each main group, one subgroup was used for analysis of cell signaling and the other was used for analysis of cell death.

Whole body heat shock. Anesthesia was administered to adult rats by intraperitoneal injection of pentobarbital sodium (50 mg/kg). Rats were then placed in a controlled environmental chamber under an infrared light while body temperature was monitored using a rectal probe. The rat remained under the light until the core temperature reached a stable temperature of 41°C for 15 min. Following the HS, rats were allowed to recover from anesthetica and recover to normal body temperature in the ambient conditions (room temperature). While recovering, rats were hydrated with an intraperitoneal bolus of normal saline (10 ml/kg). Control animals were subjected to anesthesia only. All rats were allowed to recover for 24 h, at which time they were reanesthetized with 50 mg/kg pentobarbital sodium before excision and harvest of the heart for Western blot analysis of signaling activity and/or analysis of cell injury.

Ischemic preconditioning. IP was induced using a standard 2 × 5′ protocol; i.e., two 5-min episodes of ischemia each followed by a 5-min period of reflow before the onset of the sustained period of global ischemia.

Ischemia-reperfusion. Global ischemia was induced by clamping the flow line and reducing coronary flow to 0 ml/min. The heart temperature was maintained at 37°C throughout the ischemic period by a heat-jacketed chamber and bathing the heart in perfusion buffer at 37°C. Following 25 min of global ischemia, flow was restored by unclamping the flow line and allowing the resumption of spontaneous coronary flow. Coronary flow was measured at 1-min intervals during the 30-min reperfusion phase, and the effluent was saved for analysis of cell death where indicated [lactate dehydrogenase (LDH) assay].

Western blot analysis. Left ventricular myocardium was harvested for protein analysis using standard Western blot analysis as described previously (36, 37). Ventricular tissue was homogenized with lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, and 1% Triton X-100 and 1× protease inhibitor cocktail. Sixty micrograms of sample (as determined by BCA protein assay) were loaded to each lane and subjected to SDS-protein electrophoresis. Following electrophoresis, the proteins were transferred to nitrocellulose membranes, and the membranes were incubated overnight at 4°C with one of the following primary antibodies: 1) a rabbit anti-FAK (No. 06-543, Upstate Biotechnology) or a mouse anti-FAK (No. 610087, BD Biosciences), 2) a rabbit anti-FAK (No. 44-624G, Biosource), 3) rabbit anti-Akt (No. AM1011, ECM Biosciences). The membranes were then incubated with a secondary antibody, donkey anti-rabbit IgG (Santa Cruz) for 1 h at room temperature, and the final protein expression was detected using a standard horseradish peroxidase chemiluminescence system (Amersham; Arlington, IL). In some experiments, the membranes were incubated with fluorescent-labeled donkey anti-rabbit IgG (Invitrogen) or RDye 800CW conjugated goat anti-mouse IgG (LI-COR Biosciences), and the final protein expression was detected using the Odyssey infrared imaging system (LI-COR Biosciences). Signaling data are reported in arbitrary units and/or percent elevation over control protein expression as indicated.

Cell injury assay. LDH release was used as an indication of cell death/lethal cell injury. LDH release was measured in the effluent collected at zero time (just before the onset of ischemia) and at 1, 3, 5, 10, 15, 20, and 30 min of reperfusion. LDH was assayed using a commercial kit (TOX7 kit, Sigma) and LDH release if expressed as international units per 30 min per gram wet heart weight. The presence of cell death and/or protection was confirmed by processing a separate group of hearts from each group for routine histological analysis. Hearts were fixed in buffered formalin, processed, embedded in paraffin, and stained with hematoxylin and eosin.

Statistics. All LDH data are expressed as means ± SE [in international units released per 30 min of perfusion (reperfusion)]. All Western blot data are expressed as means ± SE (in arbitrary units). Statistically significant differences between groups were tested using a paired t-test analysis. A P value ≤ 0.05 was considered statistically significant.

RESULTS

Effect of HS on protein expression and cell signaling. In the first series of experiments, we sought to determine whether whole body HS increases the expression of heat shock proteins and causes the activation of the cytoskeletal-based survival pathway previously identified in cultured NRVMs. As expected, HS caused an increase in expression of heat shock protein 27, 20, and 90 in left ventricular myocardium (Fig. 1). We next sought to determine the level of activation of FAK and Akt in whole hearts harvested from adult rats subjected to whole body HS. Confirming and extending our previous data from cultured NRVMs, we noticed that HS resulted in a significant activation of FAK (as measured by phosphorylation of FAK tyrosine residue 397) compared with non-heart shocked control hearts perfused for the same period of time before tissue sampling (P ≤ 0.04 vs. control heart tissue; Fig. 2A). Similar results were obtained when Akt signaling was examined (P ≤ 0.05 vs. control heart tissue; Fig. 2B).

Effect of HS on cell injury: LDH release. In cultured NRVMs, we have shown that HS results in the protection against oncotic cell death as measured by trypan blue exclusion (35, 37). It is well known that HS protects hearts from lethal ischemia-reperfusion injury. The current study was designed to confirm that HS would protect against ischemia-reperfusion in intact hearts subjected to global ischemia-reperfusion. Figure 2C confirms that whole body HS applied 24 h before harvest and perfusion resulted in significantly less cell death in response to 25 min of global ischemia followed by 30 min of reperfusion (P ≤ 0.05 HS vs. control; Fig. 2C).

Effect of IP on cell signaling. Previous studies have shown that IP is protective against cell death in isolated Langendorff-perfused adult rat hearts subjected to global ischemia-reperfusion (28, 38). However, the status of the cytoskeletal-based cell survival pathway in IP hearts is not known. To determine the effect of IP on FAK and Akt activity, hearts were subjected to...
a standard IP protocol in perfused rat hearts that consists of two 5-min episodes of global ischemia (at 37°C), each followed by a 5 min period of reperfusion (2 × 5’ protocol). Figure 3, A and B, shows that IP caused the activation of both FAK (a proximal member of the pathway) and Akt (distal member of the pathway) in whole adult rat hearts, confirming that myocardial stress, in this instance IP, is capable of activating the cell survival pathway.

**Effect of IP on cell injury: LDH release.** After we confirmed that IP activates both FAK and Akt, it was important to determine whether IP would protect against ischemia-reperfusion in intact hearts subjected to global ischemia-reperfusion. Figure 3C confirms that the standard IP 2 × 5’ protocol resulted in significantly less cell death in response to 25 min of global ischemia followed by 30 min of reperfusion.

**Effect of HS and IP on proline-rich tyrosine kinase 2 and ILK activity.** Proline-rich tyrosine kinase 2 (PYK2) has been shown to serve as a scaffolding protein for phosphoinositide-dependent kinase 1 (PDK1) in myocytes (7). PDK1 can phosphorylate/activate Akt at Thr308 and perhaps Ser473 and therefore could be responsible for the Akt activation we measured in response to IP. Alternatively, it is possible that IP directly activates integrin-linked kinase (ILK), which in turn activates/phosphorylates Akt. Figure 4 shows that neither IP...
nor HS caused a significant activation/tyrosine phosphorylation of PYK2. Interestingly, HS caused a significant inactivation of PYK2 activity. Similarly, to investigate the role of ILK in the activation of Akt, ILK activity was estimated by measuring GSK3\(^{\alpha/\beta}\) phosphorylation on Western blot analysis. Figure 5 shows that ILK activity was not significantly increased by either IP or HS.

**DISCUSSION**

The present results extend the conclusions of our previous studies defining a cytoskeletal-based cell survival pathway present in myocardium (35, 37) in several important ways. First, the current study demonstrates that HS activates the critical pathway members FAK and Akt in intact hearts. Our previous studies in cultured rat ventricular myocytes provided evidence of a similar cytoskeletal-based survival pathway in vitro (35). The present results offer important new insights into the cellular mechanisms underlying stress-induced cardioprotection. The results suggest that HS and IP activate the cytoskeletal pathway, which is responsible for cell survival. This pathway involves the activation of FAK and Akt, with concomitant phosphorylation of GSK3\(^{\alpha/\beta}\). The involvement of ILK in this process was also highlighted, as ILK activity was not increased by either treatment.

Fig. 3. Effect of IP on FAK activation, Akt activation, and LDH release in adult rat myocardium. LV lysates of control LV tissue and LV tissue harvested from rats subjected to standard IP without a sustained period of global ischemia were used for A and B. A: representative immunoblot showing phosphorylation of FAK at tyrosine-397 (indicative of activation). IP increased activation of FAK at time 0 (*P ≤ 0.028, Con vs. IP; n = 4 rats). Y-axis data are plotted as fraction of control LV levels. There was no difference in the total amount of FAK protein in any of the samples (lower portion of blot). B: LV tissue was probed for p-Akt\(^{\alpha/\beta}\) to detect activated Akt. When compared with control hearts, hearts harvested from rats subjected to IP showed increased phosphorylation/activation of Akt (*P = 0.007, Con vs. IP; n = 3 rats). Y-axis data are plotted as fraction of control LV levels. The amount of total FAK (to control for loading) present in each sample was not significantly different.

C: adult hearts were subjected to either control aerobic perfusion for 40 min or a standard 4 × 5” IP protocol followed by 25 min of global ischemia and 30 min of reperfusion to induce cell death. Y-axis values indicate the amount of LDH release, indicating loss of membrane integrity (dead cells). As expected, IP resulted in significant protection against oncotic cell death. (*P ≤ 0.02 vs. control hearts; n = 5 rats).

Fig. 4. Effect of HS and IP on proline-rich tyrosine kinase 2 (PYK2) activity. Adult hearts were subjected HS or IP. LV extracts were immunoprecipitated with anti-phosphotyrosine (PY20) antibody followed by probing for PYK2. Neither HS nor IP had a significant stimulatory effect of PYK2 phosphorylation. Interestingly, HS caused an unanticipated significant reduction in PYK2 activation compared with control LV tissue. Y-axis data are plotted as arbitrary units of density. Representative blot is shown. *P ≤ 0.05 vs. control; n = 4 rats. WB, Western blot.

Fig. 5. Effect of HS and IP on integrin-linked kinase (ILK) activity. Adult hearts were subjected HS or IP. LV extracts were immunoprecipitated with anti-ILK antibodies followed by an in vitro kinase assay using GSK3 fusion protein as a substrate (see MATERIALS AND METHODS for details). Activation of ILK was measured by probing for anti-phospho-GSK3\(^{\alpha/\beta}\) (Ser21/9). Total ILK protein present in each lane was detected using anti-ILK antibody after stripping the blot. Neither HS nor IP had a significant stimulatory effect of ILK phosphorylation. Y-axis data are plotted as arbitrary units of density. Representative blot is shown. *P = not significant; n = 4 rats.
convincing evidence that myocardial stress, specifically HS, resulted in a selective activation of the survival pathway as well as protection from lethal cell injury. However, the studies needed to be extended to whole hearts to validate the presence of the pathway outside of cultured myocyte systems. Second, the current study shows that the pathway is activated in adult heart tissue and is not unique to neonatal heart tissue. Third, and most important, the present study demonstrates that the activation of the survival pathway occurs in response to a different type of myocardial stress, reversible ischemia and reperfusion. This report used a well-known and reproducible form of acute cardioprotection known as IP. IP resulted in the activation of both FAK and Akt in whole heart tissue (activation was measured before the onset of lethal ischemia-reperfusion injury). As expected, the IP-induced activation of the survival pathway correlated with the expected IP-induced cardioprotection.

**IP and lethal injury.** It is well known that IP protects myocardium against subsequent lethal ischemia-reperfusion injury. Many mechanisms have been hypothesized to explain the protective role of IP including the reduction of energy utilization, the inhibition of the mitochondrial F1F0-ATPase, the reduction in lactate accumulation/intracellular calcium, and the activation of several cellular signaling pathways (19). However, the precise mechanism of cardioprotection is not known. In support of the present study, it has been suggested that the induction of a general cell stress response may be all that is necessary for cardioprotection (1). The results of the present study show for the first time that IP activates the same subcellular signaling proteins that significantly reduce cell death in cultured NRVMs and support the notion that myocardial stress activates a generalized cell survival/protection pathway.

**Role of myocardial stress in activation of a cell survival pathway.** Our previous studies have suggested that a cytoskeletal-based cell survival pathway may play an important role in cardioprotection by showing that HS causes an activation of the pathway and increased cardioprotection, whereas the inhibition of the pathway (inhibition of FAK or PI3K) resulted in an increased cell death (35, 37). However, theses studies were conducted in cultured neonatal myocytes using a nonischemic form of cardiac stress. Therefore, one of the goals of the present study was to investigate whether the survival pathway was present in intact, adult hearts and in a more clinically relevant form of stress, ischemia-reperfusion.

If cytoskeletal-based signaling is important in cell survival, then myocardial stress should result in the assembly/activation of the docking protein p130cas into a membrane/cytoskeletal-based signaling complex. HS does indeed result in increased p130cas in the membrane fraction (data not shown). This result coupled with the data from our previous study (35) showing that HS both enhanced the interaction between integrin and paxillin and increased the localization of paxillin in the membrane fraction suggests that stress results in the assembly of a signaling complex localized to the membrane fraction consisting of at least integrin, FAK, p130cas, and paxillin. Although not specifically interrogated in this study, it is likely that talin and vinculin are also localized in the membrane-based signaling complex (6).
GRANTS

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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