Reperfusion-Induced Translocation of δPKC to Cardiac Mitochondria Prevents Pyruvate Dehydrogenase Reactivation

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Abstract—Cardiac ischemia and reperfusion are associated with loss in the activity of the mitochondrial enzyme pyruvate dehydrogenase (PDH). Pharmacological stimulation of PDH activity improves recovery in contractile function during reperfusion. Signaling mechanisms that control inhibition and reactivation of PDH during reperfusion were therefore investigated. Using an isolated rat heart model, we observed ischemia-induced PDH inhibition with only partial recovery evident on reperfusion. Translocation of the redox-sensitive δ-isofrom of protein kinase C (PKC) to the mitochondria occurred during reperfusion. Inhibition of this process resulted in full recovery of PDH activity. Infusion of the δPKC activator H2O2 during normoxic perfusion, to mimic one aspect of cardiac reperfusion, resulted in loss in PDH activity that was largely attributable to translocation of δPKC to the mitochondria. Evidence indicates that reperfusion-induced translocation of δPKC is associated with phosphorylation of the αE1 subunit of PDH. A potential mechanism is provided by in vitro data demonstrating that δPKC specifically interacts with and phosphorylates pyruvate dehydrogenase kinase (PDK2). Importantly, this results in activation of PDK2, an enzyme capable of phosphorylating and inhibiting PDH. Thus, translocation of δPKC to the mitochondria during reperfusion likely results in activation of PDK2 and phosphorylation-dependent inhibition of PDH. (Circ Res. 2005;97:78-85.)

Key Words: pyruvate dehydrogenase ■ δPKC ■ pyruvate dehydrogenase kinase ■ free radicals ■ mitochondria ■ ischemia/reperfusion

Pyruvate dehydrogenase (PDH) is responsible for the conversion of pyruvate derived from glycolysis to acetyl-CoA for Krebs cycle activity. Enzyme activity is regulated, in part, by phosphorylation- and dephosphorylation-dependent inhibition and activation, respectively.1,2 Phosphorylation is catalyzed by 4 PDH-associated pyruvate dehydrogenase kinases (PDK1–4) that exhibit tissue-specific expression patterns and differences in specific activity toward 3 phosphor- nases (PDK1–4) responsible for reactivation of PDH.2–4 PDH also contains 2 pyruvate dehydrogenase phosphatases (PDP 1 and PDP 2) responsible for reactivation of PDH.2–4 PDH therefore represents a highly regulated and critical site for the control of glycolytic flux and ATP production.

Cardiac ischemia/reperfusion is associated with alterations in metabolism that, depending on the severity of the ischemic insult, can progress to irreparable myocardial damage.5 Although PDH activity in myocardial tissue has been reported to decline during flow-induced ischemia,6 this is not universally observed.7–9 The effects of reperfusion also exhibit considerable variability, with the majority of studies demonstrating a decrease in PDH activity.7–9 Despite the disparity in evidence regarding PDH activity, cardiac efficiency and recovery of contractile function in posts ischemic hearts can be improved by pharmacological stimulation of PDH8,10–16 or infusion of pyruvate.17–23 Identification of factors that regulate PDH activity during ischemia/reperfusion may therefore enhance the potential for therapeutic intervention.

Reperfusion of ischemic myocardium is associated with enhanced free radical generation.5,24 Pro-oxidants have been shown to regulate protein function either directly or indirectly through the modulation of other regulatory molecules.25–27 One such example is the novel δ-isofrom of PKC. Exposure of purified δPKC to the thiol-specific oxidant diamide and glutathione (GSH) at concentrations that induce inactivation of other PKC isozymes results in δPKC activation.28 Additionally, treatment of various cell types with H2O2, glutathione depleting agents, or the general PKC activator PMA results in tyrosine phosphorylation and/or activation and translocation of δPKC to the mitochondria where it promotes cytochrome c release and the initiation of apoptosis.29–35 In contrast, inhibition of δPKC translocation reduces...
reperfusion-induced myocardial dysfunction and apoptosis and results in improved regeneration of intracellular ATP, phosphocreatine, and pH.\textsuperscript{36–40}

In the present study, we tested the hypothesis that δPKC is involved in regulation of PDH during reperfusion. Rat hearts were perfused in a Langendorff fashion, and a specific peptide inhibitor of δPKC was used to test the contribution of δPKC to ischemia- and reperfusion-induced alterations in PDH activity. In addition, hearts were infused with H₂O₂ to gain insight into potential mechanisms responsible for concerted regulation of δPKC and PDH during ischemia/reperfusion. Finally, in vitro experiments were performed to address potential mechanisms by which δPKC influences the phosphorylation state of PDH.

Materials and Methods

Rat Heart Perfusion and Isolation of Mitochondria
Hearts isolated from male Sprague-Dawley rats (250 to 300 g, Zivic Miller, Pittsburgh, Pa) were perfused according to the Langendorff technique and after each experimental procedure, mitochondria were isolated as described.\textsuperscript{40}

Measurement of PDH and Citrate Synthase Activities
Mitochondria (100 μg/mL) in 20 mmol/L MOPS, 0.15% Triton, pH 7.4 were incubated with 200 μmol/L thiamine pyrophosphate, 40 μmol/L CoASH, 2.5 mmol/L pyruvate, 5.0 mmol/L MgCl₂, 5.0 mmol/L CaCl₂, 1.0 mmol/L NAD⁺, and ±0.5 mmol/L NaF. PDH activity was measured at 25°C as the rate of NADH production at 340 nm. Citrate synthase activity was measured as described.

Evaluation of δPKC Translocation
Mitochondrial protein (60 μg/lane) was resolved by 4% to 15% SDS-PAGE, transferred to nitrocellulose membrane, and probed with polyclonal anti-δPKC (Sigma). After incubation with alkaline phosphatase–conjugated anti-IgG rabbit antibody, binding was visualized by chemiluminescence (CSPD system, Tropix).

Analysis of PDH by 2-D Gel Electrophoresis
Mitochondria (50 μg from each of 3 independent experiments) were pooled and solubilized in a buffer containing 7.0 mol/L urea, 2.0 mol/L thio urea, 4.0% CHAPS, and 0.5% IPG electrophoresis buffer. Protein was resolved by isoelectric focusing using precast Immobiline DryStrips (pI 3 to 10, 13 cm, Amersham) followed by 10% SDS-PAGE. After transfer to nitrocellulose, blots were probed with antibodies against the subunit of PDH (Molecular Probes), HRP-conjugated secondary antibody (Amersham), and enhanced chemiluminescence (Sigma).

Assay of PKC-Dependent Phosphorylation of PDK2
Phosphorylation of recombinant PDK2 by purified brain ε and δPKC was determined by detecting the incorporation of γ\textsuperscript{32}P from [γ\textsuperscript{32}P]ATP (Amersham) in the presence of PKC activators but in the absence of Ca\textsuperscript{2+}. The reactions were conducted at room temperature (20 minutes) and terminated by boiling samples in Laemmli buffer. Proteins were then resolved by 12.5% SDS-PAGE and transferred to nitrocellulose membranes. Densitometric analyses of autoradiograms were performed using NIH ImageJ software program.

PKC and PDK2 Enzymes
Recombinant rat δPKC was cloned into the pET28 vector, transformed into Escherichia coli BL21(DE3)pLysS cells, and expressed as a His-tagged fusion protein (Dirk Bossemeyer, Heidelberg, Germany). Recombinant rat His-tagged PDK2 protein was obtained from Paresh Sanghani (Indiana University School of Medicine, Indianapolis, Ind). Rat brain PKC enzymes were purified as previously described.\textsuperscript{44} Human recombinant ε and δPKC were purchased from Invitrogen (Carlsbad, Calif).

Column Overlay Affinity Binding Assay
A partially purified brain PKC preparation (1.0 μg/mL in TBS) was incubated with 2.0 μg of polycyonal IgG antibodies against the α, βII, ε, or δPKC (Santa Cruz) and Protein G agarose beads (Santa Cruz) overnight at 4°C. Beads were washed (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.1% Triton X-100) and recombinant PDK2 protein (30 μg) was added and incubated for 1 hour at 4°C. Agarose-immobilized protein complexes were then washed and eluted by boiling the samples in Laemmli buffer. Protein was resolved (12.5% SDS-PAGE), transferred to nitrocellulose membranes, and probed with anti-His conjugated to HRP (Clontech) or with antibodies against α, βII, ε, or δPKC followed by anti-rabbit IgG antibodies linked to HRP (Amersham).

Assay of Interactions Between PDK2 and PKC by ELISA
Recombinant PDK2 (20 ng/μL) in carbonate buffer (4.0 mmol/L Na₂CO₃, 3.6 mmol/L NaHCO₃, pH 9.6) was placed in a 96-well (100 μL/well) flat bottom high binding Costar EIA/RIA plate (Corning) and incubated overnight at 4°C. Wells were washed and treated with 10 μg of partially purified brain PKC (diluted in 20 mmol/L Tris-HCl, pH 7.5) in the presence of activators (1 hour, 25°C). Binding was assessed using antibodies against ε or δPKC (Santa Cruz), alkaline phosphatase (AP)-conjugated secondary antibodies (Boehringer Mannheim), and AP substrate (Pierce).

Assay of PKC-Dependent Phosphorylation of PDK2
Phosphorylation of recombinant PDK2 by purified brain ε and δPKC was determined by detecting the incorporation of γ\textsuperscript{32}P from [γ\textsuperscript{32}P]ATP (Amersham) in the presence of PKC activators but in the absence of Ca\textsuperscript{2+}. The reactions were conducted at room temperature (20 minutes) and terminated by boiling samples in Laemmli buffer. Proteins were then resolved by 12.5% SDS-PAGE and transferred to nitrocellulose membranes. Densitometric analyses of autoradiograms were performed using NIH ImageJ software program.

PD2K Peptide Activity Assay
The activity of purified recombinant rat PDK2 was determined by measuring phosphorylation of the PDH E1α subunit tetradecapeptide substrate of PDK2 (YHGHSMSNPGVSYR, SynPep Corporation).\textsuperscript{45–47} Phosphorylation was initiated by incubation of [γ\textsuperscript{32}P]ATP (Amersham) with 1.0 μg of PDK2, 100 μmol/L peptide, and 100 ng of ε or δPKC (Invitrogen) in 20 mmol/L Tris-HCl, 10 mmol/L EGTA, 20 mmol/L ATP, 20 mmol/L MgCl₂, pH 7.5. Reactions were conducted at 25°C for 20 minutes and terminated on addition of 25 μL of 200 mmol/L ATP/EDTA. The solution was applied to chromatograph paper, dried for 15 minutes at 25°C, rinsed
for δPKC (n = 4) are represented as mean ± SD with the mean for I5R120 intensity assigned a value of 100. P values: * ≤ 0.0005, † ≤ 0.00005, ‡ ≤ 0.002. Citrate synthase (CS) activity was measured as described in Materials and Methods. D, Protein from total homogenate (T) (10 µg per lane) and isolated mitochondria (M) (10 µg per lane) were subjected to Western blot analysis using antibodies specific to δPKC, phospho-δPKC (serine/threonine), and adenine nucleotide translocase (ANT). Results of densitometric analyses of Western blots with H2O2 and 70% ethanol, and then dried for 10 minutes. Peptide phosphorylation was measured using a scintillation counter.

Results

Translocation of δPKC to Mitochondria During Reperfusion Prevents Recovery of Pyruvate Dehydrogenase Activity

No-flow ischemia (30 minutes) resulted in a 65% loss in PDH activity relative to activity measured in mitochondria isolated from perfused hearts (Figure 1B). PDH activity remained depressed during reperfusion (120 minutes), with only partial recovery in activity relative to ischemic values. The level of native lipoic acid on the E2 subunit of PDH was unaffected by ischemia or reperfusion indicating no alterations in protein content (Figure 1B). As shown in Figure 1C, δPKC translocated to the mitochondria during reperfusion. The relative level of total δPKC associated with the mitochondria is reflected by the appearance of phospho-δPKC (Figure 1C). Infusion of the δPKC specific inhibitor Tat-δV1,1 during the first 10 minutes of reperfusion (Figure 1A) reduced translocation of δPKC to the mitochondria during reperfusion (Figure 1C). Importantly, inhibition of δPKC translocation resulted in recovery of PDH activity to near control values during reperfusion (Figure 1B). Infusion of δV1,1 before ischemia failed to diminish ischemia-induced inhibition of PDH indicating that this decrease in activity is δPKC-independent. Alterations in PDH activity did not appear to be caused by global changes in mitochondrial function given that citrate synthase activity remained unchanged (Figure 1C). In addition, isolation of mitochondria did not result in significant copurification of contaminating fractions (Figure 1D), and infusion of the Tat carrier alone had no effect on PDH activity or δPKC translocation (not shown). Finally, the PKC inhibitor rottlerin, at a concentration (10 µmol/L) specific to δPKC, exhibited effects similar to those observed for Tat-δV1,1 (Figure 2). Therefore, whereas δPKC does not appear to be involved in inhibition of PDH activity during ischemia, translocation of the kinase to the mitochondria during reperfusion prevents complete reactivation of PDH.

H2O2 Induces δPKC Translocation and Inhibition of PDH

Pro-oxidants have been shown to activate δPKC, whereas other isoforms of PKC are inactivated.28,31,32,35 To further test whether δPKC translocation to the mitochondria is responsible for inhibition of PDH and to determine whether alterations in redox status may act as the stimulus for δPKC translocation in the intact heart, hearts were perfused in the absence of NaF (5.0 mmol/L), infused with H2O2 (250 µmol/L), and then subjected to 150 minutes of perfusion (P150), 30 minutes of no-flow ischemia (I50), or 30 minutes of ischemia followed by 120 minutes of reperfusion (I50R120), in the presence or absence of the δPKC specific peptide inhibitor, Tat-δV1,1 (1.0 µmol/L), infused for the first 10 minutes of reperfusion. B, The activity of PDH was measured in the presence of NaF (5.0 mmol/L). Values are presented as the mean ± SD of 4 to 6 individual experiments. P values (2-tailed t test) where like symbols indicate values compared: * ≤ 0.002, † ≤ 0.001, ‡ ≤ 0.02, § ≤ 0.001. Native lipoic acid on the E2 subunit of PDH was examined by Western blot analyses using polyclonal antilipoic acid antibodies.54 C, Mitochondrial protein was subjected to Western blot analysis using antibodies that recognize BIP (endoplasmic reticulum), GAPDH (cytosolic), the β-subunit of NaKATPase (plasma membrane),55 and ANT (mitochondria).

Phosphorylation-Dependent Inhibition of PDH during Ischemia and Reperfusion

To determine whether reperfusion induces phosphorylation-dependent inhibition of PDH, enzyme activity was measured.
in the presence and absence of the general phosphatase inhibitor NaF. In mitochondria isolated from reperfused tissue, PDH activity was ∼25% higher when measured in the absence of NaF (Figure 4A), indicating that ∼50% of the enzyme activity lost during ischemia/reperfusion may be attributable to phosphorylation. In contrast, NaF had no significant effect on activity in mitochondria isolated from perfused tissue (NaF, 84.9 ± 12.1 nmol/min/mg; ∼NaF, 79.8 ± 5.4 nmol/min/mg). Thus, whereas PDH activity remained significantly below control values indicating inhibition/dissociation of enzyme associated phosphatase(s) or alternative mechanisms of inhibition, reperfusion-induced loss in PDH activity appears due, in part, to phosphorylation of the enzyme. PDH can be inhibited to varying degrees by phosphorylation of 3 serine residues on the E1 subunit of the enzyme.2 Two-dimensional Western blot analysis using anti-E1 antibody indicates that PDH migrates at 4 distinct isoelectric points consistent with 4 phosphorylation states of the protein (Figure 4B). In mitochondria isolated from perfused control hearts, the relative abundance of the E1 subunit increased with increasing pI (Figure 4B). On ischemia/reperfusion, this distribution shifted in the acidic direction consistent with an increase in phosphorylation. Infusion of δPKC inhibitor Tat-δV1-1 during ischemia/reperfusion prevented this shift (Figure 4B). Preincubation of mitochondria from reperfused hearts with phosphatase collapsed the distribution of the E1 subunit consistent with near complete dephosphorylation (∼90% by densitometry; Figure 4B). Taken together, these results provide further evidence that the 4 isoelectric points represent different phosphorylation states of the E1 subunit, and that δPKC plays a role in phosphorylation and inhibition of PDH.

δPKC Phosphorylation and Activation of Pyruvate Dehydrogenase Kinase 2

A potential mechanism for δPKC-dependent inhibition of PDH is through the activation of specific kinases that phosphorylate and inhibit PDH. An unbiased screen of ∼20 000 λ...
Phage clones from a rat heart cDNA expression library was conducted using PKC as the bait protein. After secondary and tertiary screens to enrich and validate PKC protein/protein interactions, 2 clones were isolated, sequenced, and identified as the rat form of pyruvate dehydrogenase kinase 2. Binding was specific to the PKC isoform of PKC with no binding evident for PKC, PKC II, or PKC (Figure 5A). The interaction between PDK2 and PKC and specificity of this interaction were confirmed using affinity chromatography with immobilized PKC isoforms (Figure 5B) and ELISA with immobilized PDK2 (Figure 5C). As shown in Figure 6A, purified and PKC catalyzed the in vitro phosphorylation of PDK2, with greater levels of phosphorylation evident for PKC. Phosphorylation of PDK2 occurred on a serine/threonine residue(s), consistent with the catalytic properties of PKC (Figure 6B). To determine whether phosphorylation of PDK2 activates the kinase, a peptide analog of the phosphorylation site on PDH was used as substrate. As shown in Figure 6C, PKC-dependent phosphorylation resulted in activation of PDK2. Activation appears specific to PKC in that no appreciable phosphorylation of the peptide analog was observed in the absence of PKC or PDK2 or in the presence of PKC. It is well documented that PDK2 catalyzes the phosphorylation and inhibition of PDH. These results provide a plausible mechanism for PKC-dependent inhibition of PDH during cardiac reperfusion.
Recovery of PDH activity on inhibition of δPKC translocation may therefore, in part, provide an explanation for the previously observed cardioprotective role of the δPKC specific peptide inhibitor.36–40

Reperfusion of myocardial tissue is associated with a rapid increase in the level of various pro-oxidant species.5,24 Purified δPKC is sensitive to redox status, increasing in catalytic activity under oxidative conditions that induce inactivation of other PKC isoforms.28 In addition, treatment of cells in culture with H₂O₂ results in the translocation of δPKC to the mitochondria.33 We have demonstrated that perfusion of rat hearts with H₂O₂ induces the translocation of δPKC to the mitochondria and reduction in PDH activity. H₂O₂-dependent loss of PDH activity was largely prevented by inhibition of δPKC translocation. Treatment of mitochondria with H₂O₂ did not have an effect on PDH activity, indicating that cytosolic component(s) are necessary for inhibition of PDH. Therefore, pro-oxidants produced during cardiac reperfusion may provide the stimulus for δPKC translocation and PDH inhibition.

PDH is regulated by specific kinases and phosphatases associated with the PDH complex.2–4,49 In mitochondria isolated from reperfused tissue, PDH activity was partially recovered when assayed in the absence of the phosphatase inhibitor NaF. In addition, reperfusion-induced declines in PDH activity were associated with an acidic shift in the isoelectric point of the αE1 subunit of pyruvate dehydrogenase that was prevented on inhibition of δPKC translocation. Thus, δPKC appears to promote phosphorylation-dependent inhibition of PDH. In vitro data indicates that δPKC specifically interacts with PDK2, a kinase that can phosphorylate and inhibit PDH. Importantly, the interaction between δPKC and PDK2 leads to the phosphorylation and activation PDK2.

Endogenous dephosphorylation of PDH (assayed in the absence of NaF) partially restored the ischemia/reperfusion-induced loss in PDH activity, suggesting additional modes of δPKC-dependent inhibition. One potential mechanism is through the inhibition of PDP1 and/or PDP2. However, when mitochondrial samples isolated from reperfused tissue were incubated with alkaline phosphatase to promote dephosphorylation, no further regain in enzyme activity was observed (not shown). In addition, it has been demonstrated that treatment of L6 skeletal muscle cells and immortalized hepatocytes with insulin results in δPKC activation and interaction with PDP1/2. This leads to activation of PDP1/2 and stimulation of PDH activity.49 Nevertheless, the effects of δPKC are likely to be tissue specific.49

An alternative mechanism of PDH inhibition may involve oxidative modification of PDP1/2 or PDH. Precedence for this possibility is provided by evidence that the phosphatase PTP1B is readily inhibited on glutathionylation in response to receptor stimulated pro-oxidant production50 and that PDH is susceptible to oxidative inhibition.51–53 In the present study, H₂O₂-dependent loss of PDH activity was partially prevented by inhibition of δPKC translocation. In contrast, prevention of δPKC translocation to the mitochondria during reperfusion resulted in full reactivation of PDH. This difference may be explained by previous findings that translocation of δPKC to the mitochondria results in release of cytochrome c that could

**Discussion**

PDH was shown to decline in activity during cardiac ischemia. Although a fractional regain in PDH activity occurred on reperfusion, the activity of the enzyme remained depressed relative to control values. δPKC translocated to the mitochondria during reperfusion. Prevention of δPKC translocation resulted in complete recovery in PDH activity. Thus, δPKC prevents reactivation or promotes continued inhibition of PDH in response to cardiac reperfusion. Activation of PDH or inclusion of pyruvate during cardiac ischemia/reperfusion improves recovery of hemodynamic function.10–14,16–18,21–23
in turn amplify mitochondrial free radical production.29–35 Thus, prevention of δPKC translocation to the mitochondria during reperfusion would be expected to prevent δPKC- and redox-dependent inhibition of PDH.

Acknowledgments

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