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

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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 δ PKC (protein kinase C) inhibitor, δ V1-1, in their myocytes only were treated with or without the δ PKC inhibitor after ischemia in an *ex vivo* AMI model. δ V1-1 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— δ PKC 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 δ PKC-selective inhibitor, δ V1-1 (~250 ng/Kg), and was completely corrected by 24 hrs. Myocardial contrast echocardiography, electron microscopy studies, and TUNEL staining demonstrated δ PKC-mediated microvascular damage. δ PKC-induced preconditioning, which also reduces infarct size by >60%, did not improve microvascular function.

Conclusions— These data suggest that δ PKC 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 δ PKC inhibitor given in conjunction with removal of the coronary occlusion.

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

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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 δ PKC as a mediator of cardiac damage following ischemia and reperfusion. Vascular smooth muscle cells from δ PKC null mice exhibit decreased free radical-induced apoptosis [7], and δ PKC mediates neutrophil adhesion to vascular endothelial cells [8]. Further, after cardiac ischemia, a one-minute infusion of a rationally designed δ PKC-selective peptide inhibitor, δ V1-1 [9], reduces reperfusion-induced myocyte apoptosis and necrosis in a porcine AMI model [10]. Here, we determined whether δ PKC mediates microvascular damage. Using transgenic mouse hearts expressing δ V1-1 in their myocytes [11,12] and an *in vivo* porcine AMI model, we found that delivery of δ V1-1 inhibits microvasculature dysfunction, reducing the “no-reflow” phenomenon.

METHODS

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

Ex vivo model of global ischemia and reperfusion injury

Transgenic mice (TG) that selectively express δ V1-1 in myocytes [11] had normal hemodynamic and morphometric characteristics. The cDNA for the δ V1-1 (δ PKC 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/minute 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 TAT_{47–57}-conjugated δ V1-1 [9] (50nmol/L) or vehicle (control) during the first 20 min of reperfusion (n=5/group) and infarct size determined with triphenyltetrazolium chloride (TTC) [12]. Four hrs 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).

In vivo porcine model of regional ischemia

Yorkshire swine (30–45kg) 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, TAT_{47–57}-conjugated δ V1-1 (15 μ g/mL to pigs weighting 30–45kg represent 170–320 ng/Kg of active ingredient in the compounds, or ~250ng/kg of δ V1-1 without TAT), TAT_{47–57} (~250ng/kg) alone or saline were infused at 1 mL/minute through the guide-wire lumen of the balloon catheter (n=9 per group). (Previous studies demonstrated no differences between saline and TAT_{47–57} controls [10].) Ejection fraction (EF) and hypokinetic area were measured by left ventriculogram (LVG) (40° left anterior oblique projection, 30 frames/sec) before ischemia (baseline), 30 min, 24 hrs, 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, 10mL of TAT₄₇₋₅₇-ΨεRACK (at 15μg/mL or ~2.5 μg/kg) or TAT alone (~2.5 μg/kg) were infused during the first 10 min of the 35-minute ischemic event at 1mL/min followed by 1mL of saline or of δV1-1 (~250ng/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 15μg/mL injected to pigs of 30kg–45kg represent 1.7–3.2μg/Kg of active ingredient in the compounds, or ~2.5μg per animal of TAT-ΨεRACK.) Hypokinetic area before ischemia (baseline), 30 min and 24 hrs 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 minutes 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.

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 24hrs 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, 3μM in saline [15]) was infused into the coronary artery before ischemia and 24 hrs 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.

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 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 reperused for 24 hours. 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 minutes) 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 (365nm, Spectroline 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 minutes). 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 10mm/min. Heart samples were collected from the blue area (non-ischemic tissue), the non-stained area (AAR) but 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 were estimated using

spectrophotometric analysis. Myocardial blood flow was calculated as ml/min/g tissue, as previously described [17].

Immunohistochemistry and histomorphometry of cardiac tissues

Immunohistochemistry 2hrs after reperfusion in mouse hearts (n=5) and 4 or 24 hrs 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 1,500 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 hrs 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].

Statistical Analysis

Data are expressed as mean $\pm$ 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.

Results

Reduced coronary vascular resistance, infarct size and apoptosis during reperfusion in hearts of $\delta V1-1$ transgenic mice subjected to global ischemia, *ex vivo*

We compared the cardioprotective effect of $\delta V1-1$ following ischemia/reperfusion damage in wildtype and transgenic mice expressing the δPKC inhibitor, $\delta V1-1$, only in their cardiomyocytes [11,12]. Following 30 min of ischemia, infarct size and myocyte cytolysis (CPK release; Figs 1B-D) decreased by 68% in $\delta V1-1$ -expressing hearts, as compared to wildtype. Although both mice groups had a similar coronary vascular resistance (CVR) at baseline, transgenic hearts had an $\sim 70\%$ lower CVR as compared to wildtype mouse hearts 5 min after reperfusion had begun (Fig 1E). Delivery of $\delta V1-1$ through the coronary arteries in wildtype hearts resulted in a similar (67%) decrease in infarct size and CPK release (Figs 1B-D) and an $\sim 85\%$ improved CVR, 5 min after the onset of reperfusion (Fig 1E). Unexpectedly, although infarct size and CPK release were unaffected by further $\delta V1-1$ infusion to the transgenic hearts, $\delta V1-1$ 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 $\delta V1-1$ reduced the number of TUNEL-positive endothelial cells and myocytes by $\sim 80\%$. In transgenic mouse hearts, expression of $\delta V1-1$ almost abolished TUNEL-positive myocytes, but did not prevent endothelial cell apoptosis. However, treatment with exogenous $\delta V1-1$ in transgenic mice decreased endothelial cell apoptosis by $\sim 80\%$ (Fig 1F,G). Therefore, δPKC activation in cells other than myocytes (likely endothelial cells) also contributes to vascular dysfunction and cardiac injury.

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 $48\mu g$

adenosine, an endothelium-independent vasodilator [21]. The assay is also called coronary flow reserve (CFR). The time-course of CFR recovery in pigs receiving ~250ng/kg of $\delta V1-1$ 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; Figs 2B,C; Table 1) and did not fully recover to pre-ischemic levels even 6 days after ischemia ($CFR = 1.8 \pm 0.2$). In $\delta V1-1$ -treated pigs, however, CFR was only slightly decreased 30 min after ischemia (from 2.5 ± 0.2 to 2.0 ± 0.2 ; Figs 2B,C) and normalized within 24 hrs ($CFR = 2.5 \pm 0.2$).

To determine whether the $\delta V1-1$ -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 ± 0.1 to 1.6 ± 0.1) 24 hrs after reperfusion. However, CFR was normal in the $\delta V1-1$ -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 $\delta V1-1$ into the coronary artery (LAD) at the time of reperfusion significantly lowered infarct size (30 ± 5 vs. $4 \pm 1\%$, $P < 0.001$; control vs. $\delta V1-1$, $n = 9$ per group), completely normalized cardiac output (measured by ejection fraction; 55 ± 2 vs. $70 \pm 2\%$, $p < 0.05$), and greatly decreased wall motion abnormalities (measured as hypokinetic area; 25 ± 4 vs. $5 \pm 2\%$, $p < 0.05$) 12 days after ischemia (Figs 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 (Figs 2H,I).

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

$\delta V1-1$ decreased the no-reflow area by 64% (36 ± 7 vs. $13 \pm 6\%$; Fig 2J), measured by thioflavin S staining. Furthermore, the regional myocardial blood flow measured by microspheres showed a trend for improvement by $\delta V1-1$ treatment within both the infarcted territory and non-infarcted territory, which did not reach statistical significance (Fig 2K).

Pathological evidence for protection of the microvasculature from reperfusion injury by $\delta V1-1$ 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 $\delta V1-1$ -treated animals (Fig 3C,D), an effect that was sustained even 24 hrs after treatment (Fig 3D). Furthermore, at 4hrs, active caspase-3 levels, found both in vascular cells and in myocytes, were lower in $\delta V1-1$ treated animals (Fig 3C, bottom panels).

To assess whether $\delta V1-1$ treatment improves microvascular patency 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, were observed (Fig 4B). In contrast, there was a minimal endothelial cell swelling and only occasional endothelial cell morphological changes in $\delta V1-1$ -treated pigs. Importantly, capillary obstruction by blood cells, invariably present in control hearts (Fig 4B), was rarely seen in $\delta V1-1$ -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 were observed in control-treated MI pigs (Figs 4C,D) but not in $\delta V1-1$ -treated hearts (Figs 4E,F).

Finally, to rule out that improved microvascular function in the $\delta V1-1$ 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 δPKC before or early during ischemia greatly decreased cardiac myocyte damage by mimicking preconditioning [13]. Although the δPKC -selective activator, $\Psi eRACK$ (~ 2.5 $\mu g/kg$), delivered during the first 10 min of the ischemia, reduced infarct size by $\sim 60\%$ and reduced wall motion abnormalities 24 hrs after treatment (Figs 5B,C), there was no statistically significant improvement in coronary flow reserve in $\Psi eRACK$ -treated hearts at that time point (Fig 5D). Additionally, we found that activation of δPKC did not affect hypokinesia immediately after infarction whereas δPKC inhibition reduced it by $\sim 45\%$ (Fig. 5C) and that combination treatment with the δPKC 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 δPKC activator, alone. Therefore, δPKC activation and δPKC inhibition contribute to cardiac protection from ischemia by independent although additive mechanisms.

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 significant benefit [23]. Here we demonstrated that when given at reperfusion, the δPKC inhibitor, $\delta V1-1$, 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 $\delta V1-1$ 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 [2,26]. Our data suggest that all four are inhibited by δPKC inhibition.

Obstruction by myocyte swelling and contracture

Transgenic mice expressing $\delta V1-1$ in cardiac myocytes show reduced myocyte apoptosis and coronary vascular resistance (Fig 1F), but unaltered apoptosis of endothelial cells (Figs 1F,G). Thus, expression of $\delta V1-1$ in myocytes decreases coronary vascular resistance indirectly, by inhibiting myocyte swelling and increased muscle contracture.

Endothelial cell damage

Infusion of the δ PKC inhibitor during reperfusion in mice expressing δ V1-1 in their myocytes inhibited endothelial cell apoptosis and further improved vascular function (Fig. 1E-G). Therefore, δ PKC is activated independently in endothelial cells and in myocytes resulting in cell damage and apoptosis of both cell types (see also Fig 3).

Leukocyte obstruction of the vessel lumen

δ V1-1 delivery in a porcine model of acute MI preserved the ultrastructure of the microvessels and inhibited re-occlusion 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 has 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].

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 hrs after a one-minute delivery of δ V1-1 at reperfusion. Protection of the ischemic myocardium with the δ PKC-selective activator, a preconditioning mimetic, reduced infarct size (Fig 5B) similar to the δ PKC inhibitor, but did not significantly inhibit microvascular dysfunction (Fig 5D). Therefore, δ PKC activation causes an additional impairment of microvascular function by a mechanism that is independent of infarct size and δ PKC.

End effectors of δ PKC after MI

Reperfusion increases the translocation of δ PKC to cardiac mitochondria [20], where δ PKC 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. δ PKC 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 δ PKC substrate(s) remains to be elucidated, but our data suggest that δ PKC is upstream of apoptosis, oncosis and necrosis [31], cell death processes leading to myocardial damage following MI.

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 comorbidity 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 hrs 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 δ V1-1 [32], indicating that damage by a longer ischemic period can also be reversed by this peptide.) Also, we did not use 60 or

90 minutes 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 $\delta V1-1$ 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.

Conclusion

Administration of a δPKC -specific inhibitor for one minute 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.

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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 $\times 200$). * $p < 0.05$ 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 hrs 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).

Fig. 2. δ V1-1 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 δ V1-1-treated hearts (filled circle) (* p < 0.01 vs. control TAT; n =9/each). D: Bradykinin-induced CFR was assessed before ischemia and 24 hrs 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,

δ V1-1-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 – δ V1-1-treated hearts.). J: No-reflow area of areas-at-risk (AAR) were significantly reduced by δ V1-1 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 δ V1-1 relative to control.

Fig. 3.

Apoptosis of both myocytes and endothelial cells is inhibited by $\delta V1-1$ treatment following 30 minutes-ischemia and 4 hours- or 24 hours-reperfusion injury, *in vivo*. Immunohistochemistry was performed in pig hearts subjected to ischemia/reperfusion (A; protocol). B: Images and scheme showing that $\delta V1-1$ treatment (right panels) decreased infarct size as compared to saline-treated control hearts (left panels; black arrow and yellow area). Tissue samples (green circle) were taken from area at risk and non-ischemic area. C: Immunohistochemistry of cardiac tissues. Myocytes (MC) were identified by anti- α -actinin (blue; top), vascular endothelial cells (EC) by anti-PECAM-1 (blue; middle and bottom panels), and nuclei were counterstained with DAPI (green). Representative fields with TUNEL staining (red, yellow: overlapped with nuclei and TUNEL staining; top and middle) and active caspase-3

staining (red; bottom) from pigs treated with saline (control, left panels) or $\delta V1-1$ (right panels) following infarction. D: TUNEL-positive EC and MC (%) 4hrs and 24 hrs after reperfusion in infarcted saline- (CTL) or $\delta V1-1$ -treated pig hearts are presented. $*p < 0.05$; $n=3-4$ for each group).

Fig. 4.

Ultrastructural conservation of myocytes and endothelial cell by $\delta V1-1$ -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 $\delta V1-1$ -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 $\delta V1-1$ -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: $\delta V1-1$ -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).

Fig 5.

Correction of microvascular dysfunction is independent of reduction in infarct size following 35 minutes-ischemia and 24 hours-reperfusion injury *in vivo* (A; protocol). B: Infarct size was calculated in pig hearts subjected to ischemia/reperfusion. Pig hearts were treated with TAT (control; n=3), Ψ εRACK alone (~2.5μg/kg for the first 10 min of occlusion. n=3), δ V1-1 alone (~250ng/kg for 1 min, at the onset of reperfusion. n=4), or both Ψ εRACK and δ V1-1 (n=7), each given as above. The mean area at risk (AAR) for all treatment groups was 37±4%. C: Hypokinetic area was calculated using left ventriculogram. Pig hearts were treated with TAT (control; closed circle), Ψ εRACK alone (~2.5μg/kg for the first 10 minutes of occlusion; filled triangle), δ V1-1 alone (~250ng/kg for 1 minute, at the onset of reperfusion; filled square), or both Ψ εRACK and δ V1-1, each given as above (open circle). D: CFR was calculated 24 hrs after reperfusion.

Table 1

Hemodynamic data and CFR in the *in vivo* AMI porcine model

	Baseline			30 min R		24 hr R		6 days R		12 days R	
	δV_{I-1}	Control	δV_{I-1}	δV_{I-1}	Control	δV_{I-1}	Control	δV_{I-1}	Control	δV_{I-1}	Control
HR, bpm	98±2	97±5	90±3	103±11	92±6	98±6	92±4	98±6	92±4	90±7	94±4
BP (Sys/Dia), (mmHg)	92±2/61±4	95±2/69±3	87±4/65±3	101±8/66±13	96±10/68±13	97±5/68±3	99±3/71±3	97±5/68±3	99±3/71±3	94±6/60±3	90±3/64±4
CFR (LAD)	2.5±0.2	2.5±0.2	2.0±0.2*	2.5±0.2*	1.5±0.1 [†]	2.7±0.2*	1.8±0.2 [†]	2.7±0.2*	1.8±0.2 [†]	2.7±0.3	2.2±0.2
b-APV, cm/s	24±3	26±2	27±4	18±1	28±3	24±3	27±3	24±3	27±3	25±4	29±5
h-APV, cm/s	58±7	63±6	49±4	46±6*	39±4 [†]	67±9*	47±4 [†]	67±9*	47±4 [†]	57±6	62±6
CFR(Cx)	2.4±0.1	2.6±0.2	1.7±0.2	2.0±0.1	2.0±0.2	2.0±0.1	2.0±0.2	2.0±0.1	2.4±0.2	2.5±0.2	2.4±0.2
b-APV, cm/s	24±2	27±2	20.3±3	15±2	22±2	25±5	20±3	25±5	20±3	25±3	26±4
h-APV, cm/s	61±3	69±5	37±3	30±4	43±4	55±10	55±10	55±10	55±10	55±5	62±9

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.

* p<0.05 vs. control.

[†] p<0.05 vs. baseline.