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


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-isof orm 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 H\textsubscript{2}O\textsubscript{2} 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 (PDK\textsubscript{2}). Importantly, this results in activation of PDK\textsubscript{2}, an enzyme capable of phosphorylating and inhibiting PDH. Thus, translocation of δPKC to the mitochondria during reperfusion likely results in activation of PDK\textsubscript{2} 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.\textsuperscript{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 phosphatases (PDK1–4). The PDH complex also contains 2 pyruvate dehydrogenase phosphatases (PDP 1 and PDP 2) responsible for reactivation of PDH.\textsuperscript{3–4} 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.\textsuperscript{5} Although PDH activity in myocardial tissue has been reported to decline during flow-induced ischemia,\textsuperscript{6} this is not universally observed.\textsuperscript{7–9} The effects of reperfusion also exhibit considerable variability, with the majority of studies demonstrating a decrease in PDH activity.\textsuperscript{7–9} Despite the disparity in evidence regarding PDH activity, cardiac efficiency and recovery of contractile function in postischemic hearts can be improved by pharmacological stimulation of PDH.\textsuperscript{8,10–16} or infusion of pyruvate.\textsuperscript{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.\textsuperscript{5,24} Pro-oxidants have been shown to regulate protein function either directly or indirectly through the modulation of other regulatory molecules.\textsuperscript{25–27} One such example is the novel δ-isof orm of PKC. Exposure of purified δPKC to the thiol-specific oxidant diamide and glutathione (GSH) at concentrations that induce inactivation of other PKC isoforms results in δPKC activation.\textsuperscript{28} Additionally, treatment of various cell types with H\textsubscript{2}O\textsubscript{2}, 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.\textsuperscript{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.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.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 NaF, 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 thiourea, 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. On transfer to nitrocellulose membrane, Western blot analysis was performed using monoclonal antibody to the E1α subunit of PDH ( Molecular Probes), HRP-conjugated secondary antibody (Amersham), and enhanced chemiluminescence (Sigma). For samples incubated with phosphatase before analysis, mitochondria (50 μg) were suspended in 50 μL of 50 mmol/L Tris-HCl, 100 mmol/L NaCl, 0.1 mmol/L EGTA, 2 mmol/L dithiothreitol, 0.01% Brij 35, 2.0 mmol/L MnCl2, 0.05% Triton X-100 at pH 7.0, and lysed in a water bath sonicator (Branson 1200) with three 30-s pulses. Lambda protein phosphatase (4000 U, New England Biolabs) was then added to the mitochondria extract and incubated at 30°C for 2 hours.

δPKC Overlay Interaction Screen
Approximately 20 000 lambda plaques were screened from a Sprague-Dawley rat heart cDNA library (Strategene) as previously described.42,43 Recombinant bacterially expressed δPKC and partially purified rat brain PKC, in the presence of PKC activators (12 μg/mL phosphatidylserine, 2 μg/mL diacylglycerol) (Avanti Polar Lipids, Inc), were used as bait proteins. PKC binding was detected using rabbit polyclonal antibodies against α, βII, ε, or δPKC (Santa Cruz Biotechnology).

δPKC 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.44 Human recombinant ε and δPKC were purchased from Invitrogen (Carlsbad, Calif).

Column Overlay Affinity Binding Assay
A partially purified rat brain PKC preparation (1.0 μg/mL in TBS) was incubated with 2.0 μg of polyclonal 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 Laemml 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 like HRP (Amersham).

Assessment of Interactions Between PDK2 and PKC by ELISA
Recombinant PDK2 (20 ng/mL) 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-32P from [γ-32P]ATP (Amersham) in the presence of PKC activators but in the absence of Ca2+. 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. PDK2 (40 μg/mL) was incubated with recombinant human ε or δPKC (4.0 μg/mL) in 20 mmol/L Tris-HCl, 10 mmol/L EGTA, 20 mmol/L ATP, 20 mmol/L MgCl2, pH 7.5 in the presence of PKC activators for 20 minutes at 37°C. Reactions were terminated by boiling in Laemmli buffer and proteins resolved by 10% SDS-PAGE. After transfer to nitrocellulose, blots were probed with anti-PDK2 antibody (Abgent), anti-δPKC (Santa Cruz), or a mixture of anti-phosphorylated serine PKC substrate, anti-phosphorylated threonine, and anti-phosphorylated threonine-X-arginine antibodies (Cell Signaling). Binding was detected using HRP-conjugated anti-rabbit IgG antibodies (Amersham).

PDK2 Peptide Activity Assay
The activity of purified recombinant rat PKD2 was determined by measuring phosphorylation of the PDH E1α subunit tetradecapeptide substrate of PDK2 (YHGHSMSNPGVSVYR, SynPep Corporation).45–47 Phosphorylation was initiated by incubation of [γ-32P]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
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-δV₁₁ [1.0 μmol/L] (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 δV₁₁ 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-δV₁₁ (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. 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 and presence of H₂O₂ (250 μmol/L; Figure 3A). This resulted in translocation of δPKC to the mitochondria (Figure 3B) and an 50% loss in PDH activity relative to controls (Figure 3C). Treatment of isolated respiring mitochondria with H₂O₂ had no effect on PDH activity suggesting the requirement for cytosolic factors (not shown). Coinfusion of the δPKC inhibitor Tat-δV₁₁ with H₂O₂ resulted in inhibition of δPKC translocation to the mitochondria (Figure 3B) and significant protection of PDH from H₂O₂-induced inhibition (Figure 3C). Thus, H₂O₂-induced inhibition of PDH is due, in large part, to δPKC translocation. 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 approximately 25% higher when measured in the absence of NaF (Figure 4A), indicating that approximately 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. 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 βPKC as the bait protein. After secondary and tertiary screens to enrich and validate βPKC protein/protein interactions, 2 clones were isolated, sequenced, and identified as the rat form of pyruvate dehydrogenase kinase 2. Binding was specific to the β isoform of PKC with no binding evident for α, II, or δPKC (Figure 5A). The interaction between PDK2 and βPKC 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 δ and εPKC catalyzed the in vitro phosphorylation of PDK2, with greater levels of phosphorylation evident for δPKC. Phosphorylation of PDK2 occurred on a serine/threonine residue(s), consistent with the catalytic properties of δPKC (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, δPKC-dependent phosphorylation resulted in activation of PDK2. Activation appears specific to δPKC in that no appreciable phosphorylation of the peptide analog was observed in the absence of δPKC or PDK2 or in the presence of εPKC. It is well documented that PDK2 catalyzes the phosphorylation and inhibition of PDH. These results provide a plausible mechanism for δPKC-dependent inhibition of PDH during cardiac reperfusion.

Figure 4. Phosphorylation-dependent inhibition of PDH during ischemia/reperfusion. A, The activity of PDH was measured in the presence or absence of NaF (5.0 mmol/L) using mitochondria prepared after perfusion (P120) or ischemia/reperfusion (I30/R120) in the absence or presence of Tat-δV1-1. Values are presented as the mean±SD of 4 to 6 individual experiments. P values: * =0.03, † =0.02. B, Mitochondrial protein was resolved by 2-D gel electrophoresis followed by Western blot analysis using polyclonal antibodies to the E1α subunit of PDH. Where indicated, mitochondria were preincubated with phosphatase as described in Materials and Methods. Blots shown represent pooled mitochondrial samples from 3 separate hearts for each experimental protocol.

Figure 5. In vitro interactions between PDK2 and δPKC. A, Purified recombinant rat heart δPKC was used as bait to screen a rat heart λ phage cDNA expression library in the presence of 12 μg/mL phosphatidyserine (PS) and 2.0 μg/mL diacylglycerol (DAG). Two positive clones were isolated and identified as PDK2. The specificity of PDK2/PKC interaction(s) were further evaluated using indicated isoforms of PKC purified from rat brain. B, Partially purified rat brain PKC isoforms (α, II, δ) were incubated with polyclonal IgG antibodies against the α, II, δ, or δPKC and Protein G agarose beads. Immobilized protein was then incubated with purified recombinant His-tagged PDK2 (rat heart) in the presence of PS and DAG. PKC binding to PDK2 was detected by subjecting the eluted proteins to Western blot analysis with anti–His-HRP antibody. PKC immunoprecipitates were also probed with anti–PKC polyclonal antibodies. Blots shown are representative of 3 independent experiments. C, PDK2 was immobilized onto 96-well plates and incubated with either δ or εPKC in the presence of PS and DAG. Protein complexes were probed with polyclonal antibodies against ε and δPKC. Values are presented as the mean±SD of 6 individual experiments. P values: * =0.002, † =0.003.
Recovery of PDH activity on inhibition of \( \delta \)PKC translocation may therefore, in part, provide an explanation for the previously observed cardioprotective role of the \( \delta \)PKC 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 \( \delta \)PKC 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 \( \text{H}_2\text{O}_2 \) results in the translocation of \( \delta \)PKC to the mitochondria.\(^{33}\) We have demonstrated that perfusion of rat hearts with \( \text{H}_2\text{O}_2 \) induces the translocation of \( \delta \)PKC to the mitochondria and reduction in PDH activity. \( \text{H}_2\text{O}_2 \)-dependent loss of PDH activity was largely prevented by inhibition of \( \delta \)PKC translocation. Treatment of mitochondria with \( \text{H}_2\text{O}_2 \) 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 \( \delta \)PKC 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 E1 subunit of pyruvate dehydrogenase that was prevented on inhibition of \( \delta \)PKC translocation. Thus, \( \delta \)PKC appears to promote phosphorylation-dependent inhibition of PDH. In vitro data indicates that \( \delta \)PKC specifically interacts with PDK2, a kinase that can phosphorylate and inhibit PDH. Importantly, the interaction between \( \delta \)PKC 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 \( \delta \)PKC-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 \( \delta \)PKC activation and interaction with PDP1/2. This leads to activation of PDP1/2 and stimulation of PDH activity.\(^{49}\) Nevertheless, the effects of \( \delta \)PKC 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 production\(^{50}\) and that PDH is susceptible to oxidative inhibition.\(^{51–53}\) In the present study, \( \text{H}_2\text{O}_2 \)-dependent loss of PDH activity was partially prevented by inhibition of \( \delta \)PKC translocation. In contrast, prevention of \( \delta \)PKC translocation to the mitochondria during reperfusion resulted in full reactivation of PDH. This difference may be explained by previous findings that translocation of \( \delta \)PKC 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. \( \delta \)PKC translocated to the mitochondria during reperfusion. Prevention of \( \delta \)PKC translocation resulted in complete recovery in PDH activity. Thus, \( \delta \)PKC 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.

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References


