Aims Recent studies from our laboratory demonstrated that increased expression of the small GTP-binding protein RhoA and activation of the RhoA/ROCK pathway play an important role in the contractile dysfunction associated with diabetic cardiomyopathy in hearts from streptozotocin (STZ)-induced diabetic rats. Nitric oxide (NO) has been reported to be a positive regulator of RhoA expression in vascular smooth muscle, and we have previously found that the expression of inducible NO synthase (iNOS) is increased in hearts from STZ-diabetic rats. Therefore, in this study, we investigated the hypothesis that induction of iNOS positively regulates RhoA expression in diabetic rat hearts.

Methods and results To determine whether NO and iNOS could increase RhoA expression in the heart, cardiomyocytes from non-diabetic rats were cultured in the presence of the NO donor sodium nitroprusside (SNP) or lipopolysaccharide (LPS) in the absence and presence of the selective iNOS inhibitor, N^6-(1-iminoethyl)-L-lysine dihydrochloride (L-NIL). In a second study, 1 week after induction of diabetes with STZ, rats were treated with L-NIL (3 mg/kg/day) for 8 more weeks to determine the effect of iNOS inhibition in vivo on RhoA expression and cardiac contractile function. Expression of iNOS was elevated in cardiomyocytes isolated from diabetic rat hearts. Both SNP and LPS increased RhoA expression in non-diabetic cardiomyocytes. The LPS-induced elevation in RhoA expression was accompanied by an increase in iNOS expression and prevented by L-NIL. Treatment of diabetic rats with L-NIL led to a significant improvement in left ventricular developed pressure and rates of contraction and relaxation concomitant with normalization of total cardiac nitrite levels, RhoA expression, and phosphorylation of the ROCK targets LIM (Lin-11, Isl-1, Mec-3) kinase and ezrin/radixin/moesin.

Conclusion These data suggest that iNOS is involved in the increased expression of RhoA in diabetic hearts and that one of the mechanisms by which iNOS inhibition improves cardiac function is by preventing the upregulation of RhoA and its availability for activation.

KEYWORDS
RhoA; INOS; Diabetic cardiomyopathy; Contractility; Rho kinase

1. Introduction
Cardiomyopathy, characterized by impaired cardiac function that can be detected in the absence of hypertension or ischaemic heart disease, is a common complication of both type 1 and type 2 diabetes. This condition is associated with a series of morphological, biochemical, and functional abnormalities seen in both human patients and animal models of both type 1 and type 2 diabetes, including changes in metabolism, contractile protein composition, and intracellular Ca\(^{2+}\) transients.\(^1\)\(^2\) Despite intensive investigation, the precise mechanisms underlying the contractile dysfunction are still not completely understood.

The small GTPase RhoA and its effector rho kinase (ROCK) play important roles in physiological and pathophysiological functions in the cardiovascular system (reviewed in references 3–5). Recently, we found evidence suggesting that the RhoA/ROCK pathway is activated in hearts from diabetic rats.\(^6\) Increases in the expression and activity of RhoA, and increased phosphorylation of the downstream ROCK target, LIM (Lin-11, Isl-1, Mec-3) kinase (LIMK), were detected in hearts from rats with chronic streptozotocin (STZ)-induced diabetes. This was associated with an increase in actin polymerization that was blocked by inhibition of ROCK. Most importantly, acute administration of ROCK inhibitors improved the function of hearts from diabetic rats, both in vivo, as assessed by echocardiography, and in vitro, in the isolated working heart.\(^6\) These data suggest that the RhoA/ROCK signalling pathway plays an important role in the development of diabetic cardiomyopathy.

The regulation of RhoA activity through its interaction with guanine-nucleotide dissociation inhibitors (RhoGDIs), guanine-nucleotide exchange factors (RhoGEFs), and
Male Wistar rats weighing 250–300 g were obtained from the UBC and drug treatment was administered equivalent volumes of saline via the same route. Blood glucose was measured 72 h after STZ administration, and induction of diabetes was confirmed by the presence of hyperglycaemia (blood glucose > 18 mmol/L). To determine the effects of chronic inhibition of iNOS, 1 week after injection of STZ, control and diabetic rats were subdivided into two groups, one of which received the selective iNOS inhibitor, N\(^\text{\textsuperscript{6}}\)-(1-iminoethyl)-l-lysine dihydrochloride (L-NIL) dissolved in water at a dose of 3 mg/kg/day by oral gavage. The other group received equivalent volumes of vehicle by the same route. Eight weeks later, rats were weighed and blood was taken for measurement of glucose levels. Then rats were deeply anaesthetized with sodium pentobarbital (65 mg/kg, IP) and hearts were collected and frozen for biochemical analysis. All protocols were approved by the UBC Animal Care Committee.

### 2.2 Isolated working heart studies

The contractile function of isolated working hearts from vehicle and L-NIL-treated control and diabetic rats was measured as described in Lin et al.\(^7\). Hearts were initially perfused through the aorta at a rate of 17 mL/min with Chenoweth-Koelle (CK) solution (composition in mM: NaCl 120, KCl 5.6, CaCl\(_2\) 2.18, MgCl\(_2\) 2.1, NaHCO\(_3\) 19.2, and glucose 10) maintained at 37°C and bubbled with 95% O\(_2\)/5% CO\(_2\). Following cannulation of the pulmonary vein, cardiac work was initiated by switching the perfusion system from the retrograde mode to the working heart mode. Left ventricular developed pressure (LVDP), and the rates of contraction and relaxation (\(+\text{dP/dt}\) and \(-\text{dP/dt}\)) 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 in the left ventricle. The afterload was kept constant throughout the perfusion. The heart was paced at 300 b.p.m. using a Grass model SD 90 stimulator connected to a stainless steel electrode placed on the left atrium.

### 2.3 Preparation of isolated rat ventricular cardiomyocytes

Ca\(^2+\)-tolerant cardiomyocytes were isolated as described in Lin et al.\(^8\). Cardiomyocytes were either snap-frozen in liquid nitrogen or were maintained in primary culture in order to assess the effects of drug treatment. For the latter, cardiomyocytes were resuspended in medium 199 supplemented with 1% BSA, 100 units/mL penicillin, 100 \(\mu\)g/mL streptomycin, 1.2 mM L-carnitine, and 25 mM HEPES (pH 7.4). Cells prepared from a single rat heart were plated on laminin-coated culture dishes and allowed to recover for 3 h. Cultured cells were then incubated for 18 h at 37°C in supplemented medium 199 containing the various treatments and then snap-frozen in liquid nitrogen.

### 2.4 Western blot analysis

Frozen ventricles and isolated cardiomyocytes were homogenized as described in Lin et al.\(^8\). Both ventricular and myocyte homogenates were spun at 100,000 g for 5 min, and the protein content of the supernatants was determined by the Bradford protein assay. Equal amounts of protein (40 \(\mu\)g) from each sample were separated by 8% SDS–PAGE and transferred to a PVDF membrane. The membranes were blocked for 1 h in a solution of 5% skim milk and then incubated overnight at 4°C with primary antibodies against ROCK, RhoA, and iNOS. Samples were incubated with goat anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:10,000, Santa Cruz Biotechnology Inc.) for 1 h then exposed to chemiluminescence reagents (Amersham Inc., Cambridge, MA, USA). Densitometric analysis was performed to quantify band optical densities.

### 2.5 Assay of nitric oxide production

A commercially available assay kit (Cayman Chemical, MI, USA) was used to measure the nitrite/nitrate (NO\(_x\)) levels in whole-heart homogenates as an index of NO production.\(^9\) Samples were incubated with nitrate reductase enzyme for 3 h at room temperature to convert all nitrates to nitrites, which were quantified using the Griess reaction. Proteins were extracted from ventricular tissue according to the manufacturer’s protocol and protein concentration was determined using the Bradford method.\(^10\) Samples were measured in triplicate.
2.6 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 version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). LVDP, ±dP/dt, and −dP/dt were analysed by repeated measures ANOVA (general linear models approach) followed by the Newman–Keuls test, using NCSS 2000 (NCSS, Kaysville, UT, USA). For all results, the level of significance was set at P < 0.05.

3. Results
3.1 Expression of inducible nitric oxide synthase and RhoA in cardiomyocytes isolated from diabetic rats
We previously reported that RhoA expression was upregulated in cardiomyocytes from diabetic rats.6 In the present study, we examined whether this was associated with an increase in expression of iNOS in the same cells. As shown in Figure 1, both RhoA and iNOS expression levels were significantly increased in cardiomyocytes isolated from 12–14 week diabetic rat hearts compared with their age-matched controls.

3.2 Effect of sodium nitroprusside and lipopolysaccharide on RhoA expression in isolated cardiomyocytes
We next determined whether the NO donor, sodium nitroprusside (SNP), could increase the expression of RhoA in cardiomyocytes from non-diabetic rats, as was reported in vascular smooth muscle cells.8 Treatment of isolated cardiomyocytes in primary culture with 10 μM SNP for 18 h resulted in a significant increase in RhoA expression compared with control myocytes that were incubated for the same time in the absence of SNP (Figure 2A and B).

In order to determine whether iNOS induction was also associated with an increase in RhoA expression, the effect of treatment of isolated myocytes with LPS (Salmonella enterica serotype typhimurium) on expression of either RhoA and iNOS was determined. Treatment of myocytes with 20 μg/ml LPS for 18 h had no effect on the expression of either iNOS or RhoA (Figure 2C–E). However, a higher concentration of LPS (40 μg/ml) produced a significant increase in the expression of both iNOS and RhoA (Figures 2C–E and 3). The phosphorylation of RhoA at serine 188 was also increased in LPS-treated cardiomyocytes (Figure 3A), such that the ratio of phosphorylated to total RhoA remained unchanged compared with untreated myocytes (Figure 3B). The LPS-induced increases in both the expression and phosphorylation of RhoA were prevented by the selective, irreversible iNOS inhibitor, L-NIL (Figure 3), suggesting that they were secondary to the induction of iNOS by LPS.

3.3 Effect of 8 week N⁶-(1-iminoethyl)-L-lysine dihydrochloride treatment on RhoA and inducible nitric oxide synthase expression and RhoA, Lin-11–Isl-1–Mec-3 kinase, and ezrin/radixin/moesin phosphorylation
To investigate whether the increased expression of iNOS detected in hearts from diabetic rats was responsible for the associated increase in RhoA expression, diabetic rats were treated with L-NIL (3 mg/kg/day) by oral gavage for 8 weeks. At the end of this period, STZ-treated rats had significantly elevated blood glucose levels and reduced body weights compared with their age- and gender-matched controls, which were not altered by chronic L-NIL treatment (Table 1). The expression of iNOS was significantly elevated (Figure 4A and B), whereas the expression of eNOS was reduced and no change in levels of nNOS was detected (data not shown) in hearts from vehicle-treated diabetic rats. Immunohistochemical detection of iNOS in ventricular slices from diabetic rat hearts confirmed that it was expressed in cardiomyocytes (see Supplementary material online, Figure S1). To determine the effectiveness of L-NIL, total NOx levels were also measured in hearts from L-NIL and vehicle-treated animals as an index of NO production. In vehicle-treated diabetic rats, NOx levels in ventricular homogenates were significantly elevated (Figure 4C), consistent with the increased expression of iNOS. Although L-NIL had no significant effect on the expression of iNOS, it prevented the increase in NOx levels in diabetic hearts, suggesting that it was effectively inhibiting the enzyme (Figure 4C). Furthermore, in control rats, L-NIL treatment did not affect either mean arterial blood pressure (data not shown) or total cardiac NOx levels, suggesting that at
the dose used, L-NIL was selective for iNOS. RhoA expression was also significantly elevated in the STZ-treated rat hearts, and this increase was also prevented by treatment of diabetic rats with L-NIL ([Figure 4F]). However, the increased RhoA expression in hearts from untreated diabetic rats was not accompanied by a change in the phosphorylation of RhoA at serine 188 ([Figure 4D]), resulting in a significant decrease in the ratio of phospho-RhoA to total RhoA ([Figure 4F]). By reducing RhoA expression without affecting its phosphorylation, L-NIL treatment normalized the phospho-RhoA to total RhoA ratio ([Figure 4D–F]). L-NIL had no effect on the expression or phosphorylation of RhoA in hearts from control rats.

LIM kinase is an immediate downstream target of ROCK, and in our previous investigation, we found that the phosphorylation of LIMK was increased in hearts from diabetic rats, and that this could be abolished by inhibition of ROCK. In the present study, we confirmed the increase in LIMK phosphorylation in hearts from untreated diabetic rats and found that it was attenuated by treatment of the rats with L-NIL ([Figure 5A and B]). Similarly, the elevated phosphorylation of ERM, another downstream target of ROCK, in the diabetic rat hearts was significantly reduced by L-NIL treatment ([Figure 5C and D]).

3.4 Effect of 8 week N6-(1-iminoethyl)-L-lysine dihydrochloride treatment on contractile function of the diabetic hearts

As shown in [Figure 6], compared with controls, hearts from vehicle-treated diabetic rats showed a significant reduction in LVDP, +dP/dT, and -dP/dT at LAFP above 5 mmHg, which is characteristic of the left ventricular dysfunction of diabetic cardiomyopathy. Administration of L-NIL to diabetic rats prevented the deterioration of cardiac contractile function and significantly improved LVDP, +dP/dT, and -dP/dT compared with the diabetic untreated rats. L-NIL did not significantly alter the contractile function of hearts from control rats except for a small decrease in LVDP at the highest filling pressure (10 mmHg).

4. Discussion

The importance of the RhoA/ROCK pathway in cardiovascular physiology and pathophysiology has recently become clear. Extensive research is ongoing to elucidate role of this pathway in cardiovascular pathologies including hypertension, arteriosclerosis, and left ventricular hypertrophy and failure. We have previously reported that the RhoA/ROCK pathway is activated in diabetic...
cardiomyopathy and contributes to the pathogenesis of this complication.\textsuperscript{6} The present work provides evidence to support a role for iNOS in enhancing RhoA expression both in isolated cardiomyocytes and in diabetic rat hearts and highlights an important role for iNOS in mediating cardiac contractile dysfunction in the diabetic heart through upregulation of RhoA expression, thus contributing to increased activation of the RhoA/ROCK pathway.

A role for NO in positively regulating the expression of RhoA was first suggested by the observation that treatment of cultured vascular smooth muscle cells with the NO donor SNP produced significant increases in the expression of RhoA.\textsuperscript{8} Similarly, we have shown that a significant increase in RhoA expression can be induced in cultured cardiomyocytes by the same concentration of SNP, suggesting that NO can also positively regulate the expression of RhoA in the heart. Furthermore, treatment of cardiomyocytes with LPS induced concurrent expression of iNOS and RhoA, and the latter was prevented by L-NIL, an L-arginine analogue which acts as a selective, irreversible iNOS inhibitor,\textsuperscript{18} confirming that it is iNOS that is responsible for increasing RhoA expression.

The results of the present study confirm our previous reports of increased expression of RhoA\textsuperscript{6} and iNOS\textsuperscript{9} in hearts from chronically diabetic rats. The detection of elevated levels of iNOS in cardiomyocytes by immunoblotting of protein from isolated cardiomyocytes and by immunohistochemical detection in whole hearts suggests that cardiomyocytes rather than other cells such as inflammatory or endothelial cells are the major site of iNOS expression in the diabetic heart. Since expression of eNOS was reduced and no change in nNOS expression could be detected, it is likely that iNOS is responsible for the increase in NO\textsubscript{2} levels found in diabetic hearts. This is supported by the observation that L-NIL, at a dose previously reported to be selective for iNOS\textsuperscript{12} in vivo, completely prevented the increase in cardiac NO\textsubscript{2} levels and further suggests that iNOS activity was effectively inhibited in these hearts. The normalization of RhoA expression by L-NIL treatment suggests that in vivo, as well as in vitro, the increased levels of NO produced by iNOS are responsible for RhoA upregulation. Interestingly, we have also found that the cardiac NO\textsubscript{2} level and RhoA expression were elevated in the Goto-Kakizaki rat, a non-obese model of spontaneous type 2 diabetes (unpublished observations) at an age at which these animals have been reported to exhibit cardiomyopathy,\textsuperscript{19} suggesting that regulation of RhoA expression by iNOS may be common to both type 1 and 2 diabetes.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Effect of N\textsuperscript{6}-(1-iminoethyl)-L-lysine dihydrochloride treatment on lipopolysaccharide-induced increase in RhoA and inducible nitric oxide synthase expression in isolated cardiomyocytes. (A) A representative blot showing inducible nitric oxide synthase and RhoA expression and phosphorylation (p-RhoA), with glyceraldehyde-3-phosphate dehydrogenase as a loading control. (B) Inducible nitric oxide synthase, RhoA, and p-RhoA/RhoA ratio band optical densities were normalized by the optical densities of their corresponding glyceraldehyde-3-phosphate dehydrogenase band and expressed relative to the mean control value ($n=5-7$ in each group). Data are expressed as mean ± SEM. *$P < 0.05$ compared with control.}
\end{figure}

\begin{table}[h]
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\begin{tabular}{|l|c|c|c|c|}
\hline
Parameters & C & D & CT & DT \\
\hline
Body weight (g) Before treatment & $285.0 \pm 11.8$ & $282.6 \pm 13.6$ & $282.8 \pm 13.0$ & $285.3 \pm 10.0$ \\
After treatment & $549.3 \pm 21.8$ & $372.3 \pm 14.1^*$ & $556.5 \pm 16.5$ & $406.4 \pm 14.5^*$ \\
Blood glucose (mM) & $6.1 \pm 0.11$ & $21.2 \pm 0.5^*$ & $6.3 \pm 0.11$ & $22.1 \pm 0.87^*$ \\
\hline
\end{tabular}
\caption{Effect of N\textsuperscript{6}-(1-iminoethyl)-L-lysine dihydrochloride on body weight and blood glucose level in control and diabetic rats}
\label{tab:1}
\end{table}

Data are expressed as mean ± SEM. C, control; D, diabetic; CT, N\textsuperscript{6}-(1-iminoethyl)-L-lysine dihydrochloride-treated control; DT, N\textsuperscript{6}-(1-iminoethyl)-L-lysine dihydrochloride-treated diabetic ($n=8-9$ per group). *$P < 0.05$ compared with control.
The mechanism by which iNOS/NO might regulate RhoA expression in the heart is not known. However, the elevated levels of RhoA induced by NO production in vascular smooth muscle appear to be due to a combination of transcriptional and translational upregulation of the RhoA gene and decreased degradation of the RhoA protein. A number of reports have demonstrated that NO, acting through cGMP-dependent protein kinase (PKG), leads to phosphorylation of serine 188 in RhoA, thus protecting the protein from ubiquitin/proteasome-mediated degradation by increasing its interaction with RhoGDI.8,20,21 In addition, stimulation of the NO/PKG pathway by SNP or the cGMP analogue 8-pCPT-cGMP has also been shown to lead to increased RhoA gene transcription in cultured vascular smooth muscle cells.8,9 Chronic treatment of rats with a non-selective NOS inhibitor, N(omega)-nitro-L-arginine (L-NNA), was associated with a decrease in RhoA mRNA and protein expression in the aorta and pulmonary artery, suggesting that in vivo as well as in vitro, NO regulates RhoA expression at the level of gene transcription.9 The decrease in RhoA mRNA and protein expression induced by chronic hypoxia in the rat pulmonary artery was reversed by treatment with the phosphodiesterase 5 inhibitor, sildenafil, implicating the cGMP/PKG pathway in this process.

In addition to increasing RhoA expression in vascular smooth muscle cells by decreasing its degradation, phosphorylation at the Ser188 residue was reported to inactivate RhoA by enhancing the ability of RhoGDI to interact with and extract RhoA from the cell membrane.21,22 In the present study, consistent with studies in cultured vascular smooth muscle cells, iNOS upregulation was associated with an increase in RhoA expression and Ser188 phosphorylation in cultured cardiomyocytes treated with LPS. In contrast, in hearts from diabetic rats, the increase in RhoA expression was not associated with a corresponding increase in Ser188 phosphorylation. As a result, the ratio of phosphorylated to non-phosphorylated RhoA was significantly lower in hearts from untreated diabetic than control rats and was normalized by treatment of diabetic rats with L-NIL. This implies that iNOS induction, by positively regulating RhoA expression, may contribute to the increased RhoA activity that we previously observed in diabetic hearts, leading to an increase in the RhoA/ROCK pathway activity and contractile dysfunction. In agreement with this, we found that the
diabetes-induced increase in phosphorylation of the ROCK targets, LIMK and ERM, which were increased in hearts from untreated diabetic rats, were normalized by L-NIL treatment. Similarly, Sauzeau et al.8 showed that treatment of rats with L-NNA, in addition to reducing RhoA expression in the aorta and pulmonary artery, also inhibited RhoA-mediated calcium sensitization of the contractile apparatus. Expression and calcium sensitization were restored using the phosphodiesterase 5 inhibitor, sildenafil. On the basis of this, these authors concluded that RhoA expression is a limiting factor for RhoA-dependent functions, which include ROCK activation.

The discrepancy between our findings of a positive association between iNOS expression and RhoA/ROCK activity in diabetic rat hearts and the results of studies demonstrating that NO negatively regulates the activity of this pathway could arise from a number of different factors. However, the results of the present study and those of Sauzeau et al.8 suggest it may arise at least in part from differences between relatively acute studies in cultured cells vs. chronic studies in whole animals. In isolated cultured cells, the NO-mediated increase in RhoA phosphorylation that accompanies its increased expression has been shown to lead to accumulation of a pool of inactive GTP-bound RhoA in the cytosol that is available for translocation to its active site on the membrane.21,22 It has been pointed that this pool of RhoA may be activated independently of RhoGEF, although the mechanisms remain unknown.28 It is possible that under physiological and/or pathophysiological conditions in vivo, the mechanisms responsible for activation of this pool of cytosolic GTP-RhoA are functional.

If iNOS mediates increased RhoA expression in the diabetic hearts, then, on the basis of our previous findings that hyperactivity of the RhoA/ROCK pathway is implicated in diabetic cardiomyopathy and that normalizing this pathway significantly improves contractile function, L-NIL treatment would also be expected to improve contractile function in diabetic rat hearts. This possibility was investigated in isolated working hearts. Coinciding with the elevation of iNOS and RhoA expression as well as LIMK and ERM phosphorylation in the diabetic rat hearts, this NO is generally thought to mediate the expression of eNOS.31,33 Inhibiting iNOS activity limits its ability to upregulate RhoA and phosphorylation of LIMK and ERM, significantly improved the abovementioned variables. We have reported previously that the contractile dysfunction seen in hearts of diabetic rats was accompanied by elevation of RhoA expression and activity, and that acute inhibition of ROCK normalized the expression of RhoA and phosphorylation of LIMK and ERM, significantly improved calcium sensitization. Taken together, these data further support our hypothesis that iNOS mediates an increase in RhoA expression which contributes to an increased activity of the RhoA/ROCK pathway and contractile dysfunction. Inhibiting iNOS activity limits its ability to upregulate RhoA expression, thus diminishing the availability of the latter for subsequent activation of ROCK.

It is generally accepted that in diabetes, there is a marked decrease in the bioavailability of NO as a result of decreased eNOS function.29,30 Although the elevated levels of nitrites detected in the diabetic heart are consistent with increased production of NO from iNOS, this NO is generally thought to exert pathophysiological effects.31,32 In fact, studies have shown that increased expression of iNOS, through increased NO production, contributes to impaired function of eNOS.31,33

It is possible that, besides its effect on RhoA expression, inhibition of iNOS might have improved contractile function in diabetic rat hearts through other mechanisms involving NO or its reactive metabolites such as peroxynitrite. Nevertheless, our previous finding that normalizing the RhoA/ROCK pathway activity leads to complete recovery of left ventricular contractile function strongly suggests that preventing the upregulation of RhoA is one of the major mechanisms by which iNOS inhibition produced
Figure 6. Effect of chronic N^6-(1-iminoethyl)-L-lysine dihydrochloride treatment on the contractile function of isolated working hearts from control and diabetic rats. Increases in left ventricular developed pressure (LVDP, upper panel), \( +\frac{dP}{dt} \) (middle panel), and \( -\frac{dP}{dt} \) (lower panel) in response to increases in left atrial filling pressure (LAFP). Black squares represent untreated control hearts; open squares, N^6-(1-iminoethyl)-L-lysine dihydrochloride-treated control hearts; black triangles, untreated diabetic hearts; open triangles, N^6-(1-iminoethyl)-L-lysine dihydrochloride-treated control hearts; open squares, N^6-(1-iminoethyl)-L-lysine dihydrochloride-treated control hearts; black triangles, untreated diabetic hearts; open triangles, N^6-(1-iminoethyl)-L-lysine dihydrochloride-treated diabetic hearts (n = 6–7 in each group). Data are expressed as mean \( \pm \) SEM. *P < 0.05 compared with all other groups; **P < 0.05 compared with control group; ***P < 0.05 compared with control and diabetic groups; ****P < 0.05 compared with control and diabetic control groups. C, control; D, diabetic; CT, N^6-(1-iminoethyl)-L-lysine dihydrochloride-treated control; DT, N^6-(1-iminoethyl)-L-lysine dihydrochloride-treated diabetic.

In conclusion, the results of the present study demonstrate that iNOS induces increased expression of RhoA in isolated cardiomyocytes and that the increased expression and activity of the RhoA/ROCK pathway in the diabetic heart are secondary to induction of iNOS in these hearts. Reduction of the activity of the RhoA/ROCK pathway appears to be one of the important mechanisms by which iNOS inhibition improves contractile function in diabetic cardiomyopathy.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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