No-Flow Ischemia Inhibits Insulin Signaling in Heart by Decreasing Intracellular pH

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Abstract—Glucose-insulin-potassium (GIK) perfusions exert beneficial effects on the ischemic heart by reducing infarct size and mortality and improving postischemic left ventricular function. Insulin could be the critical protective component of this mixture, although the insulin response of the ischemic and postischemic myocardium has not been systematically investigated. The aim of this work was to study the insulin response during ischemia by analyzing insulin signaling. This was evaluated by measuring changes in activity and/or phosphorylation state of insulin signaling elements in isolated perfused rat hearts submitted to no-flow ischemia. Intracellular pH (pHi) was measured by NMR. No-flow ischemia antagonized insulin signaling including insulin receptor, insulin receptor substrate-1, phosphatidylinositol 3-kinase, protein kinase B, p70 ribosomal S6 kinase, and glycogen synthase kinase-3. These changes were concomitant with intracellular acidosis. Perfusing hearts with ouabain and amiloride in normoxic conditions decreased pH, and insulin signaling, whereas perfusing at pH 8.2 counteracted the drop in pH, and the inhibition of insulin signaling by ischemia. Incubation of cardiomyocytes in normoxic conditions, but at pH values below 6.75, mimicked the effect of ischemia and also inhibited insulin-stimulated glucose uptake. Finally, the in vitro insulin receptor tyrosine kinase activity was progressively inhibited at pH values below physiological pH, being abolished at pH 6.0. Therefore, ischemic acidosis decreases kinase activity and tyrosine phosphorylation of the insulin receptor thereby preventing activation of the downstream components of the signaling pathway. We conclude that severe ischemia inhibits insulin signaling by decreasing pH, (Circ Res. 2001;88:513-519.)

Key Words: ischemia ■ insulin ■ signal transduction ■ glucose ■ acidosis

Experimental and clinical studies have shown that glucose-insulin-potassium (GIK) perfusions exert beneficial effects on the ischemic heart by reducing infarct size and mortality and improving postischemic left ventricular function.1–4 The protection conferred by GIK could be due to insulin and/or glucose, and each one could act during ischemia and/or reperfusion. The aim of this work was not to confirm the beneficial effects of GIK solutions but rather to focus on the insulin response of the heart during ischemia. The heart response to insulin is usually studied by measuring glucose uptake and glycolysis, which are known targets of insulin on this tissue. This experimental approach is useless in ischemic hearts. Indeed, a correct balance of glucose metabolism cannot be made in no-flow ischemia. Moreover, the effects of insulin on myocardial glucose metabolism are not easily distinguished from those of ischemia, because both stimulate glucose uptake and glycolysis.5,6 Therefore, metabolic parameters such as glucose uptake and transport are not adequate to specifically measure the insulin response during ischemia. Another method of studying whether the ischemic myocardium is responsive to insulin is to analyze the insulin signaling pathway, which differs from that of ischemia. The stimulation of glycolysis resulting from anaerobic conditions seems to be mediated by AMP-activated protein kinase (AMPK).7,8 This protein kinase responds to the energy state of the cell but has not been reported to participate in insulin signaling.

Insulin signaling is initiated by its binding to the insulin receptor (IR). This activates the tyrosine kinase activity of IR leading to IR autophosphorylation and to subsequent phosphorylation of IR substrates (IRS) (for recent review see Vanhaesebroeck and Alessi9). The downstream signaling components involve phosphatidylinositol 3-kinase (PI3K), which is activated by binding to phosphorylated IRS, and protein kinase B (PKB/Akt), which is activated by phosphorylation of Thr308 and Ser473 by 3-phosphoinositide–dependent protein kinase-1 and -2. PKB mediates some of the metabolic effects of insulin, which include the following: (1) stimulation of glycogen synthesis by phosphorylation and inactivation of glycogen synthase kinase-3 (GSK-3); (2) activation of phosphodiesterase 3B, the enzyme responsible for the anti-cAMP effect of insulin; and (3) recruitment of...
GLUT4 transporters to the plasma membrane. However, PKB does not seem to be required for the insulin-induced activation of 6-phosphofructo-2-kinase, the enzyme responsible for the synthesis of fructose 2,6-bisphosphate, a potent stimulator of glycolysis. Nevertheless, it is clear that PKB participates in insulin signaling, even if it does not mediate all of its metabolic effects. Moreover, PKB could mediate some effects of insulin involved in cell protection, but which are not related to the metabolic effects. Therefore, as a first experimental approach in this study, we resorted to PKB activation to assess insulin action during ischemia.

Materials and Methods

Perfusion Protocol and Preparation of Isolated Cardiomyocytes

Hearts from male Wistar rats (weight 200 to 220 g, anesthetized with 50 mg/kg pentobarbital) were perfused retrogradely at 37°C and at a constant pressure of 60 mm Hg with a Krebs Henseleit buffer containing 1.5 mmol/L CaCl₂ and 10 mmol/L glucose and in equilibrium with a 95%O₂/5%CO₂ gas phase (except where otherwise stated). No-flow ischemia was obtained by interrupting the flow. The ischemic hearts were maintained at 37°C by immersion in a thermostated reservoir filled with buffer. The perfusion protocols are described in the legends to the figures. At the indicated times, the hearts were freeze-clamped. Perusions in “working” conditions were performed as described, with 10 cm H₂O preload and 60 cm H₂O afterload for “low-load” perfusions and with 15 cm H₂O preload and 100 cm H₂O afterload for “high-load” perfusions.

Isolated cardiomyocytes were prepared and resuspended (~50 mg wet weight/mL) in a medium containing (in mmol/L) NaCl 95, PIPES 25, MES 25, KCl 4.7, MgSO₄ 1.25, H₂PO₄ 1.2, CaCl₂ 1, and glucose 10% (wt/vol) BSA, at the indicated pH. The effect of 100 nmol/L insulin on the uptake of 2-[2-¹⁴C]deoxyglucose (1 μmol/L; 100 μCi/mmol) was measured in 20-minute incubations. For other measurements, cardiomyocytes were preincubated for 20 minutes and then incubated for 2 minutes with 100 nmol/L insulin and finally centrifuged (10 000 × g for 5 seconds). The cell pellets were frozen in liquid nitrogen and stored at −80°C.

NMR Spectroscopy

Hearts were positioned in a home-built thermostated probe head in a Biospec spectrometer (4.7 T). Intracellular pH was estimated from the shift of the inorganic phosphate signal in the spectra (81.1 MHz, 90° pulses, 2.32-second repetition time, 64 scans, or 2.5-minute total acquisition time).

Analytical Procedures

The frozen hearts or cardiomyocytes were homogenized (Ultra-Turrax) at 0°C to 4°C in 5 to 10 vol (vol/wt) of homogenization buffer, and the supernatants (10 000 g for 30 minutes) were stored at −80°C. PKB activity was measured in immunoprecipitates (BAK antibodies). The phosphorylation state of PKB and of p38 mitogen-activated protein kinase (p38) were monitored by immunoblots (anti–phospho-Thr308PKB, anti–phospho-Ser473PKB, and anti–phospho-Thr180/ phospho-Tyr183p38 antibodies; New England Biolabs). PKB activity was measured in immunoprecipitates using synthetic peptides (RRLIE-DAYAARG) or with the copolymer polyglutamate-tyrosine (4:1 molar ratio) as substrate. The blots shown in this work are representative of at least 3 experiments. One unit (U) of protein kinase activity corresponds to the formation of 1 nmol of product per minute under the assay conditions.

Results

Ischemia Inhibited PKB Activation by Insulin

Under control conditions, insulin caused a 10- to 20-fold activation of PKB, which lasted for at least 15 minutes (Figure 1A), in agreement with previous work on other tissues. Ischemia induced a biphasic change in PKB activity of insulin-treated hearts. There was a slight but significant increase in PKB activity at 2 minutes, followed by a progressive and persistent decrease in PKB activity, which was maximal after 10 minutes (Figure 1A). The loss of PKB activity corresponded to the dephosphorylation of both Thr308 and Ser473 (Figure 1A).

When insulin was added during reperfusion, PKB activation and phosphorylation were rapidly recovered, however with a slight delay compared with hearts not previously submitted to ischemia (Figure 1B). Insulin signaling was fully restored after 5 minutes of reperfusion (Figure 1B).

In normoxic hearts, half-maximal effects of insulin on PKB and on GSK-3 activity were observed at ~7 × 10⁻⁸ mol/L insulin, in agreement with values reported for adipocytes and for hepatocytes. Similar results were obtained for activation of p70s6k, another protein kinase downstream of PI3K and 3-phosphoinositide–dependent protein kinase-1 (Figure 2). In ischemic hearts, PKB activation was inhibited by 62% at

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the same insulin concentration. Similar results were obtained with GSK-3
from at least 3 different hearts. *Values significantly different taken 15 minutes after insulin addition. Values are mean SEM
(Y) hearts. Same protocol as in Figure 1A; samples were
inactivation were completely inhibited (Figure 2).
Figure 2. Dose-response curves of the effect of insulin on PKB
(A), p70s6k (B), and GSK-3β (C) activity in normoxic (○) and ischemic (●) hearts. Same protocol as in Figure 1A; samples were
taken 15 minutes after insulin addition. Values are mean±SEM
from at least 3 different hearts. *Values significantly different (P<0.05, unpaired t test) from corresponding control values at the
same insulin concentration. Similar results were obtained with GSK-3α (not shown).
saturating concentrations of insulin, whereas p70s6k activation and GSK-3 inactivation were completely inhibited (Figure 2).

Lack of Effect of Heart Function and Substrates on Insulin Signaling
We tested whether heart function and fatty acids, which are known to affect heart metabolism, also have an effect on PKB activation by insulin. Inhibition of heart contraction by removal of calcium or by increasing KCl concentration in the perfusion buffer (30 mmol/L, final concentration) did not modify the insulin response. Under these conditions, perfusion for 15 minutes with insulin increased PKB activity to the same extent (controls, 7.6±1.6 mU/g tissue [mean±SEM]; controls in calcium-free buffer, 10.2±1.7; controls with 30 mmol/L KCl, 7.2±1.2; insulin, 48.2±2.7; insulin in calcium-free buffer, 53.1±7.6; insulin with 30 mmol/L KCl, 45.8±1.8; n=4). Moreover, heart work did not affect insulin signaling. In low-load hearts, insulin increased PKB activity from 5.7±0.8 to 62.2±3.2 mU/g tissue, and in high-load hearts, from 7.5±0.7 to 57.7±1.9 mU/g tissue (n=4). The hydraulic power increased from 1.9±0.5 mW in low-load hearts to 4.9±1.2 mW in high-load hearts without insulin and from 2.1±0.3 to 5.4±1.5 mW with insulin (P<0.01). In the “working” model, a 10-minute period of no-flow ischemia inhibited the activation of PKB induced by insulin by ~50%, an effect similar to that reported in Figure 1A.

Addition of fatty acids, which are known preferred substrates for the heart, did not alter PKB activation by insulin. The activity of PKB after 15-minute perfusion with insulin was 62.6±8.4 and 50.0±6.9 mU/g tissue (n=4) without and with 0.6 mmol/L palmitate, respectively, compared with 6.9±0.4 and 4.8±0.6 mU/g tissue (n=4) in the same conditions, but in the absence of insulin. We verified that the ischemia-induced inhibition of PKB activation persisted in hearts perfused with fatty acids (data not shown). Lactate, another preferred substrate for the heart, also had no effect on the activation of PKB by insulin, as presented in the following paragraph.

Potential Mechanisms of Insulin Signaling Inhibition
No-flow ischemia blocks glucose and oxygen supply, as well as waste product removal, so that lactate and protons rapidly accumulate. We tested whether any of these changes could be responsible for, and thereby mimic, the ischemia-induced inhibition of the insulin response. No inhibition of PKB activation by insulin was observed when normoxic hearts were perfused without glucose or with 20 mmol/L lactate (PKB activity after 15 minutes of insulin in normoxic conditions, 47.3±3.5 mU/g tissue; without glucose, 42.8±4; with 20 mmol/L lactate, 65.3±12.6; with 10 minutes of ischemia, 6.3±0.25; n=3).

Moreover, no inhibition of insulin signaling was found in hearts perfused at a normal flow rate but under anoxic conditions, ie, when N2 replaced O2 in the gas phase (PKB activity after 15 minutes of insulin: normoxia, 73.8±3.6 mU/g tissue; 10 minutes of anoxia, 63.9±4; 10 minutes of ischemia, 27.9±5.2; n=3). AMPK was activated to the same extent in both anoxic and ischemic hearts (AMPK activity: in normoxic hearts, 216±13 mU/mg protein; in anoxic hearts, 1595±266; in ischemic hearts, 1223±140), in agreement with previous studies.22 The fact that AMPK activation was the same in anoxia and ischemia rules out AMPK as being responsible for the inhibition of insulin signaling that occurs in ischemia but not in anoxia. Phosphorylation of p38, known to be activated in no-flow ischemia,23 was detectable only after 10 minutes of ischemia (not shown), thus well after the ischemia-induced inhibition of PKB activation.

Inhibition of PKB Activation by Intracellular Acidosis
By contrast with anoxia,24 ischemia is known to cause an important decrease in pH,25 This phenomenon was confirmed in our model by NMR (Figure 3A). No-flow ischemia caused an immediate and rapid drop in pH, which was maximal after 15 minutes, with the half-maximal effect being obtained within 5 minutes of ischemia, in agreement with data published.25 Interestingly, the inhibition of PKB activation (Figure 1A) was only detectable after 5 minutes of ischemia, ie, when the pH was below 6.75. The importance of acidosis in the inhibition of insulin signaling was evaluated by changing the extracellular pH (pHc) under normoxic conditions. Per-

Figure 3A shows the pHc measured by NMR (left) during the duration of ischemia and its effect on PKB activity (right). During ischemia, pHc dropped progressively from 7.4 to 6.7. The corresponding PKB activity was 4.9±0.6 in normoxic hearts, 1.6±0.2 in anoxic hearts, and 0.4±0.2 in ischemic hearts (n=4). Thus, the pHc control of PKB activity was well confirmed by NMR, with a pHc drop of 0.7 inducing a 75% decrease in PKB activity. This confirms that the pHc decrease is responsible for the ischemia-induced inhibition of PKB activation.
fusing rat hearts in normoxic conditions and at pH 7.1 instead of pH 7.4 mimicked the ischemia-induced inhibition of PKB activation (Figure 4A) and decreased pH$_i$ (Figure 3B) to values observed in ischemia. Inhibition of PKB activation was also observed in hearts perfused with ouabain (Na$^+$/K$^+$-ATPase inhibitor) and amiloride (Na$^+$/H$^+$ exchanger inhibitor), a combination that decreased pH$_i$ (Figure 3B) as expected$^{26}$ without affecting pH$_e$ (not shown). This demonstrated that the change in pH$_i$ without change in pH$_e$ was sufficient to inhibit insulin signaling. Finally, perfusion at pH 8.2 diminished the ischemia-induced decrease in pH$_i$ (Figure 3A) and prevented the ischemia-induced inhibition of PKB activation and phosphorylation by insulin (Figure 4B).

**Insulin Resistance Induced by Extracellular Acidosis in Cardiomyocytes**

We used isolated cardiomyocytes incubated at various pH$_e$ values in normoxic conditions to study the links between PKB activation and a metabolic effect of insulin, namely the stimulation of glucose transport. Incubation of cardiomyocytes at pH$_e$ values below 6.75 inhibited both insulin-stimulated glucose transport and PKB activation (Figure 5B).

The insulin response was abolished at pH$_e$ values below 6.25. These data confirm that inhibition of insulin signaling corresponds to inhibition of the metabolic effect (ie, stimulation of glucose uptake).

**Inhibition of the First Steps in the Insulin Signaling Cascade**

We measured the activity of several components of the insulin signaling pathway, upstream of PKB. Changes in PKB were usually found to be concomitant with changes in the upstream PI3K: (1) the insulin-induced activation of PI3K and PKB was antagonized by ischemia (compare Figure 6B with Figure 1A), (2) this inhibition was not observed when the hearts were perfused at pH$_e$ 8.2 (not shown), and (3) reperfusion with insulin after an ischemic episode caused a slight delay in both PKB (Figure 1B) and PI3K (not shown) activity. However, these changes were not always concomitant; after 2 minutes of ischemia, PKB activity was further increased, whereas PI3K activity remained unaffected (compare Figures 1A and 6B).

Changes in PI3K activity were in turn related to a decreased phosphorylation of the upstream IRS-1, and PKB activation and glucose uptake in isolated cardiomyocytes incubated in normoxic conditions. A, T-cell content of IR (anti-IR) and IRS-1 (anti-IRS-1), measured by immunoblotting. Shown is IR and IRS-1 phosphorylation measured by immunoblotting with anti-IR and anti-IRS-1, respectively. B, Cardiomyocytes were incubated with or without insulin for 2 minutes; values are mean±SEM from 3 different cardiomyocyte preparations.

The data confirm that inhibition of insulin signaling corresponds to inhibition of the metabolic effect (ie, stimulation of glucose uptake).
Inhibition of the IR Tyrosine Kinase Activity

Inhibition of the first step of insulin signaling, namely the phosphorylation of IR, by ischemia or low pH could result from inhibition of insulin binding to its receptor, as already reported for isolated adipocytes incubated at pH 6.7.27 and/or from the inhibition of the IR tyrosine kinase activity itself. Removal of insulin from the medium by washout procedures is known to decrease insulin binding to its receptor.28 However, we found that PKB activation persisted for 6 and 10 minutes after washing out insulin by perfusing with insulin-free medium (Figure 7). This suggests that an element(s) other than the inhibition of insulin binding should be involved to explain the ischemia-induced inhibition of insulin signaling. Therefore, the effect of pH on the IR tyrosine kinase activity was studied in vitro. Incubation of IR from control or insulin-treated hearts at pH values below physiological pH inhibited its activity, which was completely abolished at pH 6.0 (Figure 8). We conclude that intracellular acidosis inhibits the activation of IR and so prevents its further phosphorylation and the activation of the downstream components of the insulin signaling pathway. Our data do not allow us to rule out a concomitant activation of tyrosine phosphatases.

Discussion

No-flow ischemia inhibits insulin signaling in the heart. All of the components of the insulin signaling cascade that we tested were inhibited (IR, IRS-1, PI3K, PKB, p70s6k, and GSK-3). The decreased IR tyrosine kinase activity is sufficient to explain the inhibition of the downstream components. However, it is not excluded that ischemia could influence other intracellular components. For instance, the discrepancy between the activity of PKB (Figure 1A) and PI3K (Figure 6B) found at 2 minutes of ischemia indicates that factors other than PI3K could modulate the phosphorylation state and the activity of PKB and in this case cause a transient activation of PKB. This suggests that ischemia-dependent events control PKB activation.

Inhibition of insulin signaling may occur at different levels of the signaling pathway, and several examples are known. For instance, tumor necrosis factor-α decreases the phosphorylation state of IRS in cultured muscle cells.29 Osmotic shock induces PKB dephosphorylation in cultured hepatocytes.
without IR dephosphorylation and PI3K inactivation. In adipose tissue, isoproterenol and cAMP inhibit IR tyrosine phosphorylation and tyrosine kinase activity. These mechanisms reflect the inherent complexity of a system that involves numerous control steps.

We propose that the inhibition of insulin signaling during ischemia results from the intracellular acidosis that this condition causes. This conclusion reflects the parallelism between intracellular acidosis and inhibition of insulin signaling and is based on the following observations. First, intracellular acidosis and inhibition of signaling followed the same time course during ischemia. Second, the extent of intracellular acidosis was related to the inhibition of insulin signaling not only in ischemia but also in normoxia at pH 7.1 and in normoxia with ouabain and amiloride. Third, perfusion at pH 8.2 prevented the ischemia-induced intracellular acidosis and inhibition of insulin signaling. Importantly, the parallelism between intracellular acidosis and inhibition of insulin signaling also holds for the pH profile of the tyrosine kinase activity of IR, thus indicating that intracellular acidosis inhibits the whole signaling pathway. However, the relationship between intracellular acidosis and inhibition of insulin signaling seems to hold only at pH values below 6.75 as indicated by the pH, (Figure 3A) at which point inhibition of PKB activation becomes detectable in ischemic hearts (Figure 1A). It is suggested that pH 6.75 represents a threshold value under which insulin signaling becomes inhibited. Therefore, inhibition of insulin signaling is expected to depend on the severity and duration of ischemia and probably reflects the extent of intracellular acidosis. Our data show side effects of ischemic acidosis on hormonal signal transduction, whereas others have emphasized its benefit on postischemic myocardial function.

Activation by insulin of the main components of the signaling pathway is transient. Hence, short experimental periods had to be used to measure insulin signaling. However, they had to be long enough to allow severe ischemia, and the resulting acidosis, to develop. The experimental protocol used (a 5-minute perfusion period with insulin followed by a 10-minute period of no-flow ischemia) ensured that both insulin signaling and ischemic acidosis would occur simultaneously.

In this simplified model of ischemia, inhibition of insulin signaling is readily demonstrable and specifically evaluates the insulin response. It is not the case for the metabolic effects of insulin, because inhibition of these effects is masked by the stimulation of glucose uptake and glycolysis by AMPK, which is activated under hypoxic conditions. Therefore, a stimulation of glucose metabolism persists, even if insulin signaling is impaired in ischemic hearts. This was confirmed by our measurements of lactate concentrations, which were the same in ischemic hearts (10 minutes of no-flow ischemia) whether insulin was present or not (lactate concentration in control hearts, 0.2±0.1 μmol/g tissue without insulin and 0.3±0.1 with insulin; in ischemic hearts, 12.6±0.1 without insulin and 14.2±1.6 with insulin; n=3). In models of low-flow ischemia, increasing insulin concentration did not further stimulate glucose uptake. In cardiomyocytes incubated in normoxic conditions but at pH values below 6.75, such an interference does not exist. In these cells, both insulin signaling and the metabolic effects of insulin (stimulation of glucose uptake) were indeed inhibited, although basal levels of glucose uptake were little affected. We therefore conclude that intracellular acidosis is expected to inhibit insulin response. Several examples of insulin resistance associated with acidosis have already been reported in other tissues.

Measurement of insulin signaling, and especially changes in PKB activity, could allow one to investigate the insulin effects on cell survival, because PKB is not only mediating metabolic effects but is also involved in cell protection. Therefore, inhibition of insulin signaling by ischemia may indicate that all insulin effects are abolished. Conversely, insulin signaling was fully restored early on reperfusion suggesting that the hormonal effects could then resume. We speculate that the slight delay in PKB activation corresponds to the time necessary to recover a normal pH.

Although we demonstrated that insulin signaling was inhibited during ischemia and restored during reperfusion, the implications of this on heart function, glucose metabolism, or other heart responses to insulin remain to be investigated. Moreover, the mechanisms by which insulin protects the postischemic heart are not clear. They could be related to a direct positive inotropic effect on postischemic heart or to the well-known metabolic effects of insulin. They could also include other effects of insulin, such as inhibition of apoptosis, control of gene expression, or mitogenic properties.

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