Suppression of Graft Coronary Artery Disease by a Brief Treatment With a Selective ePKC Activator and a δPKC Inhibitor in Murine Cardiac Allografts

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Background—Inhibiting delta protein kinase C (δPKC) during reperfusion and activating epsilon PKC (εPKC) before ischemia each limits cardiac ischemic injury. Here, we examined whether limiting ischemia–reperfusion injury inhibits graft coronary artery disease (GCAD) and improves murine cardiac allografting.

Methods and Results—Hearts of FVB mice (H-2b) were transplanted into C57BL/6 mice (H-2a). εPKC activator (ψεRACK) was injected intraperitoneal (20 nmol) into donor mice 20 minutes before procurement. Hearts were then perfused with ψεRACK (1.5 nmol) through the inferior vena cava (IVC) and subsequently submerged in ψεRACK (0.5 μmol/L) for 20 minutes at 4°C. Before reperfusion, the peritoneal cavity of recipients was irrigated with δPKC inhibitor (δV1–1, 300 nmol); control animals were treated with normal saline. The total ischemic time to the organ was 50 minutes. Two hours after transplantation, production of inflammatory cytokines and adhesion molecules, cardiomyocyte apoptosis, and caspase-3 and caspase-9 (but not caspase-8) activities were significantly reduced in the PKC regulator-treated group. Fas ligand levels (but not Fas) were also significantly reduced in this group. Importantly, GCAD indices, production of inflammatory cytokines, and adhesion molecules were significantly decreased and cardiac allograft function was significantly better as measured up to 30 days after transplantation.

Conclusions—An εPKC activator and a δPKC inhibitor together reduced GCAD. Clinically, these PKC isozyme regulators may be useful for organ preservation and prevention of ischemia–reperfusion injury and graft coronary artery disease in cardiac transplantation.

Key Words: reperfusion • apoptosis • transplantation • arteriosclerosis • protein kinase C

One-year allograft survival and patient survival after cardiac transplantation continue to improve, principally because of progress in immunosuppressive therapy and in the diagnosis and treatment of acute rejection and infectious posttransplant complications. Beyond 1 year, however, the incidence of heart failure caused by graft coronary artery disease (GCAD) has not changed. Although GCAD is the leading cause of death in patients who survive >1 year after cardiac transplantation, the pathological processes leading to GCAD are not yet understood in detail.

Ischemia–reperfusion injury has been shown to be the strongest alloantigen-independent factor for the subsequent development of GCAD in a case-control study. Ischemia–reperfusion injury produces a pro-inflammatory environment, which includes an influx of injurious cytokines and chemokines and upregulation of adhesion molecules on the vascular endothelium, leading to microcirculatory failure followed by graft necrosis. Cardiomyocyte apoptosis is an early event in cardiac ischemia–reperfusion injury; therefore, reducing cardiomyocyte apoptosis should reduce cardiac damage in transplanted hearts.

Protein kinase C (PKC) family of highly homologous enzymes are involved in a variety of cellular functions. We showed that treatment with an εPKC-selective activator peptide, ψεRACK (receptor for activated C-kinase), before the ischemic event or with a δPKC-selective inhibitor peptide, δV1–1, early during reperfusion conferred cardioprotection to the ischemic heart. Moreover, we have recently found that combined treatment with these 2 PKC regulator peptides provides a greater cardioprotection against ischemia–reperfusion injury than the treatment with each peptide alone in isolated perfused rat hearts and transgenic mice.
showed that ePKC activation mimics ischemic preconditioning, whereas δPKC activation mediates damage induced by reperfusion of the ischemic organ. Here, we determined the effects of the ePKC activator and the δPKC inhibitor on ischemia–reperfusion injury and GCAD in the transplanted heart. Our hypothesis is that acute reduction of ischemia–reperfusion injury by treatment with these PKC regulators should result in reduction of GCAD and improvement of allograft function. Using these selective PKC isozyme regulator peptides, we tested this hypothesis in a murine cardiac allograft model.

**Methods**

**Animals**

Male FVB (H-2b) and C57BL/6 (H-2d) mice, 6 to 10 weeks old, were purchased from Jackson Laboratory (Bar Harbor, Me) and housed at the animal care facility at Stanford University Medical Center (Stanford, Calif). The FVB mice were used as allograft donors, and the C57BL/6 mice were used as recipients. All mice were kept under standard temperature, humidity, and timed lighting conditions and provided mouse chow and water ad libitum. Animals were treated in compliance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press (revised 1996).

**Heterotopic Cardiac Transplantation**

Heterotopic cardiac transplantation was performed according to the method of Corry et al9 with some modifications. Anesthesia was induced with 5% inhaled isoflurane (Halocarbon Laboratories, River Edge, NJ). During surgery, the animals were maintained on 2.5% inhaled isoflurane. Donor animals were systemically heparinized (50 mg/kg) before heart procurement. The donor heart was rapidly excised after coronary perfusion with ice-cold saline. The procured hearts were kept in ice-cold saline for 20 minutes. Because standard graft implantation averages ~30 minutes, the total ischemic time was 50 minutes.

**Drug Administration**

ePKC agonist (1μM RACK) was injected intraperitoneally (20 nmol) into the donor mice 20 minutes before heart procurement. During procurement, the donor hearts were perfused with 3 mL of 1μM RACK (1.5 nmol) through the inferior vena cava. The procured hearts were then submerged in the same drug solution (0.5 μmol/L) for 20 minutes at 4°C. Before reperfusion, the peritoneal cavity of recipients was irrigated with δPKC antagonist (6VR1–1; 300 nmol) solution. Control animals were treated with normal saline.

**Experimental Groups**

The study was performed in 2 parts. First, indicators of ischemia–reperfusion injury were analyzed after 2 hours of reperfusion (PKC regulator-treated versus control mice, n=6 each group). Second, GCAD was evaluated at 30 days (PKC regulator-treated versus control mice, n=7 each group). In the 30-day follow-up (chronic study), organ recipients in both PKC regulator-treated group and control group received daily cyclosporine A (20 mg/kg per day) by intraperitoneal injection.

**In Situ Oligo Ligation Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Analysis**

Apoptotic cardiomyocyte counts in allograft tissues were determined by in situ staining of DNA strand breaks in serial sections of each specimen with the use of an ApopTag in situ oligo ligation (ISOL) kit with oligo A (Intergen, Purchase, NY), as previously described.10 Because conventional TUNEL assay can detect nonspecific DNA fragmentation caused by necrosis, a more specific in situ ligation assay for identification of apoptotic nuclei was used with hairpin oligonucleotide probes. Cardiomyocyte apoptosis was confirmed by double-staining the sections with α-sarcosomic actin (Sigma, St. Louis, Mo). The number of TUNEL-positive cardiomyocyte in each cardiac allograft was counted manually by 2 investigators (R.D.T., G.K.M) blinded to the experimental conditions. Cells were counted in 6 animals (4 fields each) at ×200 magnification. The percentage of TUNEL-stained cells was recorded, ie, the number of labeled nuclei divided by total number of nuclei.

**Enzyme-Linked Immunosorbent Assay, Caspase Activity, and MPO Assays**

Intragraft tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), monocyte/macrophage chemotactic protein-1 (MCP-1/CCL2), interferon-γ (IFN-γ), Fas, Fas ligand (FasL), IFN-γ–induced protein-10 (IP-10/CXCL10), monokine induced by IFN-γ (MIG/CXCL9), intracellular adhesion molecule-1 (ICAM-1), and vascular cellular adhesion molecule-1 (VCAM-1) and caspase-8 and caspase-9 activity assay kits were obtained from R&D Systems. Caspase-3 activity assay kit was purchased from Clontech. MPO activity as units per milligram of total protein was assessed in lysates of reperfused cardiac allografts as previously described.11

**Graft Survival and Allograft Function Analyses**

Mice in the second part of this study were monitored daily. Graft viability was assessed by direct abdominal palpation of the heterotopically transplanted heart. Cardiac graft function was expressed as the beating score, assessed by the Stanford cardiac surgery laboratory graft scoring system (0, no contraction; 1, contraction barely palpable; 2, obvious decrease in contraction strength, but still contracting in a coordinated manner; rhythm disturbance; 3, strong, coordinated beat but noticeable decrease in strength or rate and distention/stiffness; 4, strong contraction of both ventricles, regular rate, no enlargement or stiffness).

**Morphometric Analysis of GCAD**

At 30 days after transplantation, the cardiac grafts were harvested and embedded in paraffin. Elastica von Gieson staining was performed for morphometric analysis of arterial intimal proliferation, which was performed as described by Armstrong et al.12 Briefly, the neointima, media, and lumen were measured (SPOT Advanced Version 3.4.2 software; Diagnostic Instruments, Inc), with the neointima defined as the area bound by the internal elastic lamina and the lumen, the media as the region between the internal and external elastic membranes, and the lumen as the clear region in the vessel. Diseased vessels were identified as those with >10% luminal narrowing. Multiple sections from the middle of the heart were used for analysis. Middle-sized coronary arteries were analyzed (>8 arteries for each graft).

**Statistical Analysis**

Values are expressed as mean±SD. All comparisons shown are between PKC regulator-treated group and saline-treated control group. Differences in values were analyzed statistically by the unpaired Student t test and the differences in cardiac graft beating score were analyzed by 2-way repeated-measures ANOVA (StatView 5.0; SAS Institute). Significance was accepted at P<0.05.

**Results**

**Treatment With PKC Regulators Suppresses Cardiomyocyte Apoptosis, Necrosis, and Inflammation Caused by Ischemia–Reperfusion Injury in Cardiac Allografts**

We recently found that the ePKC activator exerts cardioprotection from cardiac ischemia when administered before the ischemic event and the δPKC inhibitor is cardioprotective...
when administered at the onset of reperfusion. Therefore, the donor mice were treated with the PKC activator before and during organ harvest and the recipient mice were treated with the PKC inhibitor before reperfusion.

Ischemia–reperfusion injury causes cardiomyocyte apoptosis in the cardiac grafts. Two hours after transplantation, ISOL TUNEL-positive apoptotic cardiomyocyte significantly decreased by \( \approx 65\% \) in cardiac allografts of the PKC regulator-treated group compared with that of the control group (Figure 1A). A corresponding decrease in caspase-3 and caspase-9 activities was also found in the PKC regulator-treated group when compared with the control group (Figure 1B and 1D). However, there was no significant difference in caspase-8 activity between these 2 groups (Figure 1C). Fas ligand expression significantly decreased by \( \approx 75\% \) in the cardiac allograft of PKC regulator-treated group (Figure 1E), whereas Fas expression did not differ between these 2 groups (Figure 1F). These results suggest that treatment with the PKC regulator inhibits cell apoptosis mediated by caspase-3–dependent and caspase-9–dependent pathway.

Ischemia–reperfusion injury produces a pro-inflammatory environment, which includes an influx of injurious cytokines and chemokines as well as increased expression of adhesion molecules on the vascular endothelium, leading to microcirculatory failure followed by graft necrosis. We therefore determined whether treatment with these PKC regulators reduces the inflammatory response after transplantation. We examined neutrophil-produced MPO, because neutrophils are known as predominant effector cells in the local inflammatory response. We also determined the levels of the pro-inflammatory cytokines and chemokines, TNF-\( \alpha \), IL-1\( \beta \), and MCP-1/CCL2. The levels of MPO and the tested pro-inflammatory cytokines were all significantly lower in the cardiac allografts of the PKC regulator-treated group as compared with the control group 2 hours after transplantation (Figure 2A to 2D). In addition, the levels of ICAM-1 and VCAM-1 in the cardiac allografts were also significantly decreased in PKC regulator-treated group compared with control group at the time tested (Figure 2E, 2F). Finally, the serum levels of CPK-MB were \( \approx 75\% \) lower in the PKC regulator-treated group compared with control group, indicating decreased cardiac graft necrosis (Figure 2G). Taken together, these results suggest that treatment with the PKC regulators inhibits cell apoptosis mediated by caspases and inflammation in the early phase after ischemia–reperfusion injury to cardiac allografts.

**Treatment With PKC Regulators Improves Cardiac Allograft Function and Reduces Local Cytokine Production and GCAD**

We next examined whether the reduction in ischemia–reperfusion injury early after transplantation results in improved cardiac allograft function and limits the development of GCAD over time. We first assessed cytokine production at 30 days after transplantation and found that production of IFN-\( \gamma \), the chemokines MCP-1/CCL2, IP-10/CXCL10, and MIG/CXCL9, and the expression of adhesion molecules ICAM-1 and VCAM-1 were all significantly lower in the cardiac allograft of the PKC regulator-treated group compared with control group at 30 days after transplantation (Figure 3). Importantly, graft beating scores were significantly better in the PKC regulator-treated group at both 20 and 30 days after transplantation (Figure 4). Marked fibrointimal thickening and luminal narrowing, morphologically re-
sembling typical human GCAD, were observed in the control group. In contrast, less intimal thickening and preserved vessel lumen were observed in the PKC regulator-treated group (Figure 5A). Finally, GCAD, assessed by the mean percentage of luminal narrowing, the intima-to-media ratio, and the percentage of diseased vessels, was significantly inhibited in the PKC regulator-treated group compared with the control group (n=7). Treated=PKC regulator-treated group (n=7).

**Discussion**

The goal of this study was to determine whether inhibition of ischemia–reperfusion injury by a brief treatment with εPKC activator and δPKC inhibitor during tissue procurement and transplantation would reduce GCAD in murine cardiac allografts. We recently found that treatment with the εPKC-selective activator, ψεRACK, before ischemia followed by treatment with the δPKC-selective inhibitor, δV1–1, at the onset of reperfusion protects murine heart from ischemia–reperfusion injury, as determined ex vivo. We therefore reasoned that if ischemia–reperfusion injury contributes to activation of the immune response and aggravates GCAD, then activation of εPKC before and during organ procurement and inhibition of δPKC before and early during reperfusion of the transplanted heart in the recipient should improve the outcome of cardiac transplantation. The findings described agree with this prediction: we found that treatment with these PKC-selective regulators reduced acute cytokine production (measured 2 hours after transplantation) and cardiomyocyte necrosis and apoptosis. Importantly, this treatment resulted in improved cardiac function and reduced coronary artery disease in the allograft. We therefore suggest that inhibition of ischemia–reperfusion injury reduced production of inflammatory cytokines, chemokines, and adhesion molecules in the early phase after transplantation, which in turn led to reduction of GCAD in the chronic phase.

Based on the studies here and our recent published studies, we suggest that the combined treatment with εPKC-specific activator and δPKC-specific inhibitor decreases ischemia-reperfusion injury to the allograft by 2 distinct means: an ischemic preconditioning mimetic effect of the εPKC activator, given to the donor before organ harvest and during organ procurement, and an anti-apoptotic effect of the δPKC inhibitor, given to the recipient just before the onset of reperfusion of the transplanted heart.

Apoptosis involves a complex signal transduction events including the mitochondria disruption-mediated stress pathway, on one hand, and the Fas and TNF receptor-mediated death receptor pathway, on the other. The mitochondria disruption-mediated stress pathway involves the release of...
cytochrome c from the mitochondria into the cytosol and subsequent caspase-9 and caspase-3 activation, whereas the Fas and TNF receptor-mediated death receptor pathway leads to caspase-8 and then caspase-3 activation.15 In the present study, caspase-3 and caspase-9 activities were 60% to 90% lower in PKC regulator-treated grafts during ischemia–reperfusion injury, whereas caspase-8 activation was unchanged. In addition, FasL levels decreased by ∼75%. Thus, it appears that under the treatment of PKC regulators, cardiomyocyte apoptosis is reduced mainly by inhibition of the caspase-9–mediated pathway.

We observed >50% reduction in GCAD 30 days after transplantation in animals treated with the PKC regulating peptide just during the transplantation procedure. It is highly unlikely that the peptides remain active to exert an effect in the chronic phase, because of their short half-life in vivo (unpublished data). We therefore suggest that the reduction in GCAD observed in the chronic phase is caused mainly by reduction of ischemia–reperfusion injury in the early phase after transplantation. In support of this suggestion, we found a significant decrease in IFN-γ and related chemokines production in the chronic phase.

Both apoptosis and necrosis caused by ischemia–reperfusion injury are thought to induce cytokine production by endothelial cells as well as other damaged cells. These cytokines lead to infiltration of inflammatory cells (eg, neutrophils and macrophages) into the graft and infiltration of CD4+ and CD8+ cells; the cytokines also stimulate these inflammatory cells to secrete additional pro-inflammatory cytokines, including IFN-γ and MCP-1. The association of IFN-γ with GCAD is supported by studies showing lack of GCAD in IFN-γ–knockout recipients16 and the fact that IFN-γ directly causes vascular remodeling and intimal proliferation in the absence of immune cells.17 In addition, we found a significant decrease in production of IFN-γ–related chemokines, such as IP-10/CXCL10 and MIG/CXCL9 in the chronic phase, which contribute substantially to GCAD because of their strong chemotraction to antigen-primed T cells.18 Thus, interstimulation of IFN-γ and IFN-γ–related chemokines elaborates the immune response, and thus contributes to the development of GCAD. We also found a significant decrease in MCP-1/CCL2 production, a potent chemokine secreted by activated endothelial and vascular smooth muscle cells as well as monocyte/macrophages in cardiac allografts, which contributes to the accumulation of these inflammatory cells within the expanding neointima.19 Therefore, decreased production of pro-inflammatory stimuli may result not only in inhibition of immune cell infiltration to the allograft, but may also inhibit processes directly leading to GCAD development.

Finally, we also observed a significant decrease in the production of both ICAM-1 and VCAM-1 during ischemia–reperfusion injury and in the chronic phase in mice treated with selective regulators of δPKC and ePKC. Such activation of endothelial cells provides the proper milieu for recruitment of pro-inflammatory cells and subsequent development of GCAD. Furthermore, TNF-α and MCP-1/CCL2 released from T cells, macrophages, and other cells dramatically increase ICAM-1 and VCAM-1 expression on allograft endothelial cells.20 Treatment with anti–VCAM-1 antibody induces long-term acceptance of murine cardiac allografts and treatment with anti–ICAM-1 antibody inhibits GCAD in rats.21,22 We therefore suggest that these adhesion molecules, which facilitate transmigration of inflammatory and immune cells to the graft, contribute to the development of GCAD. Accordingly, the reduced ICAM-1 and VCAM-1 production during ischemia–reperfusion injury and in the chronic phase may relate to decreased GCAD.

This study used only one protocol of treatment with the ePKC and δPKC regulators. Studies that address the time- and dose-dependent effects of these regulators and the effect of each regulator treatment alone on GCAD are needed. In addition, we determined the beneficial effects obtained by treatment with these peptides only after a 50-minute ischemia to the allograft. According to The Registry of International Heart and Lung Transplantation, the mean ischemic time of donor heart in clinical transplantation is 3.1 hours. Therefore, studies using prolonged ischemic time are required to better-mimic the clinical situation. Nevertheless, the obtained results in this study are encouraging and suggest a therapeutic potential for the ePKC activator and the δPKC inhibitor.
during organ procurement and early reperfusion of the transplanted organs, respectively, to improve both the short-term and long-term function of cardiac allografts.

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