Ischemic preconditioning, insulin, and morphine all cause hexokinase redistribution

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Submitted 25 November 2004; accepted in final form 8 March 2005

Zuurbier, Coert J., Otto Eerbeek, and Alfred J. Meijer. Ischemic preconditioning, insulin, and morphine all cause hexokinase redistribution. Am J Physiol Heart Circ Physiol 289: H496–H499, 2005. First published March 11, 2005; doi:10.1152/ajpheart.01182.2004.—Association of hexokinase (HK) with mitochondria preserves mitochondrial integrity and is an important mechanism by which cancer cells are protected against hypoxic conditions. Maintenance of mitochondrial integrity also figures prominently as a major characteristic of many cardioprotective manipulations. In this study, we provide evidence that cardioprotective interventions may promote HK redistribution from the cytosol to the mitochondria in the heart. Isolated Langendorff-perfused rat hearts (n = 6/group) were subjected to normoxic perfusion (control, Con), three 5-min ischemia-reperfusion periods (ischemic preconditioning, IPC), 1 U/l insulin (Ins), or 1 μM morphine (Mor). Hearts were immediately homogenized and centrifuged to obtain whole cell, cytosolic, and mitochondrial fractions. HK, lactate dehydrogenase (LDH), and citrate synthase (CS) enzyme activities were determined. No change in LDH or CS present in the cytosol fraction relative to whole cell activity was observed with any of the cardioprotective interventions. By contrast, HK present in the cytosol fraction relative to whole cell activity decreased significantly (P < 0.05) with all cardioprotective interventions, from 0.58 ± 0.03 (Con) to 0.46 ± 0.04 (IPC), 0.41 ± 0.01 (Ins), and 0.45 ± 0.02 (Mor). In addition, HK relative to CS activity in the mitochondrial fraction increased significantly with cardioprotection, from 0.15 ± 0.001 (Con) to 0.21 ± 0.002 (IPC), 0.18 ± 0.003 (Ins), and 0.21 ± 0.005 (Mor). Our novel data suggest that well-known cardioprotective interventions share a common end-effector mechanism of cytosolic HK translocation. Association of HK with mitochondria may promote inhibition of the mitochondrial permeability transition pore and thereby reduce cell death and apoptosis.

mitochondria; myocardial ischemia

ISCHEMIC PRECONDITIONING is the phenomenon in which transient nonlethal periods of ischemia protect the heart against a subsequent lethal ischemic period (20). Although much research has been devoted to this phenomenon, the final end-effector or mechanism of protection remains elusive. Many of the signals that can induce preconditioning seem to converge on the mitochondrion (19). In addition, glucose metabolism, especially glycolysis, also figures prominently in the setting of ischemic preconditioning (20). We (31, 32) have also noted that mitochondrial activation is delayed after a reversible period of ischemia, provided that glycolysis can take place. These data suggest that glycolysis and mitochondria converge to invoke the protective action of ischemic preconditioning. Interestingly, this interaction between glucose metabolism and mitochondria also figures prominently in the setting of cancer cells that are protected against apoptosis (21). More specifically, it was recently shown in cultured fibroblasts and hepatocytes that an integral component of protection against apoptosis consists of the translocation of hexokinase (HK), the enzyme catalyzing the first step in glycolysis, to the mitochondrial permeability transition pore (7, 8). HK is either located in the cytosol or mainly associated with the mitochondria (29).

The present study examined whether redistribution of cardiac HK occurs with ischemic preconditioning. In addition, the effects of administration of insulin and morphine (well known for their cardioprotective actions) on the redistribution of HK in the heart were studied. The data suggest that HK redistribution may constitute a common end-effector mechanism by which the heart may protect itself against cell death.

METHODS

Heart perfusion. Preparation of the heart was as reported previously (30, 31). Briefly, hearts isolated from male Wistar rats (402 ± 12 g) anesthetized with pentobarbital sodium (60 mg/kg) were cannulated in situ, with perfusion started before excision of the heart. Hearts were Langendorff perfused at a constant flow (initial perfusion pressure of 80 mmHg resulting in a flow of 16.8 ± 0.7 ml/min) at 37°C with Tyrode solution containing (in mM) 128.3 NaCl, 4.7 KCl, 1.4 CaCl2, 1.1 MgCl2, 20.2 NaHCO3, 0.4 NaH2PO4, and 11.0 glucose, gassed with 95% O2-5% CO2. End-diastolic pressure was set at 3–6 mmHg with a balloon inserted in the left ventricle. After stabilization of pressure development during the first 30 min of Langendorff perfusion, four groups of hearts (n = 6/group) were studied for the next 35 min. In the control group, hearts were kept at baseline conditions. In the ischemic preconditioning group, hearts received three 5-min periods of ischemia each followed by 5-min reperfusion except that the last reperfusion lasted for 10 min. In the insulin group, hearts received 1 U/l insulin, starting at 5 min of the 35-min perfusion period. In the morphine group, hearts received 1 μM morphine, also starting at 5 min of the 35-min perfusion period. We previously showed (30) that this ischemic preconditioning protocol resulted in protection against a 40-min ischemia period in rat hearts. Several studies have shown that morphine or insulin given before ischemia also confers cardioprotection against ischemia-reperfusion in the heart (see, e.g., Refs. 1, 5, 17, and 24). All procedures were approved by the Animal Ethical Commission of the University of Amsterdam and conformed to National Institutes of Health guidelines.

Heart homogenization and fractionation. At 35 min of perfusion, the heart was immediately minced in 8 ml of ice-cold homogenization medium (0.25 M sucrose, 0.02 M HEPES, and 1 mM β-mercaptoethanol, pH 7.4) and homogenized on ice. Part of the homogenate was immediately centrifuged at 10,000 g for 10 min at 4°C. The supernatants and pellets were recovered and represented the soluble (cytosolic) and mitochondrial fractions, respectively, whereas the part of the
homogenate that was not centrifuged represented the total whole cell fraction. Fractions were quickly frozen at −20°C until determination of enzyme activity. Two different indexes of HK redistribution were used: 1) cytosolic HK activity relative to whole cell HK activities and 2) HK activity relative to citrate synthase (CS) activity in the mitochondrial fraction.

**Determination of enzyme activities.** For measurement of whole cell enzyme activities, the homogenate was treated with 0.5% Triton X-100 and sonicated for 5 s, followed by centrifugation in an Eppendorf microcentrifuge (12,000 g, 10 s). For measurement in the mitochondrial pellet, 1 ml of homogenization medium, 50 μl of 10% Triton X-100, and 10 μl of 100 mM glucose-6-phosphate (to promote the release of mitochondrially bound HK; Ref. 29) were added to the pellet, stirred, and incubated at room temperature for 15 min, followed by 5-s sonication. After centrifugation at 4°C (10,000 g, 10 min), activities were determined in the supernatant. HK activity was measured spectrophotometrically with glucose-6-phosphate dehydrogenase (from Leuconostoc mesenteroides), glucose, ATP, and NAD+ (1) in the presence of rotenone (1 μM) to inhibit the mitochondrial respiration chain. Lactate dehydrogenase (LDH) activity, a cytosolic marker, and CS, a mitochondrial marker, were measured according to standard spectrophotometric procedures (2) and served as a control for HK translocation. Protein content was determined by the biuret method with egg albumin as the standard.

**Statistics.** Student’s t-test was used for comparison of group means with the control group (P < 0.05).

**RESULTS**

No significant differences in baseline cardiac mechanical characteristics were observed between group means. The average values across groups amounted to an end-diastolic pressure of 5 ± 1 mmHg, a peak systolic pressure of 102 ± 4 mmHg, and a perfusion pressure of 77 ± 4 mmHg. At 35 min of perfusion, ischemic preconditioning, insulin, and morphine were without effect on these characteristics except that insulin significantly increased peak systolic pressure (by 24 ± 4 mmHg) compared with control.

Whole cell enzyme activity of LDH, CS, or HK, expressed per milligram of protein, was not affected by any of the cardioprotective interventions. The averaged activities across groups amounted to 670 ± 74 nmol·min⁻¹·mg protein⁻¹ for LDH, 513 ± 48 nmol·min⁻¹·mg protein⁻¹ for CS, and 35 ± 4 nmol·min⁻¹·mg protein⁻¹ for HK.

LDH was exclusively found in the cytosol, and this was not affected by any of the cardioprotective interventions (Fig. 1A). About 30% of the mitochondrial marker CS was found in the cytosol fraction, presumably because of damage of mitochondria during homogenization of the tissue. CS distribution was not altered by the different interventions, demonstrating that the cardioprotective interventions were without effect on mitochondrial recovery (Fig. 1B). In contrast, all cardioprotective treatments significantly reduced the amount of HK activity present in the cytosol (Fig. 1C). Cytosolic HK activity relative to whole cell activity decreased significantly (P < 0.05) from 0.58 ± 0.03 for control to 0.46 ± 0.04 for the ischemic preconditioning group, 0.41 ± 0.01 for the insulin group, and 0.45 ± 0.02 for the morphine group.

Enzyme activities were also determined in the mitochondrial fraction (Fig. 2). It was demonstrated that HK relative to CS activity in the mitochondrial fraction increased significantly with all cardioprotective interventions, from 0.15 ± 0.001 (control) to 0.21 ± 0.002 (ischemic preconditioning), 0.18 ± 0.003 (insulin), and 0.21 ± 0.005 (morphine).

**DISCUSSION**

The first studies of ischemic preconditioning suggested that alterations in glycolysis were an integral component of the
endogenous protection phenomenon (20). Preconditioning results in a decreased buildup of fructose-6-phosphate and lactate, despite normal or even elevated levels of glucose and glucose-6-phosphate during the index ischemia (11, 28). We have shown that this is not due to an increased channeling of glycolytic intermediates into the pentose phosphate pathway (30). Regulation of glycolytic enzymes by ischemic preconditioning may involve enzyme translocation, with HK being a likely candidate. Surprisingly, to our knowledge, this is the first study in the heart that has examined whether short periods of ischemia may cause a redistribution of HK. The literature in the early 1970s had already shown that brief ischemia can redistribute HK in the chicken brain (12). Interestingly, the 2-h time course of mitochondrial binding of HK after ischemia in the brain (9) is similar to the window of protection offered by classic ischemic preconditioning, suggesting an important role of HK binding in the mechanism of ischemic preconditioning.

Morphine has emerged as a powerful mediator of cardioprotection, mimicking ischemic preconditioning (24). Recently, it was demonstrated that this opioid-induced cardioprotection is related to glycolytic synthase kinase-β inhibition (10). The current study shows that morphine also induces HK translocation. It is unknown at present whether this HK translocation is downstream of glycolytic synthase kinase-β inhibition or represents a parallel pathway leading to cardioprotection.

Although it is not clear whether insulin causes HK translocation in the heart (6, 23), studies in other tissues have shown that insulin, IGF-I, and several cytokines transduce signals via phosphatidylinositol-3-kinase (PI3-kinase) and protein kinase B (Akt), resulting in HK being recruited to the mitochondria (8). Interestingly, PI3-kinase was recently shown to be a prerequisite for protection to occur with ischemic preconditioning and to play a crucial role in cellular trafficking (26). Our data with insulin support the notion that insulin causes HK redistribution. Why no translocation was observed by Doenst et al. (6) is not clear but may be due to the elaborate and time-consuming handling of the tissue needed for the determination of HK activity in the mitochondrial fraction, the presence of KCl and/or MgCl2 in the isolation medium, and/or the higher workload of the heart compared with the previous work (23). In the brain, 15–30 s of ischemia already results in HK translocation (14); for this reason, we chose a fast fractionation and in situ cannulation of the heart.

How mitochondria-associated HK offers protection against cell death has not been clarified as of yet. Two mechanisms, not mutually exclusive, have been proposed (15, 16). One has a structural basis, in which HK associated with mitochondria, more specifically with the outer mitochondrial membrane (OMM) and the voltage-dependent anion channel (VDAC), restricts the ability of proapoptotic proteins such as BAX/BAK to oligomerize on the OMM. Alternatively, the mitochondrial HK associated with the VDAC provides for increased availability of cytosolic ADP (ADP is product of HK reaction), thereby stimulating flux through the adenine nucleotide translocator and thus stimulating the F1F0-ATP synthase complex. As a result, the H+ gradient, i.e., mitochondrial membrane potential, is lowered (4). A lower mitochondrial membrane potential results in decreased mitochondrial reactive oxygen species production and consequently decreased oxidative damage (13). It may also prevent remodeling of the inner mitochondrial membrane, whereby individual cristae become fused and the junctions between cristae and intermembrane space are opened with mobilization of cytochrome c stores in the intra-mitochondrial cristae (3, 25). Interestingly, prevention of hyperpolarization or increased depolarization also figures prominently as one of the proposed mechanisms of cardioprotection induced by mitochondrial ATP-sensitive K+ channel openers (18). Maintenance of mitochondrial exchanges, such as that of ADP and ATP, seems to be an important mechanism by which the cell protects itself against cell death (27). In addition, the preferential use of mitochondrially produced ATP by bound HK may result in increased coupling of oxidative phosphorylation with glycolysis, possibly preventing excessive acidosis during the index ischemic period (29).

In conclusion, renewed interest in the well-known high glycolytic rate of tumor cells (21) indicates that this may be caused, at least in part, by association of HK with mitochondria (22), which results in high resistance of cancer cells to hypoxic conditions. Whereas this endogenous protection mechanism offers a challenge to oncologists to combat, it may offer an opportunity to clinicians in the setting of cardiovascular disease for therapy. Our study showing that ischemic preconditioning, insulin, and morphine all induce translocation of cytosolic HK strongly suggests that HK redistribution may also be an important player in cardioprotection.

REFERENCES


