Decreased RhoA expression in myocardium of diabetic rats

Jiping Tang, Sharyn M. Fitzgerald, Brandi N. Boughtman, Samuel W. Cole, Michael W. Brands, and John H. Zhang

Abstract: Diabetic cardiomyopathy is one of the major causes of death in diabetic patients, but the pathogenesis is unclear. There is evidence that RhoA, a small GTPase, might be involved in cardiac function. This study, therefore, analyzed RhoA expression and activation in hearts of diabetic rats. Male Sprague–Dawley rats were divided into control and diabetic groups of 18 each. Diabetes was induced by intravenous injection of streptozotocin (55 mg/kg). Rats were studied 3 weeks after induction of diabetes. Heart rate, which was measured 24 h/day, decreased by 93 ± 7 beats/min in diabetic rats. There was a 62% decrease ($p < 0.01$) in RhoA mRNA expression in heart tissues (left ventricle) of diabetic rats (38.5 ± 6.7 × 10^6 molecules/µg total RNA) compared with controls (101 ± 10.3 × 10^6 molecules/µg total RNA). Western blot showed a 33% decrease in total RhoA protein expression in heart tissues of diabetic rats compared with controls ($p < 0.05$). A reduced RhoA translocation in heart tissues of diabetic rats was determined by a 64% decrease in membrane-bound RhoA ($p < 0.01$ vs. control group), indicating that the activation of RhoA is markedly reduced in diabetic myocardium. Our data suggest that down-regulated RhoA may be involved in cardiomyopathy in diabetic rats.

Key words: RhoA, diabetes, heart.

Introduction

I went to Hamilton, Ontario, Canada in 1992 with my husband who was a postdoctoral fellow working with Dr. Ed Daniel. I did not truly work with Ed but have attended some activities in Ed’s research group and know many of Ed’s researchers. One of the research subjects ongoing at that time was studying the role of protein kinase C (PKC) in smooth muscle contraction (Shimamoto et al. 1993). After I went to the University of Chicago in 1993, I continued my research in diabetes and began to investigate signaling pathways such as PKC and Rho kinases. This paper described the work I did later but that was inspired by the work performed in Ed’s laboratory.

Diabetes mellitus is one of the most prevalent chronic conditions that has a close association with cardiovascular...
disease. Impaired cardiac function independent of vascular disease suggests the existence of a primary myocardial defect in diabetes mellitus (Malhotra and Sanghi 1997). Animal studies have shown previously that streptozotocin (STZ)-induced diabetic animals such as rats, dogs, and rabbits have an increased ratio of heart to body mass and prolonged myocardial contraction and relaxation (Fein et al. 1985; Dai et al. 1994; Shimabukuro et al. 1995; Verma and McNeill 1994; Litwin et al. 1990). The impaired cardiac function is suggested to be the result of abnormalities of the contractile proteins and regulatory proteins in diabetic heart muscles (Malhotra and Sanghi 1997). However, the pathogenesis of diabetic cardiomyopathy still remains unclear.

A small GTPase, RhoA, has been shown to play a key role in both actin stress fiber formation and focal adhesion complex assembly in fibroblasts (Mackay and Hall 1998). RhoA belongs to the Rho family, which includes Rho, Rac, and Cdc42. There are 3 isoforms of Rho (A, B, and C). Among the 3 isoforms, RhoA is the most ubiquitously and abundantly expressed in the body, and has been extensively studied. Therefore, the following discussion about Rho is mostly related to RhoA. Recent studies have shown that RhoA is a key mediator of hypertrophic responses in ventricular myocytes through Rho-kinase and other effectors (Finkel 1999; Sah et al. 1996; Hoshijima et al. 1998; Thorburn et al. 1997; Wang et al. 1997; Aoki et al. 1998). RhoA has also been found to regulate myofibril formation and organization in neonatal rat ventricular myocytes (Kuwahara et al. 1999). However, the role of RhoA in diabetic cardiac dysfunction has not been reported.

This project is designed to test the hypothesis that RhoA contributes to the development of cardiomyopathy in diabetes. Diabetes was induced in rats by injection of STZ. Blood pressure, heart rate, and heart and body mass ratios were measured. Rats were sacrificed after 3 weeks of diabetes. Morphological changes and both RhoA expression and activation were studied in ventricles of experimental rats.

Material and methods

Animal model

Male Sprague-Dawley rats (200–250 g) were divided into diabetic (n = 18) and control groups (n = 18). Diabetes was induced by a single injection of STZ at a dose of 55 mg/kg (Sigma Chemical Co., St. Louis, Mo.), and a control group was injected with a 0.9% saline solution vehicle. All injections were given intravenously after the animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Animals were housed under identical 12 h light : 12 h dark cycles and were fed standard rat laboratory diet with free access to water. All animals were sacrificed 3 weeks after induction of diabetes. An additional group of rats (n = 9) was used for measuring the blood pressure and heart rate in STZ-induced diabetes. The protocol for our study was approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center.

Plasma glucose, serum insulin, free fatty acid, triglycerides, and cholesterol levels

Blood samples were collected into heparin-treated hemocite tubes by tail bleeding on weekly bases. Blood was separated by centrifugation (16 000g for 5 min), and plasma glucose levels were determined by a Beckman Glucose analyzer (Beckman Instruments, Inc., Fullerton, Calif.).

Three weeks after the injection of STZ, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Blood was drawn from the aorta just prior to removing the heart at the conclusion of the study, and serum was separated by centrifugation. Insulin levels were determined by a radioimmunoassay kit (Linco Research, Inc. St. Louis, Mo.), free fatty acid levels were measured by a diagnostic kit (Boehringer Mannheim, GmbH, Germany), triglycerides, and cholesterol levels were measured with a diagnostic kit (Sigma Diagnostics, Inc., St. Louis, Mo.).

Mean arterial blood pressure and heart rate

Blood pressure and heart rate were measured to assess the cardiovascular function and the experiments were conducted in 9 rats. Anesthesia was induced with sodium pentobarbital (50 mg/kg, i.p.), and an artery and a vein catheter were implanted under aseptic conditions as described previously (Brands and Fitzgerald 1998; Fitzgerald and Brands 2000). After recovery from anesthesia, the rats were placed in individual metabolic cages, and the arterial catheter was filled with heparin solution (1000 USP/mL) and connected to a pressure transducer via a hydraulic swivel (Instech). Pulsatile arterial pressure signals were amplified, sent to an analog-to-digital converter and analyzed by computer 24 h per day using customized software. After baseline measurements, STZ (55 mg/kg, i.v.) was administrated to all rats followed by a 3-week diabetic period.

Heart to body mass ratio

Heart hypertrophy was measured by the ratio of heart to body mass. Under anesthesia, hearts were quickly removed from the chest after body masses and blood samples taken. Hearts were weighed and normalized by body mass.

Histology

Hearts were fixed in 3.7% formaldehyde/PBS solution overnight and then dehydrated in 70% ethanol, embedded in paraffin wax, and sectioned. Tissue sections were stained with hematoxylin/eosin (H/E) for general morphology and Gomori’s tricrome for collagen accumulation.

Immunohistochemistry

Immunohistochemistry was performed to assess RhoA expression in heart. Rat left ventricular tissue sections were fixed in 10% neutralized formalin saline solution and embedded in paraffin wax, and sectioned. The sections were deparaffinized and antigen retrieval, the sections were immunolabelled and visualized according to an avidin-biotin complex (ABC) methods using an ABC kit and a dianobenzidine (DAB) substrate kit from Vector Laboratories (Burlingame, Calif.). In brief, the sections were treated with 0.3% hydrogen peroxide and 3% normal serum to block endogenous peroxidase activity and to reduce nonspecific background staining, primary rabbit RhoA polyclonal antibodies in phosphate buffered saline PBS solution containing 1% serum (overnight at 4 °C), biotin-labeled secondary antibody (30 min at room temperature) and then avidin-biotin-peroxidase complex. The sections
were stained using DAB and 0.01% hydrogen peroxidase for 10 min, and finally hematoxylin counter stain.

**Protein preparation and Western blot analysis**

The expression of RhoA was examined at the protein level by Western blot using total lysate. RhoA activation was determined by its translocation from cytoplasm to membrane as described previously (Aoki et al. 1998; Seasholtz et al. 1999). In brief, left ventricular tissue was isolated and washed with ice-cold PBS and then homogenized with a polytron in ice-cold buffer containing (mmol/L): 5 Tris–HCl, 5 NaCl, 1 CaCl₂, 2 EGTA, 1 MgCl₂, 2 dithiothreitol (DTT) and 1 µL/mL of proteinase inhibitor cocktail (Sigma). Total lysate was prepared by centrifugation of homogenate at 1000g for 15 min. To prepare cell fractions, nuclei and unlysed cells in homogenate were removed by low-speed centrifugation at 500g for 5 min and the samples were centrifuged at 10 000g for 60 min. The supernatant was collected as cytosolic fraction and the pellet was resuspended in the same buffer supplemented with 1% Nonidet P40 (NP-40) and then collected as membrane fraction. Protein concentrations were measured and adjusted, and then Laemmli sample buffer added. Equal amounts of proteins were loaded in each lane, electrophoresed on 12% polyacrylamide-SDS gels, and transferred to nitrocellulose membrane. Membrane was probed with 1 µg/mL of anti-RhoA antibody (Santa Cruz Biotechnology), followed by horse-radish peroxidase-conjugated protein A (Zymed) at 1:1000 dilution. Blots were exposed to film and RhoA expression in total lysate, membrane and cytosolic fractions were analyzed by densitometry.

**RNA isolation and quantitative reverse transcription PCR (RT-PCR)**

The mRNA expression of RhoA was examined by quantitative RT-PCR as described previously (Siebert and Larrick 1992, 1993). In brief, total RNA was isolated from left ventricle with Trizol reagent (GIBCO-BRL, Grand Island, N.Y.). cDNA was prepared from 1 µg of total RNA using You-Prime RT-PCR kit (Amersham Pharmacia Biotech Inc.). Specific mRNA level was determined by adding known amount of a synthetic competitor DNA into each PCR reaction. This internal standard (competitor) could be amplified using the same primers for the experimental cDNA target and as designed to generate a PCR product that could be easily distinguished from the target cDNA due to their different sizes. The thermal cycle profile for PCR amplification of 32 cycles was denaturing for 30 s at 95 °C, annealing primers for 30 s at 55 °C, extending the primers for 1 min at 72 °C, and using specific primers for RhoA (GIBCO-BRL, Grand Island, NY). A portion of 10 µL of the PCR products was electrophoresed in 2% agarose gel in TAE buffer. For the quantitative analysis of RT-PCR products, the density of bands for mRNA was determined by a densitometer.

**Densitometric analysis**

Densitometric analysis of the PCR-products was performed as described previously (Miyagi et al. 2000). In summary, the products were separated on a 2% agarose gel in Tris–Acetate/EDTA buffer. The gel was analyzed using Gel Doc 1000 and Quantity One software (Bio-Rad, Calif.). The ratio of wild-type to competitor was calculated, understanding that the product lengths varied, and the wild-type/competitor ratio was plotted on a log scale against competitor concentration. For each experiment a standard curve was calculated using linear regression and the equivalence point (log ratio = 0) was determined. Copy numbers were calculated and expressed as molecules/µg, which corresponds to the ratio of total RNA.

**Statistical analysis**

The differences between diabetic and control groups were compared by Student’s t test. Daily hemodynamic data were analyzed by ANOVA with repeated measures and Dunnett’s t test. All values are expressed as mean ± SE. The level of significance was set at p < 0.05.

**Results**

**General information of diabetes**

Diabetes was confirmed by hyperglycemia after STZ-injection. Diabetic animals had lower body and heart masses, but the heart/body mass ratio was slightly, but significantly, greater than that in the control group (Fig. 1A). Serum insulin levels were markedly decreased, and lipid levels (FAA, TG, and cholesterol) increased in diabetic rats compared with controls (Table 1).

**Hemodynamics**

Heart rate averaged 408 ± 2.0 beats/min during the baseline period and decreased significantly after the onset of diabetes (Fig. 1B), consistent with previous reports (Tomlinson et al. 1992). Mean arterial pressure averaged 95 ± 0.2 mmHg during baseline, and the average of 97 ± 0.4 mmHg during the diabetic period was not significantly different (Fig. 1C).

**RhoA mRNA expression in myocardium (RT-PCR)**

To determine whether RhoA transcription in myocardium was effected by STZ-injection, RhoA mRNA expression was measured by quantitative RT-PCR. Figure 2 shows that there was 62% of reduction of RhoA mRNA expression in left ventricular tissue of diabetic rats compared with controls (p < 0.01), indicating that RhoA transcription was down-regulated in myocardium in STZ-induced diabetic animals.

**RhoA protein expression and activation in myocardium (Western blot)**

RhoA protein expression in total lysate extracted from left ventricular tissue was significantly decreased (p < 0.05) in diabetic group compared with controls (Fig. 3A), consistent with the decreased mRNA expression found in diabetic group. These data indicates that both RhoA transcription and translation were down-regulated in myocardium in STZ-induced diabetic animals. Increases in translocation from the cytosolic to the membrane fraction are associated with RhoA activation. To further investigate the effect of STZ-induced diabetes on activation of RhoA, we measured membrane-associated RhoA and cytosolic RhoA in left ventricular tissue in diabetic and control rats. Figure 3B shows that there was a 64% reduction (p < 0.05) of membrane-associated RhoA expression and no significant change in cytosolic RhoA expression (p > 0.05, Fig. 3C) in diabetic group compared with the control group.
The ratio of membrane-bound RhoA vs. cytosolic RhoA was 67% lower \((p < 0.05)\) in diabetic group. These data indicates that RhoA activation was markedly down-regulated in myocardium of STZ-induced diabetic rats.

Light microscopy: H/E and collagen staining
Diabetes did not affect the gross appearance of the heart. No changes in the thickness of either right or left ventricular wall were noted, nor were there any apparent changes in ventricular chamber volume. Light microscopy revealed that there was no change in the amount of intercellular or extracellular matrix in the myocardium from 3-week diabetic animals (Fig. 4A). Specifically, there was no evidence of interstitial collagen accumulation seen with stain specific for collagen (Fig. 4B). These results are consistent with previous studies from other investigators (Litwin et al. 1990; Gotzsche 1982; Modrak 1980).

RhoA protein expression in myocardium (immunohistochemistry)
RhoA protein expression was examined by immunohistochemistry using anti-RhoA antibody. Figure 4C shows that there was a decreased RhoA expression in myocardium of the ventricle from diabetic rats, especially the expression of RhoA on cell membranes of the myocardium was markedly decreased in the diabetic animals, which suggests there was reduced activation of RhoA.

Discussion
This study demonstrated that (i) mRNA expression of RhoA decreased in myocardium of diabetic rats; (ii) both
protein expression and activation of RhoA decreased in myocardium of diabetic rats; and (iii) there is no marked histological difference in the cardiac tissue between control and diabetic rats, suggesting that the change in RhoA occurred before onset of gross histological abnormalities. Decreased RhoA expression and activation were accompanied by a decreased heart rate and an increased heart/body mass ratio in diabetic rats, suggesting that RhoA might be involved in the etiology of diabetic cardiomyopathy.

**Diabetic cardiomyopathy**

Diabetic cardiomyopathy is a special heart problem and is one of the major causes of death in diabetic patients (Verma and McNeill 1994). Recent studies have suggested that diabetic patients may develop myocardial dysfunction in the absence of coronary heart disease and hypertension (Kita et al. 1991). STZ-induced diabetic animals such as rats, dogs, and rabbits have an increased ratio of heart/body mass as well as prolonged myocardial contraction and relaxation (Fein et al. 1985; Dai et al. 1994; Shimabukuro et al. 1995; Verma and McNeill 1994; Litwin et al. 1990). The impaired cardiac function is suggested to result from abnormalities of the contractile and regulatory proteins in diabetic heart muscle (Malhotra and Sanghi 1997); however, the pathogenesis of diabetic cardiomyopathy remains unclear.

**Characteristics of RhoA**

RhoA is a GTPase that has been shown to play a key role in both actin stress fiber formation and focal adhesion complex assembly in fibroblasts (Mackay and Hall 1998). RhoA has been shown to regulate myofibril formation and organization in neonatal rat ventricular myocytes (Kuwahara et al. 1999), but whether the other 2 Rho isoforms, RhoB and RhoC, have distinct cellular functions is not clear (Narumiya 1996). Rho shuttles between the active GTP-bound form on the cell membrane and the inactive GDP-bound form in the cytoplasm. Direct targets for Rho have been sought by screening for molecules selectively binding to, or activated by, GTP-Rho (Narumiya 1996; Niirro et al. 1997).

Recent studies demonstrate that RhoA is an important target in the cell signaling pathway and that RhoA plays a pivotal role in cell function. In smooth muscle cells, Hirata et al. (Hirata et al. 1992) first reported that RhoA is involved in the myofilament Ca\(^{2+}\) sensitization. Later, RhoA was found to be involved in the inhibition of myosin light chain (MLC)-phosphatase and the subsequent enhancement of MLC-phosphorylation (Noda et al. 1995; Kimura et al. 1996). Inactivation of MLC-phosphatase maintains a high level of phosphorylated myosin light chain and increases smooth muscle contractility and prolongs muscle contraction. In addition, RhoA is a very important regulator of the actin cytoskeleton, and also is involved in gene regulation and cell cycle progression (Kim et al. 1996). The activated Rho-mediated pathway has been linked to hypertension in several animal models (Uehata et al. 1997), coronary and cerebral vasospasm (Miyagi et al. 2000; Shimokawa et al. 1999), higher vessel contractility response in diabetes (Sandu et al. 2000), and heart failure (Sah et al. 1999).

**RhoA in diabetic cardiomyopathy**

RhoA activates Rho-kinase, which regulates myofibril formation and organization in neonatal rat ventricular myocytes, and there is evidence that RhoA may be important in diabetic cardiomyopathy. Transgenic mice overexpressing the active form of RhoA in the heart die in a few weeks from heart failure (Sah et al. 1999). The cardiomyopathy in RhoA transgenic mice is similar to the cardiomyopathy in STZ-induced diabetic rats. However, the role of RhoA in diabetic cardiac function has not been reported. We hypothesize that RhoA plays an important role in the abnormality of cardiac function in diabetes. This study examined whether the expression and activation of RhoA are changed in heart of diabetic rats.

We have found that RhoA expression decreased at both the transcription and protein expression levels in the left ventricles of diabetic rats. Immunohistochemistry and Western blot clearly demonstrated that RhoA expression was reduced in the membrane of cardiac myocytes, indicating a decrease in activated RhoA. The changes in RhoA were accompanied by the cardiac functional abnormalities in which heart rate was markedly decreased and the heart to body mass ratio significantly increased in the diabetic rats. This appears to be the first observation that RhoA expression is reduced in cardiac myocytes in diabetes, but it is consistent with the reports demonstrating that insulin increases RhoA protein and potentiates the RhoA-dependent nuclear effect of lysophosphatidic acid in breast cancer cells (Chappell et al. 2000) and in rat adipocytes (Standaert et al. 1998). Previous studies have shown that insulin therapy reverses hemodynamic abnormalities and cardiac dysfunction in diabetic rats (Litwin et al. 1990; Fein et al. 1981), and the effect of insulin to improve cardiac function may be related to its action to increase RhoA expression. We found in our preliminary experiments using cultured cardiac myocytes that insulin increased RhoA expression in a dose-dependent manner (preliminary data). Other possible mechanisms responsible for the effect of insulin could include increasing calcium uptake and normalization of myosin isoforms (Gotzsche

### Table 1. General information of diabetes.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Control (n = 18)</th>
<th>Diabetic (n = 18)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass (g)</td>
<td>350.4±25.8</td>
<td>236.7±12.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Heart mass (g)</td>
<td>1.173±0.083</td>
<td>0.837±0.028</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Blood glucose (mg/dL)</td>
<td>121±3.5</td>
<td>645±16.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum insulin (µU/mL)</td>
<td>33.6±3.67</td>
<td>5.46±0.78</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum triglyceride (mg%)</td>
<td>66.2±7.35</td>
<td>450±95</td>
<td>0.002</td>
</tr>
<tr>
<td>Serum free fatty acid (mmol/L)</td>
<td>0.219±0.026</td>
<td>0.585±0.087</td>
<td>0.004</td>
</tr>
<tr>
<td>Serum cholesterol (mg%)</td>
<td>66.3±3.86</td>
<td>88.5±6.6</td>
<td>0.005</td>
</tr>
</tbody>
</table>

**Note:** Data are presented as mean ± SE.
In addition, hyperglycemia may directly contribute to the development of cardiomyopathy. The glycation of intracellular proteins may alter enzyme or receptor function in a manner that depresses cardiac function (Brownlee et al. 1984). However, the roles of glucose and insulin and how their interaction with RhoA could be involved in the cardiac changes observed in diabetes requires considerable further study.

It is important to note that our observation is not in complete agreement with a report that used a transgenic mouse model, in which overexpressing a constitutively activated form of RhoA in the heart caused bradycardia and significant dilation of the left ventricular chamber that was associated with decreases in left ventricular contractility. Heart rate in those animals was grossly depressed, and most animals died in a few weeks from heart failure (Sah et al. 1999). Thus, increased RhoA was associated with significant cardiac dysfunction, but our data would suggest that elevation of RhoA enhances cardiac muscle contractility and improves heart function instead of causing heart failure. Consistent with this hypothesis, elevation of RhoA has been reported in cerebral vasospasm (Miyagi et al. 2000), a prolonged contraction of smooth muscle cells (Kim et al. 1996), and a Rho-kinase inhibitor has hypotensive effect in hypertensive rats (Uehata et al. 1997). The differences in those studies could be due to the species, transgenic model, or any of several other possibilities, but there is insufficient data to resolve the issue at this time.

Even though our observation supports a view that RhoA reduction may lead to cardiac dysfunction in diabetes, the mechanisms are currently not clear. Future studies are warranted to answer the questions of whether hyperglycemia and lack of insulin, combining with other unknown factors, inhibit the expression and activation of RhoA in diabetes.
Fig. 4. Hematoxylin and eosin (H & E), collagen and immunohistochemistry staining of myocardium. (A) Light microscopy did not reveal any changes between control and diabetic animals. Samples from control (original magnification 160×) and diabetic (original magnification 160×) rats. (B) Gomori’s tricrome staining did not reveal any changes of interstitial collagen accumulation between control and diabetic animals. Control (original magnification 320×) and diabetic (original magnification 320×) rats. (C) Immunohistochemistry revealed a marked decrease of RhoA staining (brownish colour) in the diabetic sample compared with control sample. Control (original magnification 80×) and diabetic (original magnification 80×) rats.
We hypothesize that control of diabetes with insulin treatment improves cardiac function through mechanisms that may include activation of RhoA pathway.

Acknowledgements

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Reference


Fitzgerald, S.M., and Brands, M.W. 2000. Nitric oxide may be re~


