Inhibition of δ-Protein Kinase C Protects Against Reperfusion Injury of the Ischemic Heart In Vivo

Brief Rapid Communications

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Background—Current treatment for acute myocardial infarction (AMI) focuses on reestablishing blood flow (reperfusion). Paradoxically, reperfusion itself may cause additional injury to the heart. We previously found that δ-protein kinase C (δPKC) inhibition during simulated ischemia/reperfusion in isolated rat hearts is cardioprotective. We focus here on the role for δPKC during reperfusion only, using an in vivo porcine model of AMI.

Methods and Results—An intracoronary application of a selective δPKC inhibitor to the heart at the time of reperfusion reduced infarct size, improved cardiac function, inhibited troponin T release, and reduced apoptosis. Using 31P NMR in isolated perfused mouse hearts, we found a faster recovery of ATP levels in hearts treated with the δPKC inhibitor during reperfusion only.

Conclusions—Reperfusion injury after cardiac ischemia is mediated, at least in part, by δPKC activation. This study suggests that including a δPKC inhibitor at reperfusion may improve the outcome for patients with AMI. (Circulation. 2003;108:2304-2307.)

Key Words: reperfusion ■ cardioprotection ■ kinases

Current treatment for acute myocardial infarction (AMI) is aimed at limiting the duration of the ischemic period by disrupting the occlusion in the coronary artery. However, no therapeutic treatment is available to prevent reperfusion injury, which occurs after these interventions.1,2 We previously developed several isozyme-selective inhibitor and activator peptides of protein kinase C (PKC).3 Recently, we found that treatment with a δPKC-selective inhibitor during ischemia/reperfusion reduced cardiac damage in isolated perfused rat hearts.3,4 Here, we show that the δPKC inhibitor prevented reperfusion injury in an in vivo porcine model of AMI.

Methods

Peptide Synthesis

The δPKC inhibitor peptide δV1-1 was synthesized and conjugated to Tat-derived peptide4 via a cysteine S-S bond as described.3

In Vivo Local Occlusion, Peptide Delivery, and Pathological Assessment

We applied a balloon catheter into the mid left anterior descending coronary artery of female juvenile Yorkshire pigs (35 to 40 kg) under anesthesia (1% isoflurane) and inflated the balloon to produce a total occlusion for 30 minutes. The guide wire was removed, and Tat alone (Tat) or Tat–δV1-1 conjugate (δV1-1) was infused via the lumen of the balloon catheter only for the last 1 minute of ischemia (250 ng/kg, 1 mL/min). Left ventriculograms were performed to determine cardiac function. Hearts were harvested 4 hours or 5 days after ischemia. Double staining with Evans blue dye and TTC marked areas at risk for ischemia and infarcted areas, respectively, as described previously.6 Troponin T levels in blood, as an indicator of cardiac cytolysis, were also determined after 24 hours of reperfusion. Wedge biopsies of liver, spleen, lung and kidney were fixed in 10% buffered neutral formalin and embedded in paraffin, and 8-μm-thick sections were stained with hematoxylin and eosin for pathological examination.

Biochemical Analysis of Porcine Cardiac Tissue

Heart tissues were taken after a 4-hour reperfusion. Ischemic tissues from the left anterior descending coronary artery territory and...
nonischemic tissues from the posterior wall were taken after marking of area at risk with Evans blue dye (Figure 1A). Lysates were probed for active caspase-3 (Cell Signaling) and inactive caspase-3 (Santa Cruz). Tissue lysates were also fractionated into soluble and particulate fractions as described,7 and δPKC translocation was determined by Western blot analysis (100 μg/lane) using anti-δPKC antibodies (Santa Cruz).

Heart tissues were embedded in OCT compound, snap-frozen on dry ice, and sectioned (5 μm). Cardiac myocytes were identified by α-actinin (Sigma) staining with a FITC secondary antibody (Molec-

Figure 1. Intracoronary delivery of δV1-1 reduced reperfusion injury. A, Representative pictures of TTC staining in infarcted hearts (TTC stains surviving tissues red, leaving infarcted areas white). Arrows indicate area at risk. Scheme indicates that tissue samples (green) for Western blot analyses and TUNEL staining were taken from area at risk (red) and nonischemic area (blue). B, Quantification of area at risk and infarct size. C, Analysis of troponin T levels in blood samples. D, δV1-1 inhibition of ischemia/reperfusion (I/R)-induced translocation. Data are quantified as percent translocation above control. E, Western blot analysis of caspase-3 cleavage (lower arrow). Data are quantified as percentage of cleaved caspase-3 of total caspase-3 (upper arrows). F, Cardiomyocytes are identified by anti–α-actinin staining (blue), apoptotic nuclei by TUNEL (yellow), and nuclei by DAPI staining (green). Significance of differences between δV1-1–treated (n=12) and Tat-treated (n=8) hearts was *P<0.01. D, E, and F, †P<0.02, δV1-1 vs Tat; ‡P<0.02, δV1-1 vs control; n=3 for each group.
ular Probe). TUNEL staining was carried out for detection of apoptotic cells (Roche), and nuclei were counterstained with DAPI (Sigma). TUNEL-positive nuclei were counted in a total of 1500 myocytes over several randomly selected fields and expressed as a percentage of the total number of nuclei. All animal studies were approved by Stanford’s Institutional Animal Care and Use Committee.

NMR Spectroscopy
Relative changes in phosphorus metabolites during ischemia/reperfusion were measured in isolated perfused mouse hearts (C57BL/6X129sv) subjected to a 20-minute global ischemia followed by a 40-minute reperfusion by acquiring consecutive 31P NMR spectra, as described. Tat or δV1-1 (50 nmol/L) was perfused for the first 15 minutes of reperfusion.

Statistical Analysis
Data are expressed as mean±SEM. Unpaired t tests for comparisons between 2 groups and 1-way factorial ANOVA with Bonferroni’s test for multiple comparisons were used.

Results
δV1-1 Reduces Reperfusion-Induced Cardiac Damage
Although the areas at risk for ischemic insult were similar between Tat-treated and δV1-1-treated pigs, δV1-1 treatment resulted in an ~80% reduction in infarct size and an ~75% reduction in troponin T level in the blood (Figure 1, A, B, and C). Importantly, δV1-1 treatment improved cardiac ejection fraction 30 minutes after reperfusion (53.3±1.7% versus 43.2±2.4% for δV1-1 versus control, respectively; P<0.003) and normalized it by 5 days after the onset of reperfusion (67.7±3.0% versus 57.4±3.0%, respectively; P<0.02). Ejection fraction before ischemia was the same for δV1-1–treated and control groups (68.7±1.6% versus 68.8±2.9%, respectively). δV1-1 treatment also reduced the hypokinetic area by 50% (20.0±3.7% versus 38.9±1.9%, respectively; P<0.0003) 30 minutes after reperfusion and by 75% (7.3±2.5% versus 28.8±3.6%, respectively; P<0.0001) 5 days later as determined by Plus Plus (Sanders Data Systems) analysis of the left ventriculogram. As expected, ischemia/reperfusion-induced δPKC translocation (50% increase over basal) was completely inhibited by δV1-1 treatment in these hearts (Figure 1D), suggesting that ischemia/reperfusion-induced cardiac damage in vivo is mediated by δPKC.

Previous reports demonstrated that apoptosis is a component in the death of cardiomyocytes during reperfusion. Indeed, conversion of the proapoptotic proenzyme caspase-3 to the active cleaved form increased significantly after ischemia/reperfusion compared with nonischemic tissue (Figure 1E, lower arrow). However, δV1-1 treatment greatly reduced caspase-3 activation. In addition, DNA fragmentation (assessed histologically by TUNEL staining) decreased by 67% in δV1-1–treated hearts compared with Tat-treated hearts (Figure 1F). These results suggest that inhibition of δPKC translocation inhibits reperfusion injury–induced apoptosis.

We assessed possible adverse effects induced by δV1-1. The injection of δV1-1 resulted in no acute changes in blood pressure, heart rate, or cardiac function (data from all animals used in this study; not shown). Moreover, there were no pathological findings in any tissue, including kidney, lung, liver, and spleen. Therefore, δV1-1 treatment does not seem to cause acute allergic reaction or other adverse effects.

δV1-1 Improves Recovery of Myocardial ATP, Phosphocreatine, and Intracellular pH During Reperfusion
Using 31P NMR in an ex vivo model of cardiac ischemia, we determined whether the immediate improvement in cardiac contractility observed in δV1-1–treated animals could be a result of improved metabolism of the myocardium. Treatment with δV1-1 during reperfusion ex vivo resulted in a significant improvement in functional recovery (Figure 2, A and B). We showed that ATP levels decreased to ~20% of the preischemic level and remained at this level for the duration of the experiment in Tat-treated hearts (Figure 2C). However, ATP levels recovered to ~70% in δV1-1–treated hearts.
Although not statistically significant, there was also an increase in phosphocreatine levels in δV1-1–treated hearts (Figure 2D). Finally, restoration of intracellular pH was significantly faster and more complete in δV1-1–treated hearts than Tat-treated hearts (Figure 2E). Thus, δV1-1 may improve cardiac functional recovery by decreasing the time required to restore energy and pH of the myocardium during reperfusion.

Discussion

Our studies here show that δPKC inhibition reduces reperfusion injury to the myocardium at least in part by inhibiting apoptosis. (These findings are in accordance with a role for δPKC in apoptosis as previously demonstrated by overexpression of δPKC.13) We also find that δPKC inhibition greatly reduced reperfusion-induced cell necrosis, as evidenced by a 5-fold decline in troponin T release (Figure 1C). Oxidative stress seems to cause δPKC translocation to the mitochondria.14 Therefore, activated δPKC at the mitochondria may have direct effects on protein substrates involved in mitochondrial energetics and pH regulation as well as in apoptosis.

We chose the pig model of AMI to investigate the effects of δPKC inhibition during reperfusion injury because of the similarity of heart size and anatomy of the pig to human heart and our ability to use the same balloon catheters as used in angioplasty in humans. The lack of significant anterograde collateral blood flow in the pig reduced complication of the analysis because of incomplete cessation of blood flow to the affected area.15 However, although the resulting infarct size in this model is similar to that in humans,16 the occlusion time is shorter in this model than the median occlusion time in patients with AMI.17

Reperfusion injury remains an unmet need for patients with AMI.2 We show here that an intracoronary treatment with ∼250 ng/kg of the δPKC inhibitor peptide for 1 minute only at the onset of reperfusion efficiently reduced cardiac damage induced by reperfusion injury and resulted in a sustained improvement of cardiac function. These results demonstrate that reperfusion injury is preventable and suggest that inhibition of δPKC should be a target for drug development to prevent irreversible cardiac injury during reperfusion in humans.

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References

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