Impaired perfusion after myocardial infarction is due to reperfusion-induced δPKC-mediated myocardial damage

Fumiaki Ikeno a,1, Koichi Inagaki b,1, Mehrdad Rezaee a,1, Daria Mochly-Rosen b,⁎,1

a Division of Cardiovascular Medicine, Stanford University School of Medicine, Stanford, CA 94305, United States
b Department of Chemical and Systems Biology, Stanford University School of Medicine, Stanford, CA 94305, United States

Received 28 August 2006; received in revised form 10 December 2006; accepted 11 December 2006
Available online 13 December 2006
Time for primary review 28 days

Abstract

Objective: To improve myocardial flow during reperfusion after acute myocardial infarction and to elucidate the molecular and cellular basis that impedes it. According to the AHA/ACC recommendation, an ideal reperfusion treatment in patients with acute myocardial infarction (AMI) should not only focus on restoring flow in the occluded artery, but should aim to reduce microvascular damage to improve blood flow in the infarcted myocardium.

Methods: Transgenic mouse hearts expressing the δPKC (protein kinase C) inhibitor, δV1-1, in their myocytes only were treated with or without the δPKC inhibitor after ischemia in an ex vivo AMI model. δV1-1 or vehicle was also delivered at reperfusion in an in vivo porcine model of AMI. Microvascular dysfunction was assessed by physiological and histological measurements.

Results: δPKC inhibition in the endothelial cells improved myocardial perfusion in the transgenic mice. In the porcine in vivo AMI model, coronary flow reserve (CFR), which is impaired for 6 days following infarction, was improved immediately following a one-minute treatment at the end of the ischemic period with the δPKC-selective inhibitor, δV1-1 (∼250 ng/kg), and was completely corrected by 24 h. Myocardial contrast echocardiography, electron microscopy studies, and TUNEL staining demonstrated δPKC-mediated microvascular damage. εPKC-induced preconditioning, which also reduces infarct size by ~60%, did not improve microvascular function.

Conclusions: These data suggest that δPKC activation in the microvasculature impairs blood flow in the infarcted tissue after restoring flow in the occluded artery and that AMI patients with no-reflow may therefore benefit from treatment with a δPKC inhibitor given in conjunction with removal of the coronary occlusion.

© 2006 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.

Keywords: No-reflow; Coronary flow reserve; Reperfusion; δ-protein kinase C; Myocardial infarction

This article has been referred to in the Editorial by Wilson and Diaz (pages 623–625) in this issue.

1. Introduction

Acute myocardial infarction (AMI) treatments focus on limiting the duration of ischemia using either angioplasty or thrombolysis to disrupt the occlusions in the coronary arteries and establish reperfusion [1]. However, early during reperfusion AMI patients demonstrate impaired microvascular flow proposed to be due to microvascular lumen obstruction by thromboembolic debris and edema of endothelial and myocardial cells [2]. Therefore, reperfusion-induced microvascular dysfunction exacerbates cardiac damage even after the obstruction to flow in the artery is removed [3]. This phenomenon, called “no- and low-reflow”, is observed in ~30% of patients with a reperfused anterior wall MI [4] and is associated with higher incidence of death [5]. Yet, there are no treatments addressing microvascular dysfunction following reperfusion [6].
Recent studies implicated δPKC as a mediator of cardiac damage following ischemia and reperfusion. Vascular smooth muscle cells from δPKC null mice exhibit decreased free radical-induced apoptosis [7], and δPKC mediates neutrophil adhesion to vascular endothelial cells [8]. Further, after cardiac ischemia, a one-minute infusion of a rationally designed δPKC-selective peptide inhibitor, δV1-1 [9], reduces reperfusion-induced myocyte apoptosis and necrosis in a porcine AMI model [10]. Here, we determined whether δPKC mediates microvascular damage. Using transgenic mouse hearts expressing δV1-1 in their myocytes [11,12] and an in vivo porcine AMI model, we found that delivery of δV1-1 inhibits microvascular injury, reducing the “no-reflow” phenomenon.

2. Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals (NIH Publication 85-23).

2.1. Ex vivo model of global ischemia and reperfusion injury

Transgenic mice (TG) that selectively express δV1-1 in myocytes [11] had normal hemodynamic and morphometric characteristics. The cDNA for the δV1-1 (δPKC amino acids 8–17) peptide, preceded by an 8-amino acid FLAG epitope was directly cloned into exon 3 of the full length mouse α myosin heavy chain promoter. Hearts were subjected to a 30-minute global ischemia and a 120-minute reperfusion at 2.5 mL/min at 37 °C in a Langendorff system [12,13]. Creatine phosphokinase (CPK) release, coronary perfusion pressure (CPP) and coronary vascular resistance (CVR, or CPP divided by coronary flow rate) were measured. Hearts were perfused with TAT47–57 conjugated δV1-1 [9] (50 nmol/L) or vehicle (control) during the first 20 min of reperfusion (n=5/group) and infarct size was determined with triphenyltetrazolium chloride (TTC) [12]. Four hours after reperfusion, sections were stained for apoptosis (identified by TUNEL; Roche), nuclei (using 4, 6-diamidino-2-phenylindole, DAPI; Sigma-Aldrich), myocytes (using anti-α actinin; Sigma-Aldrich), and endothelial cells (using anti-PECAM-1; Santa Cruz Biochemicals).

2.2. In vivo porcine model of regional ischemia

Yorkshire swine (30–45 kg) acute MI model was induced by inflating a 10 mm over-the-wire angioplasty balloon placed in the left anterior descending artery (LAD) proximal to the first diagonal branch, as previously described [10]. At the last 1-minute of the 30-minute ischemia, TAT47–57 conjugated δV1-1 (15 μg/mL to pigs weighting 30–45 kg represents 170–320 ng/kg of active ingredient in the compounds, or ~250 ng/kg of δV1-1 without TAT), TAT47–57 (~250 ng/kg) alone or saline were infused at 1 mL/min through the guide-wire lumen of the balloon catheter (n=9 per group). (Previous studies demonstrated no differences between saline and TAT47–57 controls [10].) Ejection fraction (EF) and hypokinetic area were measured by left ventriculogram (LVG) (40° left anterior oblique projection, 30 frames/s) before ischemia (baseline), 30 min, 24 h, 6 days (5–7 days), and 12 days (10–14 days) after ischemia (n=9 per group) and infarct size by TTC staining as percent of area at risk were measured as described [10]. Blood pressure and heart rate were measured just before LVG using water-filled catheters.

Where indicated, 10 mL of TAT47–57–ψεRACK (at 15 μg/mL or ~2.5 μg/kg) or TAT alone (~2.5 μg/kg) was infused during the first 10 min of the 35-minute ischemic event at 1 mL/min followed by 1 mL of saline or of δV1-1 (~250 ng/kg), given at reperfusion (TAT alone; n=3, ψεRACK alone; n=3, δV1-1 alone; n=4, ψεRACK and δV1-1; n=7). (Ten mL of TAT–ψεRACK at 15 μg/mL injected to pigs of 30–45 kg represents 1.7–3.2 μg/kg of active ingredient in the compounds, or ~2.5 μg per animal of TAT–ψεRACK). Hypokinetic area before ischemia (baseline), 30 min and 24 h after ischemia, and infarct size by TTC staining as percent of area at risk were measured [10]. The ischemic time between the assays varied from 30 to 40 min to obtain a similar AAR that varied slightly possibly due to a higher number of collaterals in the animals. There were no premature mortalities of animals in these studies.

2.3. Coronary flow reserve (CFR) measurement in the porcine AMI in vivo model

Coronary flow was measured by a 0.014" Doppler-tipped guide wire (Flowire, JOMED Inc.) in the LAD and the unaffected, left circumflex artery (LCx). The wire tip was placed 2 cm distal from the balloon-occluded site in the LAD. After achieving stable baseline flow velocity, adenosine [48 μg [14]] was infused into the coronary artery to induce hyperemia at the same 5 time points as above (n=9 in each group) and 2 extra time points (baseline and 24 h after ischemia). An additional 12 animals underwent δV1-1 (~250 ng/kg) or saline infusion at the last 1-minute of the 30-minute occlusion through the guide-wire lumen and bradykinin (0.2 mL, 3 μM in saline [15]) was infused into the coronary artery before ischemia and 24 h after reperfusion (n=6 in each group). CFR is the average peak velocity (APV) during the hyperemic phase divided by the baseline APV [14]. Although there is a potential for a preconditioning effect by adenosine or bradykinin, we have seen no evidence for that (e.g., by comparing infarct size), likely because of the low dose of each of these agents when used in the CFR assay.

2.4. Assessment of no- and low-reflow areas and regional myocardial blood flow measurement in the porcine AMI in vivo model

A total of 12 animals that underwent δV1-1 (~250 ng/kg) or TAT were infused at the last 1-minute of the 40-minutes...
through the guide-wire lumen and reperfused for 24 h. At the end of reperfusion, 1 mL/kg of 4% thioflavin S (Sigma, dissolved in 0.9% saline and centrifuged at 1500 rpm for 5 min) was injected into the left atrium. To determine the area at risk (AAR) in porcine hearts, LAD occlusion at the balloon-occluded site was performed and Evan’s Blue (1.0%) was infused into right and left coronary arteries, in vivo. The left ventricle was then sliced into 6–7 transverse sections and photographed under ultraviolet light (365 nm, Spectrolite ENF-280-C, Spectronics) using a Y48 barrier filter (Minolta), and again photographed after incubation in 1% triphenyltetrazolium chloride (TTC, 37 °C, for approximately 15 min). The area of no-reflow, defined as the non-fluorescent area within the AAR, was traced manually from projected slides. After computerized planimetry, the percentage of the areas was multiplied by the weight of the slice. The AAR was expressed as a percentage of the weight of the left ventricle and the no-reflow area as a percentage of the weight of the risk area, as described [16].

Regional myocardial blood flow (RMBF) was measured by intraatrial injection of approximately 500,000 15 micron-diameter neutron-activated stable gold labeled microspheres (BioPAL, Worcester, MA). Simultaneously, a reference blood sample was withdrawn through the arterial catheter at a rate of 10 mm/min. Heart samples were collected from the blue area (non-ischemic tissue), the non-stained area (AAR) but

---

Fig. 1. δV1-1 treatment reduces infarct size and apoptosis of myocytes (MC) and vascular endothelial cells (EC) after ischemia/reperfusion in isolated perfused mouse hearts. A: Protocol of ex vivo model of global ischemia and reperfusion injury in the Langendorff system. The effects of treatment with δV1-1 delivery on infarct size (B; TTC staining stains live tissue red and leaves dead tissue white. Quantitated images are in C), CPK release (D) and coronary vascular resistance (CVR) (E) during reperfusion were determined in δV1-1-expressing transgenic mice (TG) and in wildtype mice (WT). *p<0.01 vs. WT (C, D), *p<0.05 vs. TG (E); n=5. F provides the number of TUNEL-positive cells, which represents apoptosis as we previously found [10,20], as a percentage of the total numbers of nuclei (magnifications ×200). *p<0.05 vs. EC with vehicle in WT, *p<0.05 vs. MC with vehicle in WT; n=5/each. G: Representative fields with TUNEL staining (red, yellow: overlapped with nuclei and TUNEL staining) in heart sections taken 4 h after reperfusion are shown. Myocytes were stained with anti-α-actinin antibody (blue; top), endothelial cells with anti-PECAM-1 (blue; bottom), and nuclei with DAPI (green).
stained by TTC (border area) and the area not stained by either dye (necrotic area). Each sample was further sliced separating the endocardium from the epicardium. All tissue and blood samples were sent to Bio PAL and the concentration of microspheres in each sample was estimated using spectrophotometric analysis. Myocardial blood flow was calculated as mL/min/g tissue, as previously described [17].

2.5. Immunohistochemistry and histomorphometry of cardiac tissues

Immunohistochemistry 2 h after reperfusion in mouse hearts (n=5) and 4 or 24 h after reperfusion in porcine hearts (n=3) was compared to samples from the non-ischemic area [18]. TUNEL-positive nuclei were counted in a total of 1500 myocytes and 500 of endothelial cells over several randomly selected fields. For the EM analysis, two samples were taken from the center part of the AAR in the LAD territory and the non-ischemic area (LCx territory) in each heart (n=3), 4 h after reperfusion. Ultra-thin sections were stained with uranyl acetate–lead citrate and examined with the H300 (Hitachi) electron microscope in a blinded fashion [19].

2.6. Statistical analysis

Data are expressed as mean±SEM. Two-way ANOVA for repeated measures, 1-way factorial ANOVA with Scheffe’s test, or unpaired or paired Student’s t-test were used to analyze significant differences.

3. Results

3.1. Reduced coronary vascular resistance, infarct size and apoptosis during reperfusion in hearts of δV1-1 transgenic mice subjected to global ischemia, ex vivo

We compared the cardioprotective effect of δV1-1 following ischemia/reperfusion damage in wildtype and transgenic mouse expressing the δPKC inhibitor, δV1-1, only in their cardiomyocytes [11,12]. Following 30 min of ischemia, infarct size and myocyte cytolyis (CPK release; Fig. 1B–D) decreased by 68% in δV1-1-expressing hearts, as compared to wildtype. Although both mice groups had a similar coronary vascular resistance (CVR) at baseline, transgenic hearts had an ~70% lower CVR as compared to wildtype mouse hearts 5 min after reperfusion had begun (Fig. 1E). Delivery of δV1-1 through the coronary arteries in wildtype hearts resulted in a similar (67%) decrease in infarct size and CPK release (Fig. 1B–D) and an ~85% improved CVR, 5 min after the onset of reperfusion (Fig. 1E).

Unexpectedly, although infarct size and CPK release were unaffected by further δV1-1 infusion to the transgenic hearts, δV1-1 treatment almost eliminated the rise of CVR in transgenic mouse hearts (Fig. 1E) suggesting that δPKC inhibition protects both cardiac myocytes and cardiac blood vessels.

Inhibiting δPKC during reperfusion greatly reduces apoptosis of cardiac myocytes as demonstrated by a variety of measures [10,20]. In wildtype hearts, exogenous δV1-1 reduced the number of TUNEL-positive endothelial cells and myocytes by ~80%. In transgenic mouse hearts, expression

---

Table 1

<table>
<thead>
<tr>
<th>Hemodynamic data and CFR in the in vivo AMI porcine model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
</tr>
<tr>
<td>δV1-1</td>
</tr>
<tr>
<td>HR, bpm</td>
</tr>
<tr>
<td>BP (Sys/Dia), (mm Hg)</td>
</tr>
<tr>
<td>CFR (LAD)</td>
</tr>
<tr>
<td>CFR (Cx)</td>
</tr>
<tr>
<td>b-APV, cm/s</td>
</tr>
<tr>
<td>h-APV, cm/s</td>
</tr>
<tr>
<td>δV1-1</td>
</tr>
</tbody>
</table>

R indicates reperfusion. b indicates baseline; h, hyperemia; APV, average peak flow velocity; Cx, circumflex artery; LAD, left anterior descending artery; CFR, coronary flow reserve.

---

Fig. 2. δV1-1 improves vascular function and cardiac function during reperfusion injury, in vivo. A: Protocol of the in vivo porcine model of regional ischemia. B: Representative Doppler recording at baseline and after intracoronary adenosine-induced vasodilation. (S and D = start of systolic and diastolic phases.) C: Coronary flow reserve (CFR) measured by adenosine-induced dilatation in control (TAT carrier, open circle) and δV1-1-treated hearts (filled circle) (⁎p<0.01 vs. control TAT; n=9/each). D: Bradykinin-induced CFR was assessed before ischemia and 24 h after the ischemic event (⁎p<0.01 vs. control TAT; n=6/each). E: Mean lumen cross-sectional area of large vessels remained unchanged after adenosine infusion in both groups. For ejection fraction (EF; panel F) and hypokinetic area (G) at each time point, δV1-1-treated hearts were compared to control TAT (⁎p<0.05 vs. control; n=9/each). Significant inverse correlations between CFR at 6 days and infarct size (H; r=−0.49, p<0.05, n=18) and EF at 12 days (I; r=0.7, p<0.01, n=18). (Open circle — control, filled circle — δV1-1-treated hearts). J: No-reflow area of areas at risk (AAR) were significantly reduced by δV1-1 treatment (p<0.05, n=6). The representative images of heart slices with Thioflavin S staining from each group. K: Blood flow measure by microsphere showing a tendency of improvement by δV1-1 relative to control.
of δV1-1 almost abolished TUNEL-positive myocytes, but did not prevent endothelial cell apoptosis. However, treatment with exogenous δV1-1 in transgenic mice decreased endothelial cell apoptosis by ~80% (Fig. 1F, G). Therefore, δPKC activation in cells other than myocytes (likely endothelial cells) also contributes to vascular dysfunction and cardiac injury.

3.2. In vivo treatment with the δPKC inhibitor preserves microvascular function after AMI

The ex vivo model of acute MI does not examine the contribution of blood cells and the energy demands on the ischemic heart in vivo. We therefore used an in vivo porcine model of AMI, where the left anterior descending artery (LAD) was occluded by inflating a balloon catheter for 30 min proximal to the first diagonal branch, and determined microvascular function by measuring the increase in coronary blood flow in response to intracoronary delivery of 48 μg adenosine, an endothelium-independent vasodilator [21]. The assay is also called coronary flow reserve (CFR). The time-course of CFR recovery in pigs receiving ~250 ng/kg of δV1-1 at the end of the ischemic period was compared to that in the control infarcted group. In saline-treated control pigs, CFR in the LAD following adenosine infusion decreased significantly (from 2.5±0.2 to 1.5±0.1 within 30 min after ischemia; Fig. 2B, C; Table 1) and did not fully recover to pre-ischemic levels even 6 days after ischemia (CFR = 1.8±0.2). In δV1-1-treated pigs, however, CFR was only slightly decreased 30 min after ischemia (from 2.5±0.2

---

![Diagram](https://www.elsevier.com)
to $2.0 \pm 0.2$; Fig. 2B, C) and normalized within 24 h (CFR $= 2.5 \pm 0.2$).

To determine whether the δV1-1-induced improvement of flow through the microvasculature reflects protection of endothelial cells, we used bradykinin, an endothelium-dependent vasodilator. In control animals, CFR following bradykinin infusion decreased significantly (from $2.7 \pm 0.1$ to $1.6 \pm 0.1$) 24 h after reperfusion. However, CFR was normal in the δV1-1-treated group at that time (CFR $= 2.7 \pm 0.1$; Fig. 2D). In all the conditions, the resting average peak velocity (APV) and coronary flow reserve in selected muscle territory outside the infarcted area (left circumflex artery; LCx) remained normal (Table 1). Similarly, there were no significant differences in blood pressure, heart rate (Table 1) and large vessel area (measured by IVUS; Fig. 2E) in all treatments. Together, these data indicate that inhibition of δPKC at reperfusion improved endocardial blood flow.

As before [10], injection of δV1-1 into the coronary artery (LAD) at the time of reperfusion significantly lowered infarct size ($30 \pm 5$ vs. $4 \pm 1\%$, $p < 0.001$; control vs. δV1-1, $n=9$ per group), completely normalized cardiac output (measured by ejection fraction; $55 \pm 2$ vs. $70 \pm 2\%$, $p < 0.05$), and greatly decreased wall motion abnormalities (measured as hypokinetic area; $25 \pm 4$ vs. $5 \pm 2\%$, $p < 0.05$) 12 days after ischemia (Fig. 2F, G). Importantly, we found an inverse correlation between CFR measured 6 days after reperfusion and infarct size ($r = -0.49$, $p < 0.05$, $n=18$), and ejection fraction ($r = 0.7$, $p < 0.05$, $n=18$) 12 days after reperfusion (Fig. 2H, I).

### 3.3. In vivo porcine treatment with δPKC inhibitor reduces no- and low-reflow area after AMI

δV1-1 decreased the no-reflow area by 64% ($36 \pm 7$ vs. $13 \pm 6\%$; Fig. 2J), measured by thioflavin S staining.

---

Fig. 4. Ultrastructural conservation of myocytes and endothelial cell by δV1-1-treatment following 30 minutes-ischemia and 4 hours-reperfusion injury in vivo. Representative EM micrographs (analyzed by a blinded observer) of control saline-treated hearts (A–D) and δV1-1-treated hearts (E, F). A: The capillary shows a red blood cell (arrowhead) and white blood cells (arrows) filling up the lumen and nuclear chromatin condensation and margination. B: The capillary shows endothelial cell swelling and nuclear chromatin condensation and margination. Mitochondria swelling, fragmentation of the cristae, and intramitochondrial amorphous dense bodies are present (arrowhead). C: A myocyte with chromatin condensation and margination. Mitochondria swelling, fragmentation of the cristae, and intramitochondrial amorphous dense bodies are also present (arrowhead). D: Contraction bands (arrow) and myofilament disarray. E: In δV1-1-treated infarcted myocardium, there was a limited endothelial cell swelling and slight condensation of chromatin and margination, but the microvascular lumen remained patent. Mitochondria appear normal and swelling is not seen (white arrowhead). F: δV1-1-treated infarcted tissue showing normal contractile element organization, lack of contraction bands and normal sized mitochondria (black arrowhead). Occasional slightly swollen mitochondria were observed, but they display cristae and lack intramitochondrial amorphous dense bodies (white arrowhead).
Furthermore, the regional myocardial blood flow measured by microspheres showed a trend for improvement by δV1-1 treatment within both the infarcted territory and non-infarcted territory, which did not reach statistical significance (Fig. 2K).

3.4. Pathological evidence for protection of the microvasculature from reperfusion injury by δV1-1 in the porcine model

δPKC inhibition reduced microvascular apoptosis in vivo. Four hours after reperfusion, apoptosis of both endothelial cells and myocytes was reduced by ~ 70% in δV1-1-treated animals (Fig. 3C, D), an effect that was sustained even 24 h after treatment (Fig. 3D). Furthermore, at 4 h, active caspase-3 levels, found both in vascular cells and in myocytes, were lower in δV1-1-treated animals (Fig. 3C, bottom panels).

To assess whether δV1-1 treatment improves microvascular potency after reperfusion, we used electron microscopy. In hearts of control-treated AMI animals, the capillary lumen was obstructed with red and white blood cells (Fig. 4A). Evidence of endothelial cell swelling and protrusion and morphological hallmarks of apoptosis, e.g., chromatin condensation and margination, was observed (Fig. 4B). In contrast, there was a minimal endothelial cell swelling and only occasional endothelial cell morphological changes in δV1-1-treated pigs. Importantly, capillary obstruction by blood cells, invariably present in control hearts (Fig. 4B), was rarely seen in δV1-1-treated animals (e.g., Fig. 4E). Furthermore, myocyte damage indicated by contraction bands and swollen mitochondria with disrupted cristae and amorphous matrix densities in the ischemic zone was observed in control-treated MI pigs (Fig. 4C, D) but not in δV1-1-treated hearts (Fig. 4E, F).

Finally, to rule out that improved microvascular function in the δV1-1-treated pigs is not simply due to inhibiting myocyte damage, tissue swelling and muscle contraction, we used another agent that inhibits myocyte damage and reduces infarct size following ischemia. We found that activation of εPKC before or early during ischemia greatly decreased cardiac myocyte damage by mimicking preconditioning [13]. Although the εPKC-selective activator, ψεRACK (~2.5 μg/kg), delivered during the first 10 min of ischemia, reduced infarct size by ~ 60% and reduced wall motion abnormalities 24 h after treatment (Fig. 5B, C), there was no statistically significant improvement in coronary flow...
reserve in δεRACK-treated hearts at that time point (Fig. 5D). Additionally, we found that activation of εPKC did not affect hypokinesis immediately after infarction whereas δPKC inhibition reduced it by ~45% (Fig. 5C) and that combination treatment with the εPKC activator early during ischemia and the δPKC inhibitor at reperfusion provided a greater reduction in infarct size (Fig. 5B), wall motion abnormalities (Fig. 5C), but not CFR (Fig. 5D), as compared with either the δPKC inhibitor or the εPKC activator, alone. Therefore, εPKC activation and δPKC inhibition contribute to cardiac protection from ischemia by independent although additive mechanisms.

4. Discussion

Reperfusion is a double-edged sword; although it restores oxygen and nutrients to the starving myocardium, reperfusion also exacerbates microvascular dysfunction in the infarcted zone [6]. The recent ACC/AHA guidelines suggest that an ideal reperfusion treatment in AMI patients should not only focus on restoring flow in the epicardial-occluded artery, but should also include treatments that minimize the amount of microvascular damage to protect the jeopardized myocardium [22]. However, none of the clinical trials using pharmacological intervention at reperfusion demonstrated a significant benefit [23]. Here we demonstrated that when given at reperfusion, the δPKC inhibitor, δV1-1, reduces both myocyte damage and microvascular dysfunction [9,10,12], thus greatly improving the outcome following an ischemic event in animal models. We suggest that improved CFR during reperfusion may be a critical component in improved cardiac function after acute MI, a suggestion supported by recent human studies [24,25].

Microvascular damage impedes normal blood flow to vulnerable areas after the main occlusion in the coronary arteries has been removed. Using CFR, thioflavin S staining (to measure the no-reflow area) and blood flow measurement (using neutron-active microspheres), we found that δV1-1 significantly improved regional blood flow in this model and inhibited microvascular dysfunction. Potential mechanisms of microvascular dysfunction and impeded regional blood flow include (I) mechanical compression of the blood vessels by myocardial cell swelling and contracture, (II) endothelial cell swelling and protrusions, (III) leukocyte obstruction of the vessel lumen and (IV) microvascular dysfunction [22,26]. Our data suggest that all four are inhibited by δPKC inhibition.

4.1. Obstruction by myocyte swelling and contracture

Transgenic mice expressing δV1-1 in cardiac myocytes show reduced myocyte apoptosis and coronary vascular resistance (Fig. 1F), but unaltered apoptosis of endothelial cells (Fig. 1F, G). Thus, expression of δV1-1 in myocytes decreases coronary vascular resistance indirectly, by inhibiting myocyte swelling and increased muscle contracture.

4.2. Endothelial cell damage

Infusion of the δPKC inhibitor during reperfusion in mice expressing δV1-1 in their myocytes inhibited endothelial cell apoptosis and further improved vascular function (Fig. 1E–G). Therefore, δPKC is activated independently in endothelial cells and in myocytes resulting in cell damage and apoptosis of both cell types (see also Fig. 3).

4.3. Leukocyte obstruction of the vessel lumen

δV1-1 delivery in a porcine model of acute MI preserved the ultrastructure of the microvessels and inhibited reocclusion of the lumen with blood cells, as seen by the EM study (Fig. 4). The common findings of microvascular obstruction with blood cells after mechanical or enzymatic removal of coronary occlusion in humans have been attributed to a shower of debris sent downstream from the main occlusion during these interventions. However, in the pig model, obstruction of the main coronary artery was mechanical — using an inflated balloon catheter, and not by clot or plaque. Therefore, the finding of microvascular occlusion with blood cells suggests that the occlusion is due to new events occurring at reperfusion and likely reflects endothelial cell damage that promotes blood cell adhesion. These data may explain why devices aimed at capturing debris downstream from the original occlusion failed recently to provide any clinical benefit even though debris was effectively captured [27].

4.4. Microvascular dysfunction

Diminished responses of the microvasculature to bradykinin, an endothelium-dependent vasodilator and to adenosine, an endothelium-independent vasodilator, following ischemia were also found in the porcine MI model (Fig. 2C, D). Importantly, these impairments were improved within minutes and were completely restored 24 h after a one-minute delivery of δV1-1 at reperfusion. Protection of the ischemic myocardium with the εPKC-selective activator, a preconditioning mimetic, reduced infarct size (Fig. 5B) similar to the δPKC inhibitor, but did not significantly inhibit microvascular dysfunction (Fig. 5D). Therefore, δPKC activation causes an additional impairment of microvascular function by a mechanism that is independent of infarct size and εPKC.

4.5. End effectors of δPKC after MI

Reperfusion increases the translocation of δPKC to cardiac mitochondria [20], where δPKC appears to increase reactive oxygen species generation [28,29], decrease the activity of Krebs cycle enzymes [30] and reduce the rate of ATP regeneration [10]. The reduced cellular ATP levels results in cellular ionic imbalances, leading to myocyte and endothelial cell swelling as well as to excessive muscle
contraction and to reduced tissue repair. δPKC translocation to the mitochondria also results in increased cytochrome-c release, increase in BAD levels, caspase-3 and caspase-9 activation and in PARP cleavage [20], which lead to cell apoptosis. The direct δPKC substrate(s) remains to be elucidated, but our data suggest that δPKC is upstream of apoptosis, oncrosis and necrosis [31], cell death processes leading to myocardial damage following MI.

4.6. Study limitations and other considerations

The animal models do not represent all aspects of the clinical setting: We used healthy young animals, whereas MI patients are older and have a variety of co-morbidity factors. In addition, a 30–40-minutes occlusion time used here is greatly shorter than the mean occlusion time in humans. Although in our porcine model a similar infarct size to that observed in humans after 4 h of MI is obtained, a short ischemic period may be more readily reversed by the treatment. (Note however, that in a recent study of stroke, infarct size after a two-hour cerebral artery occlusion was reduced by >70% after a single injection of δV1-1 [32], indicating that damage by a longer ischemic period can also be reversed by this peptide). Also, we did not use 60 or 90 min ischemic time because we found that without some ischemic preconditioning, a procedure that would greatly confound our data, the mortality rates due to arrhythmia and cardiac arrest made the study impossible to conduct. Finally, in the studies in Fig. 5, only a small number of animals were included in some of the groups. Note, however, that remarkably similar results (e.g. infarct size in control animals was 33, 30, 37 and 34%) were obtained over several years in four independent studies [10,13] and the studies reported in Figs. 2 and 5.

There are also limitations to the histological assessments of cardiac damage: Due to study cost, extensive time-course to assess apoptosis in the porcine model was not carried out, affecting the accuracy of the quantitation studies [33]. In addition, although apoptosis and oncosis represent different pathways of early cell death, leading to necrosis after ischemia and reperfusion [33], these were assessed together here. TUNEL staining as a sole measure for apoptosis can be misleading [33]. However, because we previously used multiple assays and δV1-1 inhibited them as well [10,20,30], these were not used here. Finally, although very informative regarding details of structural changes, EM analysis represents only a very small part of the myocardium.

5. Conclusion

Administration of a δPKC-specific inhibitor for 1 min at the onset of reperfusion improves microvascular function by reducing cell death in myocytes and vascular endothelial cells, occlusion of microvessels due to myocyte and endothelial cell swelling and damage, and impaired response to vasodilators. Therefore, such a δPKC-specific inhibitor may be a potent therapeutic agent when given in conjunction with an intervention that removes obstruction in the coronary arteries of patients with acute myocardial infarction.

Acknowledgments

The authors are grateful for the assistance and advice of Jennifer Lyons, Hideaki Kaneda, Leon Chen and Erik Price. This work was supported by NIH HL52141 to DM-R.

References


