Inhibition of Protein Kinase C–β by Ruboxistaurin Preserves Cardiac Function and Reduces Extracellular Matrix Production in Diabetic Cardiomyopathy

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Background—Heart failure is a common cause of morbidity and mortality in diabetic patients that frequently manifests in the absence of impaired left ventricular systolic function. In contrast to the strong evidence base for the treatment of systolic heart failure, the treatment of heart failure with preserved left ventricular function is uncertain, and therapeutic targets beyond blockade of the renin-angiotensin-aldosterone and β-adrenergic systems are being sought. One such target is the β-isoform of protein kinase C (PKC), implicated in both the complications of diabetes and in cardiac dysfunction in the nondiabetic setting.

Methods and Results—Using a hemodynamically validated rodent model of diabetic diastolic heart failure, the (mRen-2)27 transgenic rat, we sought to determine whether selective inhibition of PKC-β would preserve cardiac function and reduce structural injury. Diabetic rats were randomized to receive either vehicle or the PKC-β inhibitor, ruboxistaurin (20 mg/kg per d) and followed for 6 weeks. Compared with untreated animals, ruboxistaurin-treated diabetic rats demonstrated preserved systolic and diastolic function, as measured by the slope of preload recruitable stroke work relationship (P<0.05) and the slope of the end-diastolic pressure volume relationship (P<0.01). Collagen I deposition and cardiomyocyte hypertrophy were both reduced in diabetic animals treated with ruboxistaurin (P<0.01), as was phosphorylated-Smad2, an index of transforming growth factor-β activity (P<0.01 for all, versus untreated diabetic rats).

Conclusions—PKC-β inhibition attenuated diastolic dysfunction, myocyte hypertrophy, and collagen deposition and preserved cardiac contractility. PKC-β inhibition may represent a novel therapeutic strategy for the prevention of diabetes-associated cardiac dysfunction. (Circ Heart Fail. 2009;2:129-137.)

Key Words: cardiomyopathy ▪ diabetes mellitus ▪ pharmacology

The epidemic of diabetes mellitus foreshadows a vast increase in the number of patients with cardiac complications of the disease. In contrast to the emphasis on ischemic heart disease, heart failure in diabetic subjects has been relatively neglected, even though hospitalizations for acute myocardial infarction and heart failure are similar.1 In many such patients the etiology of heart failure will be multifactorial, arising from the “toxic triad” of coronary artery disease, hypertension, and diabetic cardiomyopathy.2 Although the existence of the latter has been debated for some time; clinical, epidemiological, pathological, and experimental studies all point to the presence of cardiac dysfunction in diabetics that is independent of hypertension and underlying coronary artery disease2,3 manifested by early, mostly asymptomatic diastolic dysfunction that ultimately leads to congestive heart failure in the absence of impaired systolic function.4,5

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Research into diabetic cardiomyopathy and diastolic function had been hampered by the absence of a hemodynamically validated rodent model. More recently, however, the diabetic (mRen-2)27 transgenic (Ren-2) rat, has been shown to not only develop the structural features of human diabetic cardiomyopathy; fibrosis, hypertrophy, and apoptosis but to also display the functional changes of its human counterpart.6 Indeed, using high fidelity conductance catheters, the gold standard for the assessment of diastolic function, both the early active and later passive components of diastolic relaxation were impaired in this rodent model, despite preserved left ventricular (LV) systolic function.3

High glucose leads to the induction of a range of intracellular modifications that have been implicated in the patho-
genesis of diabetic complications. Among these is the activation of protein kinase C (PKC), a family of intracellular signaling intermediates that induce a myriad of changes in cell function through substrate phosphorylation and as such have been a target of drug discovery programs. Recently, an orally active selective inhibitor of the β-isof orm of PKC, ruboxistaurin, has been undergoing clinical trials in humans. As in diabetic cardiomyopathy, excess matrix, hypertrophy, and apoptosis are also features of diabetic nephropathy where ruboxistaurin has been shown to attenuate histological injury, functional decline, and the expression of the profibrotic and proapoptotic growth factor, transforming growth factor (TGF)-β. Accordingly, in the present study, we took advantage of the recently validated model of diabetic cardiomyopathy to examine the consequences of diabetes on LV PKC-β activity and then to assess the effects of PKC-β inhibition on the functional, structural and molecular components of heart failure, focusing in particular, on diastolic disease. Because blockade of the renin-angiotensin system is a key component in the management of patients with heart failure, we also compared the effects of ruboxistaurin with those of an angiotensin converting enzyme inhibitor.

Methods

Animals and Procedures

Study 1
Homoygous Ren-2 rats were studied (n=8 to 10 per group). At 6 weeks of age, animals were randomized to receive either 55 mg/kg of streptozocin (Sigma, St Louis, Mo) diluted in 0.1 mol/L citrate buffer pH 4.5 or citrate buffer alone (nondiabetic) by tail vein injection after an overnight fast. Diabetic rats were randomized to receive either vehicle or the PKC-β inhibitor, ruboxistaurin (20 mg/kg body weight; gift of P. Anderson, Eli Lilly, Indianapolis, Ind) and followed for 6 weeks. Diabetic animals received 2 to 4 U of isophane insulin (Humulin NPH, Eli Lilly, New South Wales, Australia) to promote weight gain and to reduce mortality. Animal care was carried out as previously described. All animal studies were approved by the St Vincent’s Hospital Animal Ethics Committee in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23).

Study 2
In a separate cohort of homozygous Ren-2 rats (n=8 per group), streptozotocin-induced diabetic rats were randomized to receive either vehicle or perindopril erbumine (2 mg/kg daily; Pharm Chemical, Shanghai, China) in drinking water. Animals followed the same protocol as study 1.

Study 3
A further cohort of Ren-2 rats (n=8 per group) were studied to examine the role of oxidative stress and PKC-β in the pathogenesis of diastolic dysfunction. Animals followed the same protocol as outlined earlier but were studied for an 8-week duration.

Cardiac Catheterization
Cardiac catheterization was performed as previously published. In brief, a 2F miniaturized combined conductance catheter-microcatheter (Model SPR-838, Millar instruments, Houston, Tex) was inserted into the carotid artery to obtain aortic blood pressure, then advanced into the left ventricle until stable PV loops were obtained. Data were then acquired under steady state conditions and during preload reduction. The following functional parameters were then calculated (Millar analysis software PVAN 3-4); end-diastolic volume, end-diastolic pressure, end-systolic pressure, the slope of the end-diastolic pressure volume relationship, the slope of the preload recruitable stroke work relationship, defined as the relationship between stroke work and end-diastolic volume, where stroke work is the pressure–volume loop area for each beat.

Histopathology and Immunohistochemistry
Changes in cardiac structure were assessed in a masked protocol in 6 animals from each group. Sections were stained for fibrillar collagen type I using a specific antibody (antitype I collagen, Southern Biotechnology Associates Inc, Birmingham, Ala). TGF-β1 was assessed using an anti-TGF-β1 antibody (Santa Cruz Biotechnology Inc, Santa Cruz, Calif), and its activity was determined by quantifying the tissue expression of a specific downstream signaling intermediate, phosphorylated Smad2, using an antiphospho-Smad2 antibody (Cell Signaling Technology, Boston, Mass). Immunohistochemistry was performed on 3-μm sections, as previously described.

Quantitation of Matrix Deposition and Phospho-
Smad2 Expression
The extent of immunostaining of collagen I, TGF-β1, and phospho-Smad2 were quantified using computer-assisted image analysis, as previously reported.

Myocyte Hypertrophy
The extent of cardiac myocyte hypertrophy, as measured by cross-sectional area, was determined on hematoxylin-eosin stained sections as previously published.

PKC-β Activity
PKC isoforms were performed as previously described. Western blot analysis was then performed using PKC β (C-16:sc-209) and β1 (C-18:sc-210) antibodies (Santa Cruz Biotechnology Inc) and activation inferred from the relative ratio of membrane to cytosol fractions.

Sarco/Endoplasmic Reticulum Ca2+-ATPase
Sacro/endooplasmic reticulum Ca2+-ATPase (SERCA-2A) ATPase and phospholamban abundance were assessed in study 1 using specific antibodies (SERCA-2A ATPase, 1:1000 dilution, Alexis Biochemicals, San Diego, Calif; phospholamban [Ser16], 05-205, Upstate Biotechnology, Charlottesville, Va; and phospho-phospholamban [Ser16], 07-052, Upstate Biotechnology) to perform Western blot analysis, as previously described.

Real-Time Quantitative Polymerase Chain Reaction (Study 1)
Quantitative real-time polymerase chain reaction was performed, as previously described, with the abundance of transcript expressed relative to that of 18S mRNA. Nucleotide sequences of primers and probes were β-myosin heavy chain (MHC) (forward) GTCG-CAGGCGGCTGAATGAG, β-MHC (reverse) GCAAGGACCTCAGGATGCA, α-MHC (forward) GTTGAAAAGATTAACCGGAGTT and (reverse) TGTGAAAGATTTAGCGAGTATTAG, α-MHC (reverse) TCTGACTTGGGAGGGATTTCG. 18 S (forward) TCG AAG CCC TGT AAT TGG AA, (reverse) CCC TCC AAT GGA TCC TCG TT, SERCA-2A (forward) TCT GTC ATT CGG GAG TGG GG, (reverse) GCC CAC ACA GCC AAC GAA AG.

Oxidative Stress (Study 3)
Urinary excretion of 8-hydroxy deoxyguanosine was used as a marker for oxidative damage and was measured in 24-hour urine samples by Bioxytech 8-hydroxy deoxyguanosine-enzyme immunoassay assay (Oxis International Inc, Foster City, Calif) using the manufacturer’s instructions.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.
Statistical Analysis

Results were expressed as mean±SEM. Differences between groups were determined by ANOVA with Fisher protected least significant differences post hoc comparison. A value of \( P<0.05 \) was considered statistically significant.

Results

Animal Characteristics

Body weight was reduced in diabetic rats. Ruboxistaurin was accompanied by a further weight reduction in diabetic but not in normoglycemic animals. Similarly, although ruboxistaurin did not affect plasma glucose or heart rate in nondiabetic rats, it was associated with a 4 mmol/L increase in plasma glucose and a 30-bpm reduction in heart rate in hyperglycaemic animals. Blood pressure, reduced in diabetic rats, fell further among diabetic animals treated with ruboxistaurin with a trend toward a similar effect in nondiabetic rats also. LV weight, indexed to body weight, was lower in diabetic rats with a further diminution among those receiving ruboxistaurin that did not reach statistical significance (Table 1). Perindopril was associated with reductions in systolic blood pressure and heart rate. LV weight and lung weight, corrected for body weight were also reduced in both perindopril treated nondiabetic and diabetic groups when compared with their respective controls (supplementary Table 1).

PKC Activity (Study 1)

Diabetic animals demonstrated evidence of PKC activation, with a significant increase in the ratio of membrane to cytosolic PKC \( \beta \) that was reduced by ruboxistaurin. The PKC-\( \beta II \) isoform demonstrated a trend toward increased activity in the diabetic group that was significantly reduced by ruboxistaurin (Figure 1).

Cardiac Catheterization

Pressure–volume loop analysis was used to assess both load-sensitive and load-insensitive measures of systolic and diastolic function (Figure 2). Chamber compliance, a measure of diastolic function was significantly reduced in diabetic animals when compared with control. Ruboxistaurin therapy was associated with increased chamber compliance and improved diastolic function. Perindopril treatment similarly improved chamber compliance (supplementary Table 2).

The preload recruitable stroke work relationship index and volume intercept \( (V_0) \), used to assess basal cardiac contractility, were reduced in diabetic animals when compared with controls. Treatment with ruboxistaurin preserved systolic function in diabetic rats, as evidenced by improved preload recruitable stroke work relationship slope and \( V_0 \) \( (P<0.05, \text{ Table 2}) \). In perindopril-treated rats, nonsignificant improvements in systolic function were also noted with modest augmentation in cardiac output and ejection fraction. The preload recruitable stroke work was, however, unchanged with perindopril (supplementary Table 2).

### Table 1. Baseline Characteristics of Ren-2 Rats Treated With Vehicle or Ruboxistaurin

<table>
<thead>
<tr>
<th></th>
<th>Ren-2 Control</th>
<th>Ren-2 Control RBX</th>
<th>Ren-2 Diabetes</th>
<th>Ren-2 Diabetes RBX</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>293±12</td>
<td>302±10</td>
<td>241±7*</td>
<td>216±8†</td>
</tr>
<tr>
<td>Plasma glucose, mmol/L</td>
<td>6±0.5</td>
<td>6±0.1</td>
<td>26±0.5*</td>
<td>30±0.44†</td>
</tr>
<tr>
<td>LV, g</td>
<td>1.1±0.05</td>
<td>0.95±0.03</td>
<td>0.71±0.04*</td>
<td>0.6±0.04*†</td>
</tr>
<tr>
<td>LV:BW</td>
<td>3.7±0.1</td>
<td>3.2±0.05</td>
<td>3.0±0.1*</td>
<td>2.7±0.08*</td>
</tr>
<tr>
<td>Lung:BW</td>
<td>0.43±0.02</td>
<td>0.38±0.02</td>
<td>0.49±0.02*</td>
<td>0.48±0.02</td>
</tr>
</tbody>
</table>

RBX indicates ruboxistaurin.

*\( P<0.01 \) when compared with Ren-2 C rats.

†\( P<0.01 \) when compared with diabetic rats.

Figure 1. PKC activity. Representative Western blots of cytosolic and membranous fractions of PKC-\( \beta I \) and PKC-\( \beta II \) are presented. PKC-\( \beta I \) activity (ratio of membrane bound to cytosolic fraction of PKC \( \beta I \) was increased by diabetes and reduced with treatment by ruboxistaurin, whereas PKC-\( \beta II \) showed a trend to being increased with diabetes but was significantly reduced with ruboxistaurin treatment. *\( P<0.01 \) when compared with control rats; †\( P<0.01 \) when compared with diabetic rats.
Tissue Structure
Immunostaining demonstrated a marked increase in collagen I in diabetic rat hearts that was reduced with ruboxistaurin (Figure 3). Similarly, diabetic rats also displayed myocyte hypertrophy with increased cardiomyocyte cross-sectional area when compared with their nondiabetic counterparts that was prevented by treatment with ruboxistaurin (Figure 4).

Immunostaining for the prosclerotic and prohypertrophic cytokine TGF-β1 demonstrated increased immunolabeled TGF-β1 in diabetic animals (0.03±0.002 versus 0.02±0.0015, diabetic versus control, P<0.001). Treatment with ruboxistaurin significantly reduced the level of immunostainable TGF-β1 (0.022±0.002 versus 0.03±0.002, ruboxistaurin treated diabetic animals versus Ren-2 diabetic, P<0.05). Similarly, the presence of diabetes resulted in marked elevation of nuclear phosphoSmad-2 immunostaining, which was also attenuated by ruboxistaurin (Figure 5).

Gene Expression
Perindopril also reduced collagen I abundance, cellular hypertrophy, and prevented Smad 2 activation in diabetic rat hearts (P<0.01, see supplementary Figures 1 through 3).

SERCA-2A ATPase and Phospholamban
The presence of diabetes resulted in a significant reduction in the content of SERCA-2A ATPase in the hearts of diabetic animals that was prevented by ruboxistaurin. Although total phospholamban was unchanged, phosphorylated-phospholamban, was modestly reduced in diabetic Ren-2 animals. Ruboxistaurin treated animals showed increased phosphorylated-phospholamban, such that the ratio of phosphorylated-phospholamban to phospholamban was reduced in diabetes and improved with ruboxistaurin therapy (Figure 6).

**Figure 2.** Representative pressure-volume loops during preload reduction. Note the steeper slope of the end-diastolic pressure volume relationship in the Ren-2 diabetic group (C) compared with control (A), which was attenuated by treatment with ruboxistaurin (D). Diastolic function was not changed in control animals treated with ruboxistaurin (B). *P<0.01 when compared with control rats; †P<0.01 when compared with diabetic rats.
strated an increase in β-MHC mRNA, with a concomitant reduction in α-MHC mRNA, such that the ratio of β-MHC:α-MHC ratio was significantly altered in diabetic Ren-2 animals when compared with control \(P<0.01\). Ruboxistaurin had no effect on the ratio of β MHC:α MHC mRNA expression. SERCA-2 mRNA tended to be lower in untreated diabetics and treatment with ruboxistaurin significantly lowered SERCA-2 mRNA relative to controls (Table 3).

**Study 3**

**Role of Oxidative Stress**

Diabetes was associated with a 3-fold rise in urinary 8-hydroxy deoxyguanosine (control versus diabetic: 345±16 versus 777±47 ng/mL, \(P<0.001\)) that was unaffected by ruboxistaurin treatment.

**Discussion**

Large scale clinical trials have provided a strong evidence base for the treatment of heart failure with impaired systolic function in which diabetic subgroups benefit similarly. Such studies contrast the relative dearth of information on the treatment of heart failure with preserved LV function where a beneficial response to angiotensin converting enzyme inhibition or angiotensin receptor blockade remains conjectural.\(^\text{15,17}\) Evidence accumulated over the past 10 years has suggested that enhanced PKC activity may play a central role

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**Table 2. Baseline Hemodynamic Variables in Diabetic and Control Ren-2 Rats Treated With Vehicle or Ruboxistaurin**

<table>
<thead>
<tr>
<th></th>
<th>Ren-2 Control</th>
<th>Ren-2 Control RBX</th>
<th>Ren-2 Diabetes</th>
<th>Ren-2 Diabetes RBX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>375±14</td>
<td>375±10</td>
<td>328±6*</td>
<td>290±9†</td>
</tr>
<tr>
<td>EDV, μL</td>
<td>329±48</td>
<td>320±19</td>
<td>268±28</td>
<td>284±20</td>
</tr>
<tr>
<td>EF, %</td>
<td>57±9</td>
<td>68±6</td>
<td>51±4</td>
<td>70±6†</td>
</tr>
<tr>
<td>Stroke volume, μL</td>
<td>170±19</td>
<td>217±16</td>
<td>128±15*</td>
<td>210±18†</td>
</tr>
<tr>
<td>CO, μL/min</td>
<td>63017±7032</td>
<td>81 609±6442*</td>
<td>38 605±3106*</td>
<td>60 635±5038†</td>
</tr>
<tr>
<td>ESP, mm Hg</td>
<td>137±16</td>
<td>125±12</td>
<td>116±4</td>
<td>101±8*</td>
</tr>
<tr>
<td>EDP, mm Hg</td>
<td>6.6±0.5</td>
<td>5.2±1.1</td>
<td>7.4±0.6</td>
<td>6.6±0.7</td>
</tr>
<tr>
<td>PRSW, mm Hg/μL</td>
<td>106±14</td>
<td>102±9</td>
<td>57±5*</td>
<td>94±11†</td>
</tr>
<tr>
<td>Vo</td>
<td>129±19</td>
<td>42±27</td>
<td>57±22*</td>
<td>76±22</td>
</tr>
<tr>
<td>EDPVR, mm Hg/μL</td>
<td>0.032±0.004</td>
<td>0.028±0.002</td>
<td>0.06±0.007*</td>
<td>0.03±0.004†</td>
</tr>
</tbody>
</table>

RBX indicates ruboxistaurin, EDV, end diastolic volume; EF, ejection fraction; CO, cardiac output; ESP, end systolic pressure; EDP, end diastolic pressure; PRSW, preload recruitable stroke work relationship; Vo, volume intercept of the slope of the PRSW relationship; EDPVR, end diastolic pressure volume relationship.

*\(P<0.01\) when compared with control rats.

†\(P<0.01\) when compared with diabetic rats.

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**Figure 3.** Immunohistochemistry (brown) for interstitial collagen type I. This demonstrated marked increases in collagen I in the Ren-2 diabetic animals (C) compared with control (A), which was reduced by treatment with 20 mg/kg of the PKC-β inhibitor in Ren-2 diabetic animals (D). Magnification ×320. *\(P<0.01\) when compared with control rats; †\(P<0.01\) when compared with diabetic rats.
in the development of heart failure. Of the 12 member PKC family, the \(\beta\)-isoform, in particular, is elevated in humans with dilated cardiomyopathy.\(^{18,19}\) This isoform is also increased in the postmyocardial infarction setting where its inhibition attenuates LV dysfunction and adverse remodeling.\(^{16}\) Moreover, cardiac-specific overexpression of PKC-\(\beta\) leads to heart failure in mice.\(^{20}\) In the present study, we show that inhibition of PKC-\(\beta\) activity in experimental diastolic heart failure due to diabetes led not only to improved cardiac function and structure but also ameliorated pathological changes in the molecular substrates of lusitropy and fibrosis.

Although increased PKC-\(\beta\) activity is clearly demonstrable in hearts from patients with dilated cardiomyopathy,\(^{18,19}\) previous studies in diabetic rodents have not been as convincing. For instance, Inoguchi et al\(^{21}\) reported a 21\% increase in PKC-\(\beta\) in the membranous fractions of hearts from Sprague-Dawley rats 2 weeks after streptozotocin-induced diabetes, whereas others have reported no change after 8 weeks of diabetes in the same model.\(^{22}\) These findings contrast the diabetic Ren-2 rat used in this report, which displays a 5-fold increase in PKC-\(\beta\) activity, more reminiscent of the 4-fold increase seen in the human setting.\(^{18}\) The current findings in an animal model of diabetes and heart failure suggest that PKC-\(\beta\) activity may be an attribute of both the failing heart and hyperglycaemia per se. For instance, although PKC-\(\beta\) activation by hyperglycaemia is well

Figure 4. Cellular hypertrophy. Diabetic Ren-2 rats demonstrated cellular hypertrophy (C), as evidenced by increased cross-sectional area when compared with control animals (A). Treatment with ruboxistaurin normalized cross-sectional area in the diabetic Ren-2 animals (D) but had no effect on control animals (B, ruboxistaurin-treated animals). Magnification \(\times 200\). *\(P<0.01\) when compared with control rats; †\(P<0.01\) when compared with diabetic rats.

Figure 5. TGF-\(\beta\) activation, as measured by nuclear Smad2 activation (brown), was enhanced in the Ren-2 diabetic animals (C) compared with control (A). Treatment with ruboxistaurin significantly reduced TGF-\(\beta\) activity in the diabetic Ren-2 animals (D) but had no effect on control animals (B). Magnification \(\times 320\). *\(P<0.01\) when compared with control animals; †\(P<0.01\) when compared with diabetic rats.
documented, recent studies have also shown that PKC-β is activated in the postmyocardial setting in nondiabetic animals in which ruboxistaurin has been shown to attenuate adverse remodeling and heart failure.16

Assessment of cardiac function was performed in the in vivo context using high fidelity conductance catheters, a critical component for validating cardiac dysfunction in diabetic rodent models23 where echocardiography and ex vivo-based measurements may be misleading.12 The ability of the heart to relax in diastole, critically dependent on its passive-elastic properties, was assessed in this study by measuring the end-diastolic pressure volume relationship over a range of loading conditions, thereby enabling chamber compliance to be determined.24 As might be expected given the associated cardiac fibrosis and hypertrophy, the end-diastolic pressure volume relationship was substantially elevated in diabetic animals but was reduced by ruboxistaurin and perindopril in conjunction with improved histology. The preload recruitable stroke work relationship and its volume intercept, a sensitive, load-independent measure of cardiac systolic function, was reduced in diabetic animals but was reduced by ruboxistaurin and perindopril in conjunction with improved histology. The preload recruitable stroke work relationship and its volume intercept, a sensitive, load-independent measure of cardiac systolic function, was reduced in diabetic animals but was reduced by ruboxistaurin and perindopril in conjunction with improved histology.

Excessive matrix accumulation is a common histopathologic feature of heart failure, almost regardless of etiology, correlating closely with the extent of cardiac dysfunction25 suggesting that its reduction should improve diastolic stiffness and dysfunction.26 In the present study, commensurate with their impaired function, diabetic rats developed substantial interstitial fibrosis that was attenuated by both PKC-β inhibition and angiotensin-converting enzyme inhibition.

Consistent with findings in other tissues, such as the kidney, not only was the expression of the profibrotic growth factor, TGF-β, elevated in the hearts of diabetic rats but its biological activity, as evidenced by the presence of nuclear phosphorylated-Smad2, was also increased. These findings are consistent with the report by Wakasaki et al20 who demonstrated that TGF-β protein was increased in a PKC-βII transgenic mouse, suggesting that PKC-β overexpression upregulates TGF-β transcription. Of note, the promoter region of the TGF-β gene contains a consensus sequence for the transcription factor activating protein-1 that is exquisitely sensitive to the PKC-β activation,27 such that PKC-β inhibition prevents hyperglycaemia-induced induction of TGF-β expression.28

The finding that TGF-β and fibrosis were both attenuated by ruboxistaurin is also consistent with previous reports showing the role of PKC in high glucose-induced TGF-β

Table 3. Activation of the Fetal Gene Program

<table>
<thead>
<tr>
<th></th>
<th>Ren-2 Control</th>
<th>Ren-2 Control RBX</th>
<th>Ren-2 Diabetes</th>
<th>Ren-2 Diabetes RBX</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Myosin HCM ratio</td>
<td>0.96±0.24</td>
<td>1.05±0.58</td>
<td>4±0.4*</td>
<td>4.7±1.2*</td>
</tr>
<tr>
<td>SERCA-2A</td>
<td>1.27±0.24</td>
<td>1.11±0.20</td>
<td>0.80±0.17</td>
<td>0.56±0.11*</td>
</tr>
</tbody>
</table>

Data has been normalized to control animals. RBX indicates ruboxistaurin; MHC, myosin heavy chain. *P<0.01 when compared with control rats.
overexpression and extracellular matrix production in the diabetic kidney, suggesting common pathogenetic processes in the diabetic kidney and heart.

In addition to the extramyocardial effects of tissue fibrosis, abnormalities within the cardiac myocyte also contribute to diastolic dysfunction. As previously demonstrated, we found activation of a “fetal” gene program in diabetic animals, with atrial natriuretic factor activation and a decrease in α-MHC mRNA that were unaffected by ruboxistaurin. These findings suggest that although PKC-β inhibition improves several attributes of the disordered structure and function found in experimental diabetic diastolic heart failure, it does not reverse the pathological process in its entirety.

The modulation of intracellular calcium concentration, mediated by its transport in and out of the sarcoplasmic reticulum, is a key determinant for actin-myosin dissociation. The sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA-2A ATPase) accounts for most of this process and is itself regulated by the abundance of phospho-phospholamban. In the present study, both SERCA-2A ATPase and phosphophospholamban were reduced in the hearts of diabetic animals and normalized by ruboxistaurin. The mechanisms accounting for these changes are uncertain. For SERCA-2A ATPase, our studies suggest that the observed changes are not the result of reduced gene expression and the change observed in SERCA-2A protein might be the result of altered protein stability or translation of the mRNA. With regard to the reduction in phospholamban phosphorylation, we speculate that this may reflect the previously reported interaction between PKC and protein phosphatase 1, the enzyme responsible for phospholamban dephosphorylation.

Ruboxistaurin treatment was associated with reduced body weight, higher plasma glucose, reduced heart rate, and lower blood pressure when compared with vehicle-treated diabetic animals. These findings are similar to that reported by Arikawa et al\(^\text{33}\) who demonstrated similar nonsignificant reductions in body weight and higher glucose levels in ruboxistaurin-treated diabetic animals. However, in clinical trials of patients with diabetic nephropathy and diabetic retinopathy no change in body mass index, glycemic control, heart rate, or blood pressure were reported.\(^\text{34}\)

### Study Limitations

Although this study has focused on PKC-β, other isoforms, including PKC-α and PKC-ε, have been implicated in the pathogenesis of diabetic heart disease in the experimental setting.\(^\text{35,36}\) However, studies examining tissue from humans with dilated cardiomyopathy have not shown changes in PKC εpsilon\(^\text{19}\) whereas others localized PKC-α primarily to intercalated discs contrasting the predominant myocyte expression of PKC-β.\(^\text{18}\)

Although the present study demonstrated that diabetes and PKC-β inhibition had a range of effects on cardiac function, structure, growth factors, and lusitropic intermediates, the role of PKC-β in diabetes-associated alterations in cardiac fuel metabolism, the subject of a recent in-depth study, were not explored in this report.\(^\text{33}\)

To compare the effects of PKC-β inhibition with current first-line treatment for heart failure, we also examined the effects of angiotensin-converting enzyme inhibition. Both ruboxistaurin and perindopril reduced the abnormal structure and diastolic dysfunction in diabetic rats to a similar extent. However, whether ruboxistaurin might provide additional therapeutic effects when added to angiotensin-converting enzyme inhibition, a pivotal issue clinically, was not assessed in this study.

In conclusion, inhibition of PKC-β with ruboxistaurin resulted in improved diastolic function, preservation of systolic function, reduction in type I collagen, attenuation of TGF-β activity, amelioration of disease-associated changes in myocyte calcium pump proteins, and also reduced myocyte hypertrophy despite the persistence of continuing hyperglycaemia. In light of the apparent safety in phase II–III studies, ruboxistaurin-mediated PKC-β inhibition may represent a novel, clinically applicable therapy for diastolic dysfunction that develops as a consequence of diabetes.

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### References


**CLINICAL PERSPECTIVE**

Diabetes mellitus has reached epidemic proportions and will be accompanied by a vast increase in its long-term complications. Despite improved medical care and the use of agents that block the renin-angiotensin-aldosterone and beta-adrenergic systems, mortality remains high. More recently, the syndrome of heart failure with preserved ejection fraction has also been identified and found to have mortality rates approaching that of systolic heart failure. However, unlike the latter, no therapies for preserved heart failure with preserved ejection fraction have been shown to reduce morbidity and mortality, so novel therapeutic targets are being actively explored. One such target is protein kinase C, the beta isoform of which is preferentially activated by hyperglycaemia and is upregulated in both human heart failure and in animal models. In the present study, using a hemodynamically validated model of diabetes-induced heart failure with preserved ejection fraction, we demonstrated activation of the protein kinase C beta isoform, which was associated with abnormal active and passive relaxation, along with the structural and molecular substrates for abnormal lusitropy. Inhibition of this isoform with ruboxistaurin normalized diastolic function and improved cardiac structure. In the light of recent clinical trials demonstrating safety of ruboxistaurin in diabetic renal and kidney disease, inhibition of protein kinase C-beta with ruboxistaurin may represent a novel therapeutic strategy in the treatment of diabetic subjects with heart failure and preserved ejection fraction.
Inhibition of Protein Kinase C−β by Ruboxistaurin Preserves Cardiac Function and Reduces Extracellular Matrix Production in Diabetic Cardiomyopathy
Kim A. Connelly, Darren J. Kelly, Yuan Zhang, David L. Prior, Andrew Advani, Alison J. Cox, Kerri Thai, Henry Krum and Richard E. Gilbert

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SUPPLEMENTARY MATERIAL.
Table 1:

<table>
<thead>
<tr>
<th></th>
<th>Ren-2 Control</th>
<th>Ren-2 Control</th>
<th>Ren-2 Diabetes</th>
<th>Ren-2 Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Perindopril</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BW (g)</strong></td>
<td>285 ± 15</td>
<td>257 ± 9 *</td>
<td>244 ± 26 *</td>
<td>252 ± 11 *</td>
</tr>
<tr>
<td><strong>Plasma glucose</strong> (mmol/l)</td>
<td>6 ± 0.5</td>
<td>6.2 ± 0.5</td>
<td>26 ± 0.5 *</td>
<td>27 ± 0.2 *</td>
</tr>
<tr>
<td><strong>LV (g)</strong></td>
<td>1.07 ± 0.05</td>
<td>0.65 ± 0.02 *</td>
<td>0.72 ± 0.03 *</td>
<td>0.46 ± 0.02 *</td>
</tr>
<tr>
<td><strong>LV:BW</strong></td>
<td>3.8 ± 0.1</td>
<td>2.5 ± 0.1 *</td>
<td>2.9 ± 0.5 *</td>
<td>1.8 ± 0.1 *</td>
</tr>
<tr>
<td><strong>Lung:BW</strong></td>
<td>0.43 ± 0.05</td>
<td>0.31 ± 0.01 *</td>
<td>0.48 ± 0.07 *</td>
<td>0.41 ± 0.04</td>
</tr>
</tbody>
</table>

BW = Body weight, LV = left ventricular weight, LV:BW is LV weight corrected for body weight, Lung:BW is lung corrected for BW; * p <0.01 when compared to control rats † p <0.01 when compared to diabetic rats
Table 2:

<table>
<thead>
<tr>
<th></th>
<th>Ren-2 Control</th>
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<tbody>
<tr>
<td></td>
<td>Perindopril</td>
<td>Perindopril</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate (BPM)</td>
<td>381 ±13</td>
<td>363 ± 10</td>
<td>326 ±6 *</td>
<td>299 ± 7 *</td>
</tr>
<tr>
<td>EDV (µl)</td>
<td>348±55</td>
<td>265±27</td>
<td>274±27</td>
<td>248±33</td>
</tr>
<tr>
<td>EF (%)</td>
<td>55 ± 7</td>
<td>54 ± 9</td>
<td>52 ± 4</td>
<td>62 ± 6</td>
</tr>
<tr>
<td>Stroke volume (µl)</td>
<td>140 ± 18</td>
<td>147 ± 20 *</td>
<td>130 ± 8</td>
<td>168 ± 23 *</td>
</tr>
<tr>
<td>CO (µl/min)</td>
<td>54702 ± 8902</td>
<td>53880 ± 8434</td>
<td>42533 ± 3297</td>
<td>50772 ± 7544</td>
</tr>
<tr>
<td>ESP (mmHg)</td>
<td>123±5</td>
<td>99±5 *</td>
<td>114±4</td>
<td>87±7 *†</td>
</tr>
<tr>
<td>EDP (mmHg)</td>
<td>5.8±0.7</td>
<td>5.6±0.66</td>
<td>6.3±2.3</td>
<td>6.3±1.2</td>
</tr>
<tr>
<td>PRSW (mmHg)</td>
<td>90±10</td>
<td>87±13</td>
<td>65±6 *</td>
<td>68±8</td>
</tr>
<tr>
<td>$V_0$</td>
<td>124±24</td>
<td>110±35</td>
<td>45±25</td>
<td>51±25</td>
</tr>
</tbody>
</table>

EDV is end diastolic volume, EF is ejection fraction, CO is cardiac output, ESP is end systolic pressure, EDP is end diastolic pressure, PRSW is preload recruitable stroke work relationship, $V_0$ is volume intercept of the slope of the PRSW relationship, EDPVR is end diastolic pressure volume relationship * p <0.01 when compared to control rats † p <0.01 when compared to diabetic rats.
Figure Legend:

Figure 1: Immunohistochemistry (brown) for interstitial collagen type I. This demonstrated marked increases in collagen I in the Ren-2 diabetic animals (C) compared to control (A), which was reduced by treatment with perindopril in Ren-2 diabetic animals (D). x320 magnification (* p <0.01 when compared to control rats, † p <0.01 when compared to diabetic rats).

Figure 2: Cellular hypertrophy. Diabetic Ren-2 rats demonstrated cellular hypertrophy as evidenced by increased cross sectional area, when compared to control animals. Treatment with perindopril normalised both cross sectional area in the diabetic Ren-2 animals, and had a modest effect upon control animals x200 magnification. (* p <0.01 when compared to control rats, † p <0.01 when compared to diabetic rats).

Figure 3: TGF-β activation, as measured by nuclear Smad2 activation (brown), was enhanced in the Ren-2 diabetic (C) animals compared to control (A). Treatment with perindopril significantly reduced TGF β activity in the diabetic Ren-2 animals (D), but had no effect upon control animals (B). x320 magnification. (* p <0.01 when compared to control rats, † p <0.01 when compared to diabetic rats.)
Figure 1:
Figure 3: