Acute inhibition of Rho-kinase improves cardiac contractile function in streptozotocin-diabetic rats

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Abstract

Objective: The purpose of the present study was to determine whether increased activation of the RhoA/Rho-kinase (ROCK) pathway occurs in diabetic cardiomyopathy and whether acute inhibition of this pathway improves contractile function of the diabetic heart.

Methods: Male Wistar rats were made diabetic with streptozotocin. Twelve to fourteen weeks later, the effects of acute administration of the ROCK inhibitors Y-27632 and H-1152 on cardiac contractile function were measured both in vitro, in isolated working hearts, and in vivo, using echocardiography. Changes in the expression and activity of RhoA, and the effect of ROCK inhibition on changes in the phosphorylation of the downstream target of ROCK, LIM kinase 2, and on actin polymerization in diabetic hearts were also determined.

Results: Perfusion of isolated working hearts from diabetic rats with Y-27632 or H-1152 acutely improved left ventricle developed pressure and the rates of contraction and relaxation. Acute administration of H-1152 also significantly improved the percent fraction shortening, an index of left ventricle contractility, in vivo in diabetic rats. The expression and activity of RhoA in cardiomyocytes from diabetic rats were significantly increased, as was the phosphorylation of LIM kinase 2. This was associated with an increase in actin polymerization (the F-actin to G-actin ratio). Both the increase in LIM kinase 2 phosphorylation and actin polymerization were attenuated by ROCK inhibition.

Conclusions: These data suggest that activation of the RhoA/ROCK signaling pathway plays a critical role in the development of diabetic cardiomyopathy, and that ROCK is an excellent therapeutic target in the treatment of this condition.

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This article is referred to in the Editorial by Peters and Michel (pages 3–4) in this issue.

1. Introduction

Diabetic cardiomyopathy, distinct from coronary ischemic heart disease, includes a constellation of morphological, biochemical and functional abnormalities that are seen in both human patients and in animal models of type 1 and type 2 diabetes [1,2]. Cardiac abnormalities, including diastolic dysfunction, manifested by decreased compliance, prolonged myocardial relaxation and impaired left ventricular filling, can be detected by echocardiography early in the course of diabetes in both humans and animal models [3–6]. With progression of the cardiac dysfunction, systolic dysfunction develops, which contributes to the eventual development of heart failure, one of the fatal complications of diabetes [7]. At present, there are no specific drugs available for the prevention or treatment of diabetic cardiomyopathy.

The RhoA/Rho-kinase pathway plays an important role in physiological function [8], and in the pathophysiology of many diseases, including hypertension, myocardial hypertrophy and heart failure [7,9,10]. Rho-kinase (ROCK) includes two isoforms, ROCKI (also known as p160ROCK or ROKβ) and ROCKII (Rho-kinase or ROKα) [11]. ROCK is a serine/
threonine protein kinase that is activated by RhoA, a member of the Rho family of small G-proteins. ROCK activity can be selectively blocked by the competitive inhibitor Y-27632, as well as the isoxquinoline sulfonyl derivative HA1077 and its more potent and selective derivative, H-1152 [12,13]. Treatment with ROCK inhibitors has been shown to effectively reduce blood pressure in rats with spontaneous or angiotensin II (Ag-II)-mediated hypertension [12,14], and in hypertensive patients in a clinical trial [15]. RhoA-ROCK activation has been also implicated in endothelin-1 (ET-1)-mediated neonatal rat cardiomyocyte hypertrophy [16], and in left ventricular hypertrophy and heart failure in dogs and in Dahl salt-sensitive hypertensive rats [10,17]. Chronic administration of the ROCK inhibitor Y-27632 effectively improved heart function in Dahl salt-sensitive hypertensive rats and prevented the progression to failure [10,18]. Increased activation of the RhoA/ROCK pathway has been demonstrated in vascular tissue from type 2 diabetic db/db mice [19] and in erectile tissue from rats with streptozotocin (STZ)-induced diabetes [20], but whether this also occurs in the heart and contributes to contractile dysfunction is not known.

In the present study, we provide evidence that the RhoA/ROCK pathway is activated in hearts from rats with STZ-induced diabetes, the most widely used model of type 1 diabetes. Furthermore, we show that acute inhibition of ROCK improves cardiac function during chronic diabetes, both in vitro and in vivo.

2. Methods

2.1. Induction of diabetes mellitus in Wistar rats

Male Wistar rats (200–250 g) were lightly anesthetized with halothane and given a single tail vein injection of 60 mg/kg STZ in 0.1 M citrate buffer (pH 4.5) or citrate buffer alone. STZ-treated rats with blood glucose levels over 13 mmol/l 1 week after injection were considered diabetic. All animals were housed under identical conditions and were given free access to food and water for 12 to 14 weeks. Just prior to experiments, rats were weighed and blood was taken for measurement of glucose and insulin levels. Plasma glucose levels were measured using a Periodochrom glucose assay kit (Boehringer Mannheim) and serum insulin was measured using a rat insulin radioimmunoassay kit (Linco Research Inc. St. Charles, MO). This investigation conforms with the Canadian Council on Animal Care Guidelines on the Care and Use of Experimental Animals, and the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996).

2.2. Isolated working heart studies in vitro

Rats were deeply anesthetized with sodium pentobarbital (65 mg/kg, IP) and hearts were excised and quickly cleaned in ice-cold oxygenated Chenoweth–Koelle (CK) solution (composition in mM: NaCl 120, KCl 5.6, CaCl2 2.18, MgCl2 2.1, NaHCO3 19.2 and glucose 10). Hearts were initially perfused through the aorta in a retrograde manner in the Langendorff mode with oxygenated CK buffer alone, or buffer containing Y-27632 ((R)(+-)-trans-N-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide), or H-1152 ((S)(+-)-2-Methyl-1-[(4-methyl-5-isoquinolynyl) sulfonyl] homopiperazine, both from Calbiochem, San Diego, CA). Drugs were dissolved directly in the CK buffer. The pulmonary vein was then cannulated and perfusion was switched to the working heart mode. Left ventricular developed pressure (LVDP), and the rates of contraction and relaxation (+dP/dT and −dP/dT) in response to increases in left atrial filling pressure, produced by pre-determined stepped increases in the rate of perfusion of the CK buffer, were measured with a pressure transducer attached to a 20-gauge needle inserted through the apex of the heart into the left ventricle. Hearts were paced at 300 beats per minute. Hearts were perfused with buffer containing the inhibitors for 15 min in the Langendorff mode throughout the experiment of the coronary artery effluent into a graduate cylinder, just prior to switching from the Langendorff to the working heart mode.

2.3. Measurement of cardiac function by transthoracic echocardiograms in vivo

The Agilent SONOS 4500 Cardiology System equipped with a high-frequency linear array transducer (model 21390A UltraBand 15.0–6.0 MHz, Andover, MA) was used to measure cardiac function in vivo. Rats were anesthetized with an IM injection of midazolam (2.5 mg/kg) plus ketamine (80 mg/kg). M-mode echocardiograms were recorded through the anterior and posterior left ventricular (LV) walls at the papillary muscle level to measure left ventricular end-systolic (LVESD) and end-diastolic (LVEDD) dimensions. Percentage fraction shortening (%FS) was calculated using the formula: FS(%) = [(LVEDD – LVESD)/LVEDD] × 100. The heart rate was monitored simultaneously by ECG. H-1152 dissolved in saline, or saline alone, was administered as a single bolus dose via the tail vein. Based on preliminary dose-finding experiments, the effects of 100 μg/kg (408 nmol/kg) H-1152 were determined 5 min after administration, the time at which the response to the drug was found to reach its peak. Heart function was found to return to pre-treatment status by 30 min after H-1152 injection, at which point a second bolus dose of H-1152 or saline was administered, animals were deeply anesthetized with pentobarbital, hearts were removed and left ventricles quickly frozen in liquid nitrogen for biochemical studies.

2.4. Preparation of isolated rat ventricular myocytes

Ca2+-tolerant ventricular myocytes were isolated using a modification of the procedure of Huang et al. [21]. Rats were anesthetized with sodium pentobarbital (65 mg/kg, IP) co-
administered with heparin (1000 units/kg). Hearts were rapidly excised and perfused in the Langendorff mode with Ca\(^{2+}\)-free Tyrode’s solution (composition in mM: NaCl 100, KCl 10, KH\(_2\)PO\(_4\) 1.2, MgSO\(_4\) 5, taurine 50, glucose 10, HEPES 10), followed by Tyrode’s solution containing 0.05 mM Ca\(^{2+}\), 0.8 mg/ml type II collagenase (Worthington Biochem Corp, NJ) and 0.1% BSA. The ventricles were removed and minced, and the resulting cell suspension filtered and centrifuged briefly at 60 × g. The cell pellet was washed 3 times in Tyrode’s solution containing increasing amounts of Ca\(^{2+}\) (0.2 mM, 0.5 mM and 1 mM Ca\(^{2+}\)). Cardiomyocyte counts were taken using a haemocytometer and viability was determined by assessing the percentage of cells that excluded trypan blue dye. Cell viability was greater than 65% in all groups.

2.5. Western blotting analysis

Frozen ventricles were powdered and homogenized in buffer containing 20 mM Tris–HCl (pH 7.5), 50 mM β-mercaptoethanol, 5 mM EGTA, 2 mM EDTA, 10 mM NaF, 1 mM PMSF, 25 μg/ml leupeptin, 2 μg/ml aprotinin, 0.1% NP40, 0.1% SDS, 0.1% deoxycholic acid and 1% phosphatase inhibitor cocktail. Isolated myocytes were homogenized via fine needle and brief sonication in lysis buffer (10 mM Tris–HCl pH 7.5, 50 mM NaF, 5 mM sodium pyrophosphate, 0.5 mM EDTA, 2 mM EGTA, 1% (v/v) glycerol, 2% sodium azide, 1% NP40, 0.1% SDS, 2 μg/ml aprotinin, 25 μg/ml leupeptin and 1% phosphatase inhibitor cocktail). Both ventricular and myocyte homogenates were spun at 700 × g for 5 min, and the protein content of the supernatants was determined by the Bradford protein assay.

Equal amounts of protein (40 μg) from each sample were separated by 12% SDS PAGE and transferred to nitrocellulose or PDVF membrane. The membranes were blocked for 1 h in a solution of 5% skim milk powder and then incubated overnight at 4 °C with primary antibody against RhoA (1:2000, Santa Cruz Biotechnology Inc, CA), LIM kinase or phosphorylated LIM kinase (both 1:2000 dilution, Cell Signaling Technology Inc, MA). Membranes were washed, incubated with secondary antibody conjugated to HRP (1:10 K-20 K dilution) for 1 h then exposed to chemiluminescence reagents (Amersham Inc, Québec, Canada) and developed on film. Densitometric analysis was performed to quantify band optical density values.

2.6. RhoA activity assay

A commercially available RhoA activation assay kit (Cytoskeleton Inc, CO) was used to determine the relative amount of active RhoA in 0.5 × 10\(^6\) freshly isolated myocytes. Each sample was measured in triplicate.

2.7. F-actin/G-actin assay

Freshly isolated control and diabetic myocytes were divided into two groups, one of which remained untreated and the other of which was treated with 1 μM Y-27632 for 15 min. Myocytes were then processed for the isolation and determination of free globular actin (G-actin) and filamentous actin (F-actin) content using a commercially available assay kit (Cytoskeleton Inc, CO).

2.8. Statistical analysis

All values are expressed as means±SEM; n denotes the number of animals in each group. Statistical analysis of all data except LVDP, +dP/dt and −dP/dt was performed using one-way ANOVA, followed by the Newman–Keuls test when more than two groups were compared, using GraphPad Prism (GraphPad Software). LVDP, +dP/dt and −dP/dt were analyzed by repeated measures ANOVA (general linear models approach) followed by the Newman–Keuls test, using NCSS statistical analysis system (NCSS). For all results the level of significance was set at P < 0.05.

3. Results

Twelve to fourteen weeks after induction of diabetes, STZ-treated rats had greatly elevated blood glucose levels (25.7 ± 0.6 mmol/l, mean±SEM, n = 30) and much lower serum insulin levels (0.5 ± 0.09 ng/ml) than their age and gender-matched controls (9.5 ± 0.2 mmol/l and 8.9 ± 0.7 ng/ml, respectively). The body weights of the diabetic rats (365 ± 26 g) were significantly less than those of the control animals (582 ± 57 g). The diabetic rats also exhibited other symptoms of diabetes, including polydipsia, polyuria and increased food intake.

3.1. Effect of ROCK inhibitors on the function of isolated working hearts

The effects of acute perfusion with 1 μM Y-27632 on the function of isolated working hearts from 12- to 14-week diabetic and age-matched control rats are shown in Fig. 1. Y-27632 had no effect on the function of hearts from control rats. Compared to controls, untreated diabetic hearts showed significantly reduced LVDP, +dP/dT and −dP/dT and furthermore, 3 of 7 hearts failed at left atrial filling pressures above 10 mm Hg, an indication of severely insufficient cardiac mechanical contractile force. Perfusion with 1 μM Y-27632 significantly improved LVDP, +dP/dT and −dP/dT in the working hearts from diabetic rats. In the presence of Y-27632, these parameters in working hearts from 12- to 14-week diabetic rats were not significantly different from those in control hearts, and none of the treated diabetic hearts failed at high perfusion pressures, indicating that the Y-27632 effectively improved the function of hearts from diabetic rats. This was not associated with a change in coronary flow rate; coronary flow in hearts treated with Y-27632 (19.0 ± 0.1 ml/min in control and 18.4 ± 0.2 in diabetic hearts) was not significantly different from that in untreated hearts (18.2 ± 0.1 in control and 17.6 ± 0.2 in diabetic hearts). Perfusion with
μM H-1152, chemically unrelated to Y-27632, also improved the LVDP, +dP/dt and −dP/dt in the working hearts from diabetic animals, while having no effect on these parameters in the control hearts (Fig. 2). As was found with Y-27632, there were no significant differences in LVDP, +dP/dt and −dP/dt between hearts from control and diabetic rats in the presence of H-1152. Thus, two structurally different ROCK inhibitors acutely improved cardiac function in isolated working hearts from diabetic rats to levels similar to control rats.

3.2. Effect of ROCK inhibitors in vivo

We then investigated by M-mode echocardiography whether acute administration of H-1152 could improve cardiac function of diabetic rats in vivo (Fig. 3). Before treatment, the percentage of left ventricle fractional shortening (% FS) was significantly lower in diabetic (47.6 ± 2.4) than in control animals (62.0 ± 1.8). Bolus injection of 160 μg/kg H-1152 had no significant effect on the % FS in control rat hearts (63.2 ± 2.6), but produced an improvement in % FS in the diabetic rat hearts, to a level (55.6 ± 2.4) that
was significantly greater than that in untreated diabetic hearts, and was not significantly different from control. The improvement in % FS in diabetic hearts by H-1152 was not associated with a change in heart rate, which was 350±15 beats/min before and 345±15 beats/min after treatment in control rats, and 310±10 beats/min before and 305±10 beats/min after treatment in diabetic rats.

3.3. Activation of RhoA and LIM kinase 2

The status of the RhoA/ROCK pathway in hearts from diabetic rats was next examined. Both the expression (Fig. 4A) and activity (Fig. 4B) of RhoA in cardiomyocytes isolated from diabetic hearts were significantly greater than in myocytes from control hearts. RhoA expression was increased to 1.62±0.18 of control, while activity was increased to 1.51±0.21 of control, in diabetic cardiomyocytes. One of the immediate downstream targets of ROCK is LIM kinase 2. Therefore, as a measure of ROCK activation, we assessed the phosphorylation of LIM kinase 2 in hearts from untreated and H-1152-treated control and diabetic rats by Western blotting. There was no significant difference between hearts from control and diabetic rats in total LIM kinase 2 levels (Fig. 5). However, LIM kinase 2 phosphorylation was significantly increased in untreated diabetic hearts to 1.39±0.14 of control, and inhibition of ROCK reduced the phosphorylation to 0.92±0.08 of control, which was not significantly different from levels in hearts from untreated or H-1152-treated (0.99±0.08) control rats (Fig. 5).

Fig. 3. Effect of H-1152 on percent fractional shortening (% FS) of control and diabetic left ventricles determined by transthoracic echocardiogram in vivo. Control before (C), control following treatment (C+H), diabetic before (D), and diabetic following treatment (D+H). #, P<0.05 compared to C; @, P<0.05 compared to D (n=7 in each group).

Fig. 4. Expression and activity of RhoA in control and 12-week STZ diabetic rat ventricular myocytes. (A) Above: representative Western blot showing RhoA expression, with actin shown as a loading control, in freshly isolated control and diabetic cardiomyocytes. Below: RhoA band optical densities (O.D.) were corrected by the O.D. of their corresponding actin band, and expressed relative to the mean control value (n=8 in each group.) (B) Comparison of the relative amount of active RhoA in protein extracts from freshly isolated control and diabetic cardiomyocytes (n=12 in each group). *, P<0.05 compared to control.

Fig. 5. Effect of ROCK inhibition on LIM-kinase 2 phosphorylation in control and STZ diabetic heart tissue. Above: representative Western blot showing phosphorylated LIM-kinase 2 (p-LIMK) and total LIM-kinase 2 (LIMK) in hearts from untreated controls (C), control rats treated with H-1152 (C+H), untreated diabetics (D) and diabetic rats treated with H-1152 (D+H). Below: phosphorylated LIM-kinase (p-LIMK) expressed relative to the mean untreated control (n=5 in each group). #, P<0.05 compared to all other groups.
The factors responsible for the development of diabetic cardiomyopathy have been the subject of intense investigation, but are still incompletely understood. Studies in animal models have demonstrated that defects in systolic and diastolic function can be detected at the level of the whole heart and in isolated cardiomyocytes [22,23], indicating that altered contractile function is due to changes occurring at the cellular and molecular levels. These changes include alterations in energy metabolism, with a decrease in glucose uptake and utilization paralleled by an increase in fatty acid oxidation [24], changes in contractile proteins [25] and in intracellular Ca$^{2+}$ signaling [26], all of which have been implicated in the development of contractile dysfunction. To date, there are no specific drugs available to treat this condition. ROCK inhibitors have been used clinically in the treatment of both hypertension and angina [27,28]. In fact, recently the vasodilatory effect of the ROCK inhibitor, fasudil, was reported to be greater than that of nitroglycerin at sites of coronary stenosis in angina patients [29]. Because diabetic cardiomyopathy frequently coexists and synergistically interacts with coronary heart disease and hypertension, it would be ideal to have a drug that treats these conditions concurrently.

Our observation that hearts from untreated 12–14-week STZ diabetic rats showed significantly reduced contractility both in vivo and in vitro is consistent with previous reports in this model. Impaired contractile function of the heart has been reported as early as 5–6 weeks after the onset of diabetes in STZ diabetic rats [30] and is well established by 12–14 weeks [31]. This is further substantiated by the finding that some untreated isolated working hearts from diabetic rats failed at higher atrial filling pressures, an indication of severely insufficient cardiac mechanical contractile force. In the present study, the impaired contractile function of the diabetic heart was associated with significant increases in both the expression and activity of RhoA in cardiomyocytes. ROCK is the best-known effector of RhoA. Our observation that acute treatment of isolated working hearts from diabetic rats with two structurally unrelated ROCK inhibitors, Y-27632 and H-1152, acutely improved % FS of the heart in vivo, while having no effect on the heart rate. The ROCK inhibitors had no effect on the contractile function of hearts from control animals, in fact they tended to have a slight depressant effect, the mechanism of which is unknown. Therefore, the improvement in the function of hearts from diabetic rats failed at higher atrial filling pressures, an indication of severely insufficient cardiac mechanical contractile force. In the present study, the impaired contractile function of the diabetic heart was associated with significant increases in both the expression and activity of RhoA in cardiomyocytes. ROCK is the best-known effector of RhoA. Our observation that acute treatment of isolated working hearts from diabetic rats with two structurally unrelated ROCK inhibitors, Y-27632 and H-1152, restored their function to levels similar to the control group strongly implicates increased activation of the RhoA/ROCK pathway in the development of the impaired contractile function. Since the ROCK inhibitors had no effect on coronary flow rate in either control or diabetic hearts, the restoration of cardiac function likely resulted from a direct effect on the myocardium. Consistent with results obtained in isolated working hearts, treatment of diabetic rats with H-1152 acutely improved % FS of the heart in vivo, while having no effect on the heart rate. The ROCK inhibitors had no effect on the contractile function of hearts from control animals, in fact they tended to have a slight depressant effect, the mechanism of which is unknown. Therefore, the improvement in the function of hearts from diabetic rats produced by these agents cannot be attributed to a non-specific positive inotropic effect. The ability of ROCK inhibitors to reduce blood pressure in hypertensive animals and in human patients with hypertension is well known. However, ROCK inhibitors have little or no effect on blood pressure in normotensive animals [12,32]. We have previously shown that rats with chronic STZ-induced diabetes are not hypertensive, in fact they develop mild hypotension by 3 weeks after induction of diabetes, that is sustained for at
least 12 weeks [33]. Therefore, it seems unlikely that the improvement in the function of the diabetic hearts in vivo is secondary to a blood pressure lowering effect produced by acute inhibition of ROCK activity. Rather it appears that by selectively inhibiting increased activation of the RhoA/ROCK pathway in the myocardium, ROCK inhibitors are able to acutely improve contractile function of the diabetic heart both in vitro and in vivo.

Our observation that RhoA expression is increased in cardiomyocytes from diabetic rat hearts is at odds with a recent report that RhoA mRNA and protein expression are decreased in hearts from diabetic rats [34]. The reason for this difference is not clear, but it may be due to differences in the duration of diabetes between the two studies. The latter was conducted in hearts from 3-week diabetic rats, a time that is prior to the development of overt impaired contractile function in most investigations.

The increase in RhoA expression and activity in the present study was associated with increased phosphorylation of the ROCK target, LIM-kinase 2. Phosphorylated LIM-kinase 2 acts as a positive regulator of actin polymerization, and the levels of polymerized actin in cardiomyocytes from diabetic hearts were also found to be significantly elevated. The attenuation of both the increase in LIM-kinase 2 phosphorylation and actin polymerization by ROCK inhibition implicates increased activation of the RhoA/ROCK pathway in mediating these changes in the diabetic heart. A number of studies have implicated a role for the cytoskeleton in conditions of impaired cardiac performance [35]. For instance, increased actin polymerization in cardiomyocytes has been associated with decreased contractility due to disruption of intracellular Ca2+ release [36]. Furthermore, RhoA-induced increases in actin polymerization can promote the exocytosis of lipoprotein lipase and increase fatty acid metabolism [37], which is associated with impaired cardiac function and lipotoxicity in chronic diabetes [38]. Therefore, normalization of actin polymerization may contribute to ROCK inhibitor-mediated improvement in contractile function of hearts from diabetic rats by a number of different mechanisms. Studies are ongoing in this laboratory to further explore the specific mechanism(s) underlying this effect.

As mentioned in the Introduction section, chronic administration of ROCK inhibitors has been shown to prevent the progression of left ventricular hypertrophy to cardiac failure in Dahl-salt sensitive hypertensive rats [10,18]. In those studies, the improvement in contractile function was attributed to reduction of myocardial hypertrophy, in association with upregulation of eNOS and downregulation of the oxidative stress–LOX-1 pathway [10,39]. However, it is unlikely that the improvement in contractile function of diabetic hearts produced by ROCK inhibitors in the present study results from similar mechanisms, since the onset of their action was very rapid.

In conclusion, the present study demonstrates, for the first time to our knowledge, that the RhoA/ROCK pathway is activated in hearts from rats with chronic STZ-induced diabetes. Furthermore, despite the diverse biochemical and physiological changes that occur in the diabetic heart, acute inhibition of the RhoA/ROCK pathway improves cardiac function, both in vitro and in vivo. This suggests a central role for this pathway in contributing to impaired contractility in the diabetic heart, and that ROCK is an excellent therapeutic target in the treatment of diabetic cardiomyopathy.

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