PKC-ε-dependent survival signals in diabetic hearts

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Diabetes mellitus is complicated by the development of a primary cardiomyopathy, which contributes to the excess morbidity and mortality of this disorder. The protein kinase C (PKC) family of isozymes plays a key role in the cardiac phenotype expressed during postnatal development and in response to pathological stimuli. Hyperglycemia is an activating signal for cardiac PKC isozymes that modulate a myriad of cell events including cell death and survival. The ε-isozyme of the PKC family transmits a powerful survival signal in cardiac muscle cells. Accordingly, to test the hypothesis that endogenous activation of cardiac PKC-ε will protect against hyperglycemic cell injury and left ventricular dysfunction, diabetes mellitus was induced using streptozotocin in genetically engineered mice with cardiac-specific expression of the PKC-ε translocation activator [ε-receptors for activated C kinase (ε-RACK)]. The results demonstrate a striking PKC-ε cardioprotective phenotype in diabetic ε-RACK mice that is characterized by inhibition of the hyperglycemia apoptosis signal, attenuation of hyperglycemia-mediated oxidative stress, and preservation of parameters of left ventricular pump function. Hearts of diabetic ε-agonist mice exhibited selective trafficking of PKC-ε to membrane and mitochondrial compartments, phosphorylation/inactivation of the mitochondrial Bad protein, and inhibition of cytochrome c release. We conclude that activation of endogenous PKC-ε in hearts of diabetic ε-agonist mice promotes the survival phenotype, attenuates markers of oxidative stress, and inhibits the negative inotropic properties of chronic hyperglycemia.

hyperglycemia; diabetes mellitus; protein kinase C; isozymes; translocation activator; cardioprotection

STRATEGIES THAT INTERRUPT OR SUPPRESS INITIATION OF THE APOPTOSIS PROGRAM OFFER A NOVEL APPROACH TO PRESERVE CELL NUMBER AND ORGAN FUNCTION. MULTIPLE LINES OF EVIDENCE SUPPORT THE CONTENTION THAT DIABETES MELLITUS (DM) IS A RISK FACTOR FOR DEVELOPMENT OF CARDIOVASCULAR DISEASE (17, 24). IN ADDITION, THIS DISORDER IS COMPROMISED BY A PRIMARY CARDIOMYOPATHY IN WHICH Apoptotic myocyte cell death and progressive deterioration of left ventricular (LV) contractile parameters result in heart failure (5, 40). AN EMERGING AREA OF CELL BIOLOGY INVOLVES THE APPLICATION OF GENE-BASED STRATEGIES TO INHIBIT PROGRAMMED CELL DEATH (5, 30). AN EMERGING AREA OF CELL BIOLOGY INVOLVES THE APPLICATION OF GENE-BASED STRATEGIES TO INHIBIT PROGRAMMED CELL DEATH (5, 40). THE GLOBAL OBJECTIVE OF THIS APPROACH IS TO PRESERVE OR RESTORE CELL NUMBER AND PREVENT OR ATTENUATE REMODELING. ACTIVATION OF THE PROTEIN KINASE C (PKC) FAMILY OF SERINE/THREONINE KINASES HAS BEEN WELL DOCUMENTED IN DIABETIC MYOCARDIUM (20, 31) AND IS IMPLICATED IN THE MODULATION OF NUMEROUS BIOLOGICAL RESPONSES (38, 41, 42).

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mouse oocytes (35). Myosin heavy chain-FLAG ε-ε-RACK founders were identified by genomic Southern analysis of tail-clip DNA.

**Induction of DM with STZ.** Nontransgenic (NTG) and ε-ε-RACK (ε-agonist) mice at 3 mo of age were injected with STZ (200 mg/kg of body wt ip) dissolved in a citrate-saline buffer. Mice were entered into the diabetic arm of the Euglycemic control NTG and ε-agonist) mice at 3 mo of age were injected with STZ (200 mg/kg (ε-agonist). Early genetic studies indicated that the ε-agonist promoter was expressed in the heart, but not in the liver or skeletal muscle (27). More recently, we have shown that the ε-agonist promoter is active in cardiac myocytes (28). Briefly, cardiac muscle was homogenized in 0.5 ml of buffer A that contained 20 mM Tris-HCl, 0.33 M sucrose, 2 mM EGTA, 2 mM EDTA (pH 7.5), 0.1 mM sodium vanadate, 20 mM NaF, 20 μM leupeptin, 200 mM PMSF, a protease inhibitor cocktail set (types I and III), 5 mM DTT, and phosphatase inhibitors calyculin A (1 nM) and okadaic acid (5 nM). The cytosolic and membrane fractions were separated by high-speed centrifugation (40,000 g). To quantify the immunoreactivity of total PKC isoforms, the cardiac muscle was homogenized in buffer A in the presence of 0.1% Triton X-100, and supernatant was collected. The samples were processed for SDS-PAGE and subsequent immunoelectrophoresis.

**Immunoblotting of PKC fractions.** Protein samples (15–20 μg; 2 mg/ml) were separated by SDS-PAGE (Bio-Rad) using an 8 or 10% (wt/vol) acrylamide gel (27, 31). Proteins were transferred to nitrocellulose membranes using a Semi-Dry Transfer Cell (Bio-Rad). Nonspecific sites were blocked with 5% nonfat milk, and membranes were incubated with primary rabbit polyclonal antibodies against PKC-ε (Santa Cruz Biotechnology) and PKC-δ (GIBCO-BRL Life Technologies) at dilutions of 1:500 and 1:1,000, respectively, in the blocking solution before being washed and treated with horseradish peroxidase-linked secondary antibodies at dilutions of 1:5,000 and 1:10,000, respectively (Boehringer Mannheim). The bound antibody was detected by autoradiography using an enhanced chemiluminescence (ECL) kit (Amersham). The specificity of the antibody was confirmed by incubation with the appropriate blocking peptide. Densitometric analysis for the translocation of PKC isozymes was performed using Quantity One image-analysis software (Bio-Rad).

**Preparation of subcellular fractions in PKC mice.** Mouse hearts were fractionated into cytosol and mitochondria by differential centrifugation as described (4). Briefly, hearts were homogenized with a tissue grinder (Tekmar) in a buffer that contained (in mM) 250 sucrose, 10 Tris-HCl, pH 7.4, 2 EDTA, 1 Na3VO4, 10 NaF, and protease inhibitor cocktail I and were centrifuged at 700 g for 10 min at 4°C. The pellet was discarded, and the supernatant was further centrifuged at 10,000 g for 25 min at 4°C. The supernatant (cytosolic fraction) and pellet (mitochondrial fraction) were collected separately, and protein concentrations in these fractions were determined by Bio-Rad assay.

**Immunoblotting of PKC subcellular fractions.** Anti-cytochrome c oxidase subunit IV (COX IV) monoclonal antibody (1:1,000 dilution; Molecular Probes) was used to probe the protein COX IV as a marker in mitochondria, and monoclonal Akt1 antibody (1:500 dilution; Santa Cruz Biotechnology) was used as a marker of cytosol in Western blot analysis. Catalase and superoxide dismutase (SOD) activities were determined by immunoblot analysis using specific anti-catalase and anti-SOD antibodies (1:1,000 dilution; Calbiochem). Fractions obtained from cytosolic and mitochondrial compartments were also probed with cytochrome c antibody by immunoelectrophoresis.

**Statistical analysis.** Data are expressed as means ± SE. Comparisons between two values were performed by unpaired Student’s t-test. For multiple comparisons among different groups of data, the significant differences were determined by the Bonferroni method (44). Significance was defined at P < 0.05.

### RESULTS

**Physiological and hemodynamic parameters of NTG and ε-agonist mice.** The characteristics of control and experimental groups are shown in Table 1. Twelve weeks after induction of DM with STZ, blood glucose values were three- to fourfold higher in NTG and ε-agonist mice. Heart rates were higher in the diabetic ε-agonist mice; however, experimental and control groups did not differ with respect to body weight, heart weight, heart weight-to-body weight ratio, mean blood pressure (MBP), or LV systolic pressure (LVSP). PKC-ε has been well-characterized by immunoblotting and functional analyses, and the expression of PKC-ε is upregulated in response to various stimuli, including diabetes. In the present study, we demonstrated that PKC-ε is increased in diabetic hearts, and that targeting PKC-ε decreases cardiac remodeling and improves cardiac function in diabetic mice. These findings support the role of PKC-ε in diabetes-induced cardiac dysfunction and provide a potential therapeutic target for the treatment of diabetic heart disease.

### Table 1. Characteristics of control and experimental groups

<table>
<thead>
<tr>
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<th>Nontransgenic Mice</th>
<th>ε-AGONIST Mice</th>
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<tbody>
<tr>
<td></td>
<td>Euglycemic Control</td>
<td>STZ-Induced Diabetic</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>30±1.2</td>
<td>28±1.2</td>
</tr>
<tr>
<td>Heart wt, mg</td>
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<td>Heart rate, beats/min</td>
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<td>238±19</td>
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<tr>
<td>Blood glucose, mg/dl</td>
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<td></td>
<td>+d/dt, mmHg/g</td>
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<td></td>
<td>−d/dt, mmHg/g</td>
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<tr>
<td>LV systolic pressure, mmHg</td>
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<td>76±5</td>
</tr>
<tr>
<td>Mean blood pressure, mmHg</td>
<td>59±10</td>
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</table>

Values are means ± SE for 6 or 7 independent observations. Diabetes mellitus was induced using streptozotocin (STZ) in genetically engineered mice with cardiac-specific expression of ε-ε-receptors for activated C kinase (RACK; ε-agonist). +d/dt, rate of pressure rise; −d/dt, rate of pressure decay; LV, left ventricular. *P < 0.05 vs. control nontransgenic mice.
reported to modulate the contractile properties of cardiac muscle cells (14, 25, 35), but whether sustained activation of endogenous PKC-ε offsets the negative inotropic properties of chronic hyperglycemia has not been determined. Accordingly, left heart catheterization was performed in NTG and ε-agonist mice to evaluate the effects of hyperglycemia on LV pump function. As shown in Table 1, the rates of pressure rise (+dP/dt) and decay (−dP/dt) were decreased by 23–27% (P ≤ 0.05) in NTG diabetic mice. Conversely, cardiac-specific expression of ε-RACK in ε-agonist diabetic mice prevented deterioration of the LV contractile parameters. Taken together, cardiac-specific expression of the ε-RACK peptide prevents the negative inotropic effect of chronic hyperglycemia in ε-agonist mice.

Effects of ε-RACK peptide on hyperglycemia-induced apoptosis. We next asked whether cardiac-specific expression of the ε-RACK peptide inhibits hyperglycemia-induced apoptosis. After 12 wk of hyperglycemia, the percentage of TUNEL-positive nuclei was significantly greater (Fig. 1) in hearts of diabetic NTG mice (threefold over nondiabetic mice). In contrast, the percent of TUNEL-positive nuclei did not increase above baseline in hearts from diabetic ε-agonist mice. Parallel investigations in genetically engineered mice with cardiac-specific expression of the translocation inhibitor (ε-V1) could not be performed due to excess mortality (85–90%). Although not documented here, previous investigations (35) indicate lethal cardiomyopathy phenotype as the cause of death in ε-V1 mice. Taken together, cardiac-specific expression of ε-RACK is cardioprotective in mice with DM.

Cardiac-specific expression of ε-RACK peptide attenuates hyperglycemia-induced oxidative stress. Hyperglycemia and DM are associated with an exponential increase in superoxide anion (O2•−) production (21, 36). Reactive oxygen species (ROS) are important mediators of biological responses including cell death by apoptosis (29). Cu/Zn-SOD and catalase expression are known to upregulate in response to oxidant stress (22, 32, 37, 39). Accordingly, Cu/Zn-SOD and catalase were examined by immunoblot analysis in lysates prepared from hearts of NTG and ε-agonist mice (Fig. 2, A and B). Expression of Cu/Zn-SOD and catalase was increased in NTG diabetic hearts, whereas chronic hyperglycemia did not up-regulate expression of Cu/Zn-SOD or catalase in hearts of diabetic ε-agonist mice. Taken together, cardiac muscle cells of ε-agonist diabetic mice do not exhibit a compensatory increase in the oxidant-response genes Cu/Zn-SOD and catalase.

Effects of cardiac-specific expression of ε-RACK peptide on PKC isozyme redistribution. An essential component of the PKC-ε signaling cascade is the translocation to anchoring proteins located in distinct subcellular compartments (34). To evaluate the in vivo specificity of the ε-RACK peptide and its effect on PKC-ε trafficking, immunoblot analyses were performed on cytosolic, membrane, and mitochondria-enriched fractions of hearts from NTG and ε-agonist mice. As shown in Fig. 3, top, PKC-ε immunoreactivity was increased in membrane fractions prepared from hearts of NTG and ε-agonist diabetic mice. The upregulation of PKC-ε was most apparent in ε-agonist diabetic mice. Conversely, PKC-δ immunoreactivity (Fig. 3, bottom) was downregulated in ε-agonist diabetic mice, whereas PKC-δ increased in the membrane fraction of NTG diabetic hearts. The data indicate that in ε-agonist mice, chronic hyperglycemia has opposing effects on the translocation of PKC-ε and PKC-δ to the membrane fraction of cardiac muscle cells.

Mitochondria are critical determinants of cell death and survival (13). To determine whether the ε-RACK peptide traffics PKC-ε to the mitochondrial compartment, cytosolic and mitochondria-enriched subfractions were prepared from NTG and ε-agonist hearts. To confirm the absence of contaminants, cytosolic and mitochondrial lysates were probed for the cytosolic protein Akt1 and the mitochondria-specific marker COX IV (Fig. 4A). Akt1 was detected in cytosol; however, COX IV was absent. Conversely, COX IV was present in the mitochondrial subfraction, but Akt1 was not detected. As shown in Fig. 4, B and C; PKC-ε immunoreactivity in mitochondrial lysates from NTG diabetic hearts was decreased by 50% (P ≤ 0.05), whereas PKC-ε content was preserved in mitochondria from ε-agonist hearts. Total PKC-ε content remained constant in all groups (Fig. 4B). An identical analysis for PKC-δ did not detect an alteration in the mitochondrial content of PKC-δ (NTG mice: control, 29 ± 3; diabetic, 24 ± 4; ε-agonist mice: control, 21 ± 1; diabetic, 26 ± 3 arbitrary units; n = 4). Taken together, cardiac-specific expression of ε-RACK selectively traffics PKC-ε to the membrane and...
mitochondrial compartments of cardiac muscle cells in diabetic mice.

Effects of cardiac-specific expression of ε-ε-RACK peptide on phospho-ERK activity in mitochondria. PKC-ε has been reported to form signaling modules with the ERK subfamily of MAPKs (18), which have been implicated in the activation of the mitochondrial survival program (3, 4). To determine whether the trafficking of PKC-ε to the mitochondrial compartment by ε-ε-RACK is coupled to increased ERK activity, mitochondrial subfractions of cardiac tissue were probed with phospho-ERK antibody. As shown in Fig. 5, A and B, phospho-ERK content was decreased in mitochondria from NTG diabetic hearts, whereas phospho-ERK content was preserved in mitochondria from hearts of ε-agonist mice. Total ERK and COX IV contents remained constant for all groups. Of note, phospho-ERK activity in mitochondrial lysates paralleled that of mitochondrial PKC-ε content (Fig. 4), which is indicative of the formation of functional PKC-ε-ERK signaling complexes. We next asked whether differences in phospho-ERK activity detected in mitochondria from NTG and ε-agonist hearts was linked to the expression of the survival program. The proapoptosis protein Bad is a target for PKC-ε-ERK signaling modules (4, 23). Accordingly, the phosphorylation status of Bad at Ser112 was determined in mitochondrial lysates from NTG and diabetic groups. As shown in Fig. 6, A–C, phosphorylation of Bad at Ser112 was decreased by threefold in mitochondrial lysates from NTG and ε-agonist hearts. Total Bad content remained constant in all groups (Fig. 6D). Taken together, the induction of DM with STZ in genetically engineered mice with cardiac-specific expression of ε-ε-RACK peptide results in the formation of PKC-ε-ERK signaling modules that target Ser112 of the Bad protein to promote the survival phenotype.

Effects of cardiac-specific expression of ε-ε-RACK peptide on cytochrome c release. The release of cytochrome c from mitochondria is tightly regulated by protein-protein interactions among Bcl-2, Bax, and Bad (13). Phosphorylation of the Bad protein increases the availability of the antiapoptotic Bcl-2 and Bcl-xL (13, 16, 46). To determine whether the increased phospho-Bad content of ε-ε-RACK hearts is correlated with inhibition of the mitochondrial apoptosis program, immunoblots were performed on cytosolic fractions to detect the release of cytochrome c from mitochondria. As shown in Fig. 7A, chronic hyperglycemia induced a twofold increase in cytochrome c content of NTG hearts, whereas the ε-ε-RACK peptide prevented hyperglycemia-induced cytochrome c release. Total cytochrome c content (Fig. 7B) remained constant in all groups. Taken together, the aggregate data indicate that cardiac-specific expression of ε-ε-RACK peptide activates a survival program in hearts of diabetic mice that is characterized by the phosphorylation and/or inhibition of Bad protein and the prevention of hyperglycemia-induced cytochrome c release.

DISCUSSION

In the present study, we demonstrate that cardiac-specific expression of the PKC-ε translocation activator ε-ε-RACK (an ε-agonist) protects cardiac muscle cells from hyperglycemia-induced apoptosis signals. The survival phenotype was coupled with preserved parameters of LV function and resistance to hyperglycemia-mediated oxidative stress. Finally, we demonstrate that PKC-ERK signaling modules at the level of mitochondria preserved the integrity and function of this critical organelle via phosphorylation and/or inactivation of Bad and inhibition of hyperglycemia-induced cytochrome c release.

Cardiac-specific expression of ε-ε-RACK activates survival program in diabetic myocardium. Hyperglycemia has been identified as a potent stimulus for apoptosis in cardiac muscle...
cells (10, 21, 43). In vitro and in vivo studies have identified PKC-ε as an essential signaling element in the expression of the cardioprotective phenotype (4, 29, 35). Activation-induced translocation of PKC isozymes is postulated to be the primary determinant of isozyme-specific function. The unique binding interactions of individual PKC isozymes with their respective RACKs has made it possible to design isozyme-specific peptides that may induce or inhibit kinase activity (29, 35). A decided advantage of this approach is the selective modification of PKC activity in an isozyme-specific manner. The octopeptide \( /H9274^\varepsilon\)RACK was developed as the first isozyme-selective PKC agonist (9). Here we report for the first time that endogenous activation of PKC-ε by the \( /H9274^\varepsilon\)RACK peptide interrupts the death signal of chronic hyperglycemia. Moreover, this effect was all the more impressive when viewed in the context of a modest 20% increase in active PKC-ε in \( /H9274^\varepsilon\)RACK mice (35). An important limitation of the present study is the absence of an experimental group with cardiac-specific expression of the peptide PKC-ε translocation inhibitor ε-V1. The development of severe cardiomyopathy in mice

Fig. 4. Characterization of subcellular fractions (A). Immunoblots were performed with cytosolic and mitochondrial lysates from NTG and ε-agonist control and diabetic murine hearts. Blots were probed with monoclonal Akt1 and monoclonal cytochrome c oxidase type IV (COX IV) antibodies. Effects of chronic hyperglycemia on mitochondrial PKC-ε expression are shown (B). Immunoblots were performed with isozyme-specific PKC-ε antibody in mitochondrial lysates from NTG and ε-agonist control and diabetic murine hearts. Blots were probed with PKC-ε antibody. PKC-ε immunoreactivity in mitochondrial isolates (A, top) and total PKC-ε content (A, bottom) are shown. Densitometric analysis was performed of mitochondrial PKC-ε expression (C). *P < 0.05; n = 4 independent observations.

Fig. 5. Effects of hyperglycemia on mitochondrial ERK expression. Immunoblots were performed with cytosolic and mitochondrial lysates from NTG and ε-agonist control and diabetic murine hearts. Blots were probed with phospho-ERK (1:1,000 dilution), ERK (1:1,000 dilution), and COX IV (1:1,000 dilution) antibodies (A). Densitometric analysis of mitochondrial phospho-ERK/ERK expression is shown (B). *P < 0.05 vs. control; n = 3 independent observations.

Fig. 6. Effects of hyperglycemia on mitochondrial phospho-Bad expression. Representative immunoblot of phospho-Bad (Ser112) in mitochondrial lysates from NTG and ε-agonist control and diabetic murine hearts (A). Total Bad content in NTG and ε-agonist hearts (B) and ratio of phospho-Bad/Bad in NTG and ε-agonist hearts (C) are shown. *P < 0.05; n = 5 independent observations.
expressing the ε-V1 genotype (35) precluded analysis of this group. However, recent investigations in our laboratory strongly support the notion of PKC-ε-dependent cardioprotection in DM. PKC peptides were delivered to primary cultures of serum-starved adult rat ventricular myocytes by conjugating peptides to the homeodomain of Drosophila antennapedia (9, 29). As expected, hyperglycemia induced a 35% increase in apoptosis in adult rat ventricular myocytes. Peptide inhibitors of PKC-β1/-β2 and -ζ blocked transmission of the hyperglycemia apoptosis signal, whereas the isoform-specific inhibitor of PKC-ε (ε-V1-2) did not alter the magnitude of apoptosis (29). Alternatively, ε-RACK abolished hyperglycemia-induced apoptosis, which is indicative of a cardioprotective role for PKC-ε in this system. Taken together, the cumulative data are consistent with the induction of a novel PKC-ε-dependent survival program in hearts of diabetic ε-RACK mice.

Cardiac-specific expression of ε-RACK peptide protects against hyperglycemia-induced LV dysfunction. DM is complicated by a primary cardiomyopathy that contributes to the excess morbidity and mortality in this disorder. Multiple factors including impaired transmission of β-adrenergic receptor signals (8, 11), oxidative stress (21), and activation of the cardiac renin-angiotensin system (21, 27, 31) have been implicated as causal factors in the progressive deterioration of LV pump function. The present study is the first report in an in vivo model to document that activation of endogenous PKC-ε preserves the mechanical properties of cardiac muscle cells in diabetic mice. The molecular basis for this cardioprotection was not investigated here. However, the sarcomeric protein troponin I (TnI) is a known substrate for PKC isozymes (27), and PKC-ε and PKC-β2 have been reported to preferentially target TnI in diabetic myocardium (31). This modification of the TnI protein is known to be coupled with changes in myofilament Ca2+ sensitivity (27, 30), which may have been operative in the hearts of diabetic ε-RACK mice. Alternatively, as might be anticipated, strategies that enhance β-adrenergic receptor signaling in diabetic myocardium may offset the negative inotropic state associated with chronic hyperglycemia. Recent investigations in our laboratory (28) in which DM was induced with STZ in genetically engineered mice with cardiac-specific overexpression of Goα document the salutary effect of targeted expression of the hyperadrenergic state on LV contractile performance. Taken together, the PKC-ε survival program was coupled with protection of LV contractile performance in ε-agonist diabetic mice. Future investigations will be directed at the downstream effectors by which activated PKC-ε modules contractile performance.

Cardiac-specific expression of ψε-RACK and hyperglycemia-induced oxidative stress. Multiple lines of evidence have established a role for ROS as important mediators of cell biology including cell death by apoptosis (13, 22, 23, 45). The antioxidant enzymes Cu/Zn-SOD and catalase protect cells from attack by ROS and have been used as surrogate markers of oxidative stress (19, 22, 33, 39). In the present study, the increased expression of Cu/Zn-SOD and catalase in hearts of NTG diabetic mice is suggestive of increased ROS production triggered by hyperglycemia (21, 22). Conversely, in ε-agonist diabetic mice, hyperglycemia did not induce a compensatory increase in the oxidant stress-response genes Cu/Zn-SOD and catalase. Taken together, cardiac-specific expression of ψε-RACK peptide prevented the induction of surrogate markers of oxidant stress in diabetic mice.

Chronic hyperglycemia, ψε-RACK, and compartmentalization of PKC-ε. An essential feature of PKC-ε cardioprotection is redistribution to subcellular compartments (4). The ψε-RACK peptide induced a robust increase of PKC-ε immunoreactivity in membrane fractions prepared from hearts of diabetic mice. PKC-δ immunoreactivity did not change significantly in these samples, which is an indicator of the specificity of the translocation activator for the ε-isozyme.

Mitochondria have received considerable attention in the development of cardioprotection, and this critical organelle is a target for PKC-ε-dependent signals (3, 4). Interestingly, trafficking of PKC-ε to mitochondria was decreased by threefold in hearts from diabetic NTG mice, whereas the ψε-RACK peptide prevented the hyperglycemia-induced decrease in mitochondrial PKC-ε immunoreactivity. Taken together, cardiac-specific expression of the ψε-RACK peptide facilitates the translocation of PKC-ε to distinct subcellular compartments, which is a pivotal event in the induction of the cardioprotection phenotype.

Mitochondrial PKC-ε and phospho-ERK activity. Recent work has provided evidence that cardiac PKC isozymes form signaling modules with subfamilies of MAPK cascades and by this mechanism modulate a wide variety of biological responses (4, 7, 18). The formation of signaling modules between PKC-ε and the ERK subfamily of MAPK has been linked to the activation of the survival program in cardiac muscle cells (4). In the present study, NTG diabetic hearts exhibited a decrease in mitochondrial PKC-ε immunoreactivity and phospho-ERK activity. Conversely, cardiac-specific expression of the ψε-RACK peptide prevented the hyperglycemia-induced decrease in mitochondrial PKC-ε immunoreactivity and phospho-ERK activity. Although ERKs, JNKs, and p38 MAPKs are expressed in cardiac mitochondria (4), only PKC-ERK signaling modules have been shown to induce cardioprotection by activating a survival program in mitochondria (4). Taken together, cardiac-specific expression of ψε-RACK in...
diabetic mice prevented hyperglycemia-induced decreases in the mitochondrial trafficking of PKC-ε and phospho-ERK activity.

PKCe-ERK modules phosphorylate Bad and inhibit hyperglycemia-induced cytochrome c release. The proapoptotic protein Bad is a known substrate for PKC-ε and ERK (4, 23). PKC-ε-ERK-dependent signals inactivate the Bad protein by phosphorylating Ser112 (4). The phosphorylated Bad protein is incapable of forming heterodimers with Bcl-2 and Bel-xL, which increases the availability of Bcl-2 for antioxidant and antiapoptotic functions (16, 45). In the present study, immunoblot analysis of mitochondrial lysates from ε-agonist diabetic hearts revealed sustained levels of PKC-ε, phospho-ERK, and phospho-Bad. Parallel investigations on diabetic NTG hearts demonstrate a striking decrease in the expression of each of these proteins. We speculate that a dynamic equilibrium exists between cytosolic and mitochondrial stores of phospho-Bad and Bad. The dephosphorylated Bad protein translocates from the cytosol to mitochondria, where it is either phosphorylated/inactivated or it participates in the apoptosis pathway (45, 46). Our data indicate that phospho-Bad can be detected in mitochondria, and the ratio of phospho-Bad to Bad is modulated by hyperglycemia and PKC-ε-ERK signaling. Finally, emerging concepts suggest that the release of cytochrome c commits a cell to die by either apoptosis or necrosis (45). Our results document that cardiaccelar expression of PKCe isoforms in PKC-ε translocation activator exhibit protection from hyperglycemia-induced apoptosis and LV dysfunction. The ε-RACK peptide facilitated the intracellular trafficking of PKC-ε, and thereby prevented hyperglycemia-mediated decreases in immunoreactivity in both membrane and mitochondrial compartments. Importantly, PKC-ε-ERK signaling modules targeted the mitochondrial Bad protein to result in inactivated proapoptotic function and inhibited cytochrome c release. The present study has certain limitations; among these was the excess mortality in mice with cardiac-specific expression of the PKC-ε translocation inhibitor, which precludes detailed analysis of this group. Finally, it must be acknowledged that modest activation of cardiac PKC-ε may recruit additional survival programs (3) that are not explored here. The application of therapeutic interventions directed at selective activation of PKC-ε may offer a novel approach to preserve cell number and function in the hearts of diabetic patients.

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DISCLOSURES

Part of this work was presented at the American Heart Association meeting in November 2003 in Orlando, Florida (26a).

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