Diabetes-induced increased oxidative stress in cardiomyocytes is sustained by a positive feedback loop involving Rho kinase and PKCβ2

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Diabetes-induced increased oxidative stress in cardiomyocytes is sustained by a positive feedback loop involving Rho kinase and PKCβ2. We previously reported that chronic inhibition of the RhoA/Rho kinase (ROCK) pathway normalized contractile function of diabetic rat hearts, but the underlying mechanism is unclear. Protein kinase C (PKC) β2 has been proposed to play a major role in diabetic cardiomyopathy at least in part by increasing oxidative stress. Further evidence suggests that PKC positively regulates RhoA expression through induction of inducible nitric oxide synthase (iNOS) in diabetes. However, in preliminary studies, we found that inhibition of ROCK itself reduced RhoA expression in diabetic hearts. We hypothesized that there is an interaction between RhoA/ROCK and PKCβ2 in the form of a positive feedback loop that sustains their activation and the production of reactive oxygen species (ROS). This was investigated in cardiomyocytes isolated from diabetic and control rat hearts, incubated with or without cytochalasin D or inhibitors of ROCK, RhoA, PKCβ2, or iNOS. Inhibition of RhoA and ROCK markedly attenuated the diabetes-induced increases in PKCβ2 activity and iNOS and RhoA expression in diabetic cardiomyocytes, while having no effect in control cells. Inhibition of PKCβ2 and iNOS also normalized RhoA expression and ROCK overactivation, whereas iNOS inhibition reversed the increase in PKCβ2 activity. Each of these treatments also normalized the diabetes-induced increase in production of ROS. Actin cytoskeleton disruption attenuated the increased expression and/or activity of all of these targets in diabetic cardiomyocytes. These data suggest that, in the diabetic heart, the RhoA/ROCK pathway contributes to contractile dysfunction at least in part by sustaining PKCβ2 activation and ROS production via a positive feedback loop that requires an intact cytoskeleton.

Diabetic cardiomyopathy; RhoA; inducible nitric oxide synthase; adult cardiomyocytes

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MATERIALS AND METHODS

Materials. Y-27632, H-1152, and 1400W (Calbiochem), cytochalasin D, and LY-333531 (ruboxistaurin; Enzo Life Sciences), cell
permeable C3 transferase (Cytoskeleton), streptozotocin (STZ) and medium 199 (Sigma-Aldrich), collagenase II (Worthington Biochem), laminin (Roche), and pentobarbital sodium (Bimeda) were used. **Induction of diabetes mellitus in Wistar rats.** Male Wistar rats (170–200 g) were lightly anesthetized with isoflurane 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 >18 mmol/L, measured with a One Touch glucometer (Life Scan) 1 wk after injection, were considered diabetic. All animals were housed under identical conditions and were given free access to food and water. 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 United States National Institutes of Health (NIH publication no. 85–23, revised 1996). All protocols were approved by the University of British Columbia Animal Care Committee.

**Isolation of adult rat ventricular cardiomyocytes.** Calcium-tolerant adult ventricular cardiomyocytes were isolated as detailed previously (32). Rats were anesthetized with pentobarbital sodium (100 mg/kg ip). Once the stage of deep surgical anesthesia was reached, confirmed adult ventricular cardiomyocytes were isolated as detailed previously by the United States National Institutes of Health (NIH published by the United States National Institutes of Health (NIH publication no. 85–23, revised 1996). All protocols were approved by the University of British Columbia Animal Care Committee.

The concentration of the PKC\(\text{\textsuperscript{2}}\) inhibitor peptide was determined by assessing the percentage of cells that excluded trypan blue dye. Cell viability was >70% in all groups.

**Cell culture and treatment studies.** Cardiomyocytes were resuspended in medium 199 and allowed to attach to laminin-coated plates (20 \(\mu\)g/ml) for 2 h before treatment. Afterwards, cells were incubated in 5.5 mM glucose (low glucose), 5.5 mM glucose plus 19.5 mM mannitol (osmotic control), or 25 mM glucose (high glucose) for 24 h to allow time for expression changes to occur. A time course of the effect of high glucose on ROCK activity was also performed by incubating cardiomyocytes in high glucose for different time periods.

Cardiomyocytes isolated from diabetic rats and their age-matched controls were incubated in low glucose medium 199 for up to 8 h. Cells were treated with the ROCK inhibitor Y-27632 (1 \(\mu\)M), the PKC\(\text{\textsuperscript{2}}\) translocation inhibitor peptide (2 \(\mu\)g/ml), the PKCB\(\text{\textsuperscript{2}}\) inhibitor LY-333531 (20 nM), the iNOS inhibitor 1400W (10 \(\mu\)M), the PKC\(\text{\textsuperscript{2}}\) inhibitor peptide synthesis and treatment. Based on the work of Stebbins and Mochly-Rosen (53) we had a selective PKCB\(\text{\textsuperscript{2}}\) translocation inhibitor peptide with the sequence QEVIRN synthesized (GenBio, Shanghai, China). This sequence was conjugated to the C-terminus of the peptide (the complete peptide sequence was RRRQRKK KRGYC-SS-CQEVIRN), which becomes reduced once the peptide enters the cell liberating the free PKCB\(\text{\textsuperscript{2}}\) inhibitor sequence. The peptide sequence is present in the V5 region of PKCB\(\text{\textsuperscript{2}}\) but not PKCB\(\text{\textsuperscript{1}}\) and is responsible for the selective binding of PKCB\(\text{\textsuperscript{2}}\) to RACK1 (53). Cardiomyocytes from control and diabetic rat hearts were cultured in medium 199 and treated with either the PKCB\(\text{\textsuperscript{2}}\) inhibitor peptide or the PKCB\(\text{\textsuperscript{2}}\) translocation and at the same time was devoid of cytotoxicity.

**Determination of PKC\(\text{\textsuperscript{2}}\) translocation.** Cardiomyocytes from control and diabetic rat hearts were mechanically lysed in a detergent-free MOPS buffer. Cell lysates were then centrifuged at 2,000 g for 5 min, and the supernatant was ultracentrifuged at 100,000 g for 1 h. Afterwards, the supernatant (S1) was collected (cytosolic fraction), and the pellet was resuspended in Triton X-100 buffer and ultracentrifuged again at 100,000 g for 1 h. The supernatant (S2) was collected (membrane fraction). Both S1 and S2 were snap-frozen in liquid nitrogen and stored at −80°C.

**ROCK activity assay.** The activity of ROCK was measured by determining the extent of Thr\(\text{\textsuperscript{696}}\) phosphorylation of MYPT1 in an in vitro assay as described in Liu and Liao (33). Briefly, cell lysates were added to a reaction mixture containing 50 mM Tris (pH = 7.5), 0.1 mM EGTA, 10 mM magnesium acetate, 1 mM ATP, 0.1% β-mercaptoethanol, and 500 ng truncated MYPT1 \(^{(554-880)}\) and incubated at 30°C for 30 min. The level of Thr\(\text{\textsuperscript{696}}\) phosphorylation of MYPT1 was determined by Western blotting.

**Western blot.** Proteins from each sample were separated by 8–12% SDS-PAGE and immunoblotted using primary antibodies against iNOS, GAPDH, Thr\(\text{\textsuperscript{696}}\)-MYPT1, Ser\(\text{\textsuperscript{188}}\)-Rhoa, Rhoa, PKCB\(\text{\textsuperscript{2}}\) (Santa Cruz Biotechnology), Thr\(\text{\textsuperscript{502}}\) pLIMK1/2 (Cell Signaling Technology), or Thr\(\text{\textsuperscript{622}}\)-pPKCB\(\text{\textsuperscript{2}}\) (Life Technologies). The intensity of the protein bands was determined by densitometry and normalized to GAPDH or its corresponding total protein in the same preparation.

**Rhoa activity assay.** A commercially available Rhoa activation assay kit (Cytoskeleton) was used to determine the relative amount of active Rhoa in 5 \(\times\) 10\(\text{\textsuperscript{5}}\) freshly isolated cardiomyocytes.

**Adenoviral infection of adult rat ventricular cardiomyocytes.** Cardiomyocytes were isolated from nondiabetic rat hearts and plated on laminin-coated culture dishes at a cell density of 5 \(\times\) 10\(\text{\textsuperscript{4}}\) cells/cm². After attachment, cells were transduced with replication incompetent infections of the adenovirus type 5 encoding dominant-negative Rhoa mutant Rhoa N19 (Ad-Rhoa N19) or green fluorescent protein (Ad-GFP) as control, driven by CMV promoter at a multiplicity of infection of 40 (Applied Biological Materials), for 90 min. Afterwards, the medium was changed to medium 199 containing either 5.5 or 25 mM glucose, and cells were incubated for 24 h.

**Determination of ROS levels.** The levels of ROS were measured using live cell imaging of dihydroethidium (DHE)-loaded cardiomyocytes. After treatment for the specified periods, cells were incubated in Hanks’ balanced salt solution containing 5 \(\mu\)M DHE for 10 min at 37°C. Cells were then immediately imaged using a FX10i LIV Laser Scanning Confocal Microscope (Olympus Canada). Controls were used to correct for autofluorescence. After entering the cell, DHE is oxidized by ROS, mainly superoxide, to red fluorescent products that accumulate in the nucleus (27).

**F/G-actin assay.** Freshly isolated cardiomyocytes were plated on laminin-coated culture dishes. Cells were treated with C3 exoenzyme (2 \(\mu\)g/ml) or cytochalasin D (1, 2, or 5 \(\mu\)M) for 24 h or left untreated. Myocytes were then processed for the isolation and determination of globular actin (G-actin) and filamentous actin (F-actin) content using a commercially available assay kit (Cytoskeleton).

**Statistical analysis.** All values are expressed as means ± SE; \(n\) denotes the no. of animals in each group. Statistical analysis of all data was performed using one-way ANOVA followed by the Newman-Keuls test when more than two groups were compared, using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

**RESULTS**

High glucose increases Rhoa/ROCK pathway activity in isolated cardiomyocytes. We first investigated whether the increased activation of the Rhoa/ROCK pathway that we previously detected in hearts of diabetic rats (32) could be attributed to exposure of cardiomyocytes to high glucose. Incubation of adult cardiomyocytes isolated from nondiabetic rat hearts in 25 mM glucose produced a time-dependent increase in ROCK activity that reached statistical significance.
after 12 h or more of incubation (Fig. 1A). To further confirm the effect of high glucose on ROCK activity, we determined the level of Thr565/560 phosphorylation of LIM kinase (LIMK) 1/2, another ROCK downstream target. Indeed, we found that LIMK phosphorylation increased significantly with incubation of cardiomyocytes in high glucose for 24 h (Fig. 1B). The increase in ROCK activity was blocked by treatment with Y-27632 (Fig. 1C). An equimolar concentration of mannitol did not alter ROCK activity (Fig. 1C). Additionally, RhoA expression (Fig. 1D) and activity (Fig. 2C) were significantly elevated in cells incubated in high glucose. The elevated RhoA expression was blocked by the ROCK inhibitors Y-27632 and H-1152 (Fig. 1D).

High glucose-induced PKCβ2 activation is inhibited by RhoA/ROCK inhibition. We next determined whether the RhoA/ROCK pathway regulated PKCβ2 activity under high glucose conditions. In agreement with our previous results (38), incubation in high glucose had no significant effect on total PKCβ2 levels but increased its Thr641 phosphorylation. The latter was significantly attenuated by inhibition of ROCK with Y-27632 or H-1152 (Fig. 2B). Phosphorylation of Thr641 is essential for the appropriate subcellular localization and catalytic function of PKCβ2 (16, 28) and is used as an index of PKCβ2 activation (1, 9, 35, 45). To confirm involvement of the pathway, cardiomyocytes were also treated with the Rho inhibitor C3 transferase at a concentration (2 μg/ml) that effectively inhibited ROCK activity (Fig. 2A), which significantly decreased PKCβ2 phosphorylation (Fig. 2B). Last, cardiomyocytes were infected with Ad-RhoA N19 and incubated in high or low glucose for 24 h. In preliminary experiments using Ad-GFP, we found that the cardiomyocyte transduction efficiency was 85% after 24 h (data not shown). Ad-RhoA N19 treatment completely prevented the high glucose-induced increase in RhoA activity (Fig. 2C), and this was associated with loss of the high glucose-induced increase in PKCβ2 Thr641 phosphorylation (Fig. 2D).

Fig. 1. Effect of duration of incubation of isolated cardiomyocytes in 25 mM glucose on phosphorylation of MYPT1 (A) and LIM kinase (LIMK) 1/2 (B) as indexes of Rho kinase (ROCK) activity. *p < 0.05 compared with the first group (n = 4 – 6). C: effect of the ROCK inhibitor Y-27632 (1 μM) treatment on ROCK activity in cardiomyocytes incubated in 25 mM glucose for 24 h. Mannitol was used as an osmotic control. *p < 0.05 compared with the other groups (n = 4 – 6). D: effect of the ROCK inhibitors Y-27632 (1 μM) and H-1152 (1 μM) treatment for 24 h on RhoA expression in cardiomyocytes incubated in 25 mM glucose. *p < 0.05 compared with the other groups (n = 7).
RhoA/ROCK inhibition attenuates PKCβ2 Thr641 phosphorylation in cardiomyocytes isolated from diabetic hearts. We next investigated whether RhoA/ROCK regulates PKCβ2 activity in vivo in hearts of diabetic rats. Cardiomyocytes isolated from diabetic and control rat hearts were treated with C3 transferase or Y-27632 for 8 h (longer periods of incubation caused rapid decline in the viability of cardiomyocytes from diabetic rat hearts). It is noteworthy that ROCK activity in cardiomyocytes isolated from diabetic rat hearts remained significantly elevated for up to 16 h after isolation (Fig. 3A). The diabetes-induced elevation of PKCβ2 Thr641 phosphorylation was attenuated to control levels by both Y-27632 and by C3 transferase (2 μg/ml) treatment for 24 h on protein kinase C (PKC) β2 Thr641 phosphorylation in cardiomyocytes incubated in 25 mM glucose. *P < 0.05 compared with the other groups (n = 6). Effect of human adenovirus type 5 encoding dominant-negative RhoA mutant RhoA N19 (Ad-RhoA N19) on RhoA activity (C) and PKCβ2 Thr641 phosphorylation (D) in cardiomyocytes incubated in 5.5 or 25 mM glucose for 24 h. Ad-GFP, human adenovirus type 5 encoding green fluorescent protein. *P < 0.05 compared with the other groups (n = 3).

RhoA/ROCK inhibition attenuates PKCβ2 Thr641 phosphorylation in cardiomyocytes isolated from diabetic hearts. We next investigated whether RhoA/ROCK regulates PKCβ2 activity in vivo in hearts of diabetic rats. Cardiomyocytes isolated from diabetic and control rat hearts were treated with C3 transferase or Y-27632 for 8 h (longer periods of incubation caused rapid decline in the viability of cardiomyocytes from diabetic rat hearts). It is noteworthy that ROCK activity in cardiomyocytes isolated from diabetic rat hearts remained significantly elevated for up to 16 h after isolation (Fig. 3A). The diabetes-induced elevation of PKCβ2 Thr641 phosphorylation was attenuated to control levels by both Y-27632 and by C3 transferase (Fig. 3B), which had no effect on PKCβ2 phosphorylation in control cardiomyocytes (data not shown). The inhibitory effect of Y-27632 on PKCβ2 Thr641 phosphorylation in diabetic cardiomyocytes was evident even after short-term (30 min) treatment (Fig. 3C).

We also determined the effect of C3 transferase and Y-27632 treatment on translocation of PKCβ2 to the plasma membrane, a hallmark of PKC activation (16). There was a marked increase in the level of PKCβ2 in the membrane fraction in diabetic cardiomyocytes that was normalized by C3 transferase and Y-27632 as well as by the selective PKCβ2 translocation inhibitor peptide (Tat-β2V5; Fig. 3E), which also normalized PKCβ2 Thr641 phosphorylation in cardiomyocytes from diabetic rats (Fig. 3D). These inhibitors had no effect on PKCβ2 translocation in control cardiomyocytes (Fig. 3F).

ROCK inhibition attenuates the PKCβ2-mediated iNOS induction in cardiomyocytes isolated from diabetic rat hearts. The RhoA/ROCK pathway regulation of PKCβ2 activation...
would also be expected to affect the expression of its downstream target, iNOS. Indeed, treatment with either Y-27632 or the PKC/θ/H9252 inhibitor LY-333531, at a concentration that we have previously shown to be effective in inhibiting PKC/θ/H9252 activity (38) and that is selective for PKC/θ/H9252 (26), significantly reduced the diabetes-induced upregulation of iNOS (Fig. 4A).

Additionally, Tat-θ/2V5 also significantly attenuated increased iNOS expression in cardiomyocytes from diabetic rat hearts (Fig. 4B). On the other hand, C3 transferase failed to significantly alter iNOS expression in diabetic cardiomyocytes, although there was a slight downward trend (Fig. 4A). For this reason and since C3 inhibits not only RhoA, but also RhoB and -C, we determined the effect of Ad-RhoA N19 on iNOS expression in cardiomyocytes incubated in high glucose for 24 h. Ad-RhoA N19 treatment abolished the high-glucose-induced increase in iNOS expression (Fig. 4C). Interestingly, Ad-RhoA N19 significantly increased iNOS expression in cardiomyocytes incubated in low glucose (Fig. 4C). We did not infect diabetic cardiomyocytes with Ad-RhoA N19 since it was not possible to maintain the cells viable for a time period sufficient for loss of RhoA activity.

Inhibition of PKC/θ, iNOS, RhoA, or ROCK disrupts the positive feedback loop and abrogates the sustained RhoA/ROCK pathway activation in diabetes. We next tested whether the hypothesized feedforward loop could be detected in diabetic cardiomyocytes. We found that the diabetes-induced increase in ROCK activity was not only blocked by Y-27632 and C3 transferase but also by treatment with the iNOS inhibitor 1400W for 8 h (Fig. 5A). The latter also normalized PKC/θ phosphorylation and translocation (Fig. 3, B and E), consistent with the proposed feedback loop. ROCK activity was also markedly attenuated by LY-333531 (Fig. 5A) and Tat-θV5 treatment for 8 h in diabetic cardiomyocytes (Fig. 5B). In contrast, treatment with Tat-θV5 for 30 min did not significantly alter ROCK activity (Fig. 5C).

To establish that ROCK positively reinforces RhoA through the PKC/θ/iNOS pathway, we determined the effect of the abovementioned inhibitors on RhoA expression and inhibitory Ser188 phosphorylation. In vehicle-treated cardiomyocytes from diabetic rats, RhoA expression but not Ser188 phosphorylation was significantly increased (Fig. 6A). The resulting diminished p-RhoA-to-RhoA ratio leads to a larger pool of readily acti-
vatable RhoA, which contributes to the elevated ROCK activity in the diabetic state. Treatment with Y-27632, 1400W, or LY-333531 failed to significantly alter RhoA Ser188 phosphorylation (Fig. 6A). On the other hand, these treatments were able to significantly decrease RhoA expression (Fig. 6A), normalizing the p-RhoA-to-RhoA ratio (Fig. 6B). The same treatments had no effect on RhoA in control cardiomyocytes (data not shown). Additionally, Tat-β2V5 significantly reduced RhoA expression in cardiomyocytes from diabetic animals compared with cardiomyocytes treated with Tat47–57 (Fig. 6C).

Actin cytoskeleton disruption mimics the effects of ROCK inhibition on the positive feedback loop. The actin cytoskeleton is a major downstream target of the RhoA/ROCK pathway (8), and, in diabetic cardiomyocytes, actin polymerization is significantly increased (32). Therefore, we investigated whether increased actin polymerization contributes to the effects of ROCK on PKCβ2 and in turn on iNOS and RhoA. Treatment of cardiomyocytes from diabetic rats with the actin depolymerizer cytochalasin D mimicked the effects of ROCK inhibition on PKCβ2 Thr641 phosphorylation and translocation, and iNOS and RhoA expression (Fig. 7, A, B, D, and E). This was associated with loss of ROCK activity and normalization of Ser188 p-RhoA-to-total RhoA ratio (Fig. 7, C and F). The concentration of cytochalasin D used in this study did not alter cardiomyocyte morphology and produced similar extent of actin depolymerization as C3 transferase (Fig. 7G). These results suggest a role for the actin cytoskeleton in the positive feedback loop that sustains RhoA and ROCK activation.

Disruption of the positive feedback loop normalizes ROS production. Oxidative stress plays an important role in mediating the deleterious effects of PKCβ2 in diabetes. Therefore, we determined the effect of disruption of the loop on diabetes-induced oxidative stress. Incubation of cardiomyocytes from diabetic rats with C3 or Y-26732 for 1 h produced a significantly greater reduction in ROS production than did either Tat-β2V5 or 1400W (Fig. 8B). However, following 8 h incu-
bation, all inhibitors markedly attenuated ROS to near control levels (Fig. 8D).

DISCUSSION

The RhoA/ROCK pathway has been shown by numerous researchers to play a pivotal role in cardiovascular pathologies, and there is great interest in ROCK inhibitors as potential therapies for many of these diseases (reviewed in Ref. 15). We reported previously that the activity of the RhoA/ROCK pathway is elevated in diabetic cardiomyopathy and that acute treatment with ROCK inhibitors significantly improves the contractile function of hearts from diabetic rats (32). The novel findings of the present study are that, in the diabetic heart, 1) activation of the RhoA/ROCK pathway leads to increased PKC\textsubscript{\(H9252\)} activity; 2) the increased activity of PKC\textsubscript{\(H9252\)} and the RhoA/ROCK pathway is sustained by a positive feedback loop; and 3) disrupting the loop markedly attenuates diabetes-induced increases in oxidative stress. These data suggest that the mechanism by which the RhoA/ROCK pathway contributes to contractile dysfunction in the diabetic heart is at least in part through activation of PKC\textsubscript{\(H9252\)} and promotion of the production of ROS.

There is much evidence to suggest that hyperglycemia-induced activation of PKC\textsubscript{\(H9252\)} in the heart contributes to the development of diabetic cardiomyopathy (22, 47, 56). The results of the present study show that treatment of cardiomyocytes incubated in high glucose or isolated from diabetic rat hearts with a concentration of Y-27632 or H-1152 that has been shown to be selective for ROCK over other kinases, including PKC (13), as well as inhibition of ROCK activation with either C3 exoenzyme or by overexpression of Ad-RhoA N19, prevented the increase in PKC\textsubscript{\(H9252\)} activity. These data strongly implicate the RhoA/ROCK pathway in the activation of PKC\textsubscript{\(H9252\)} in the diabetic heart. Our further observation that inhibition of ROCK or PKC\textsubscript{\(H9252\)} prevented the diabetes-induced increase in RhoA expression supports the hypothesis that the
interaction between the RhoA/ROCK pathway and PKCβ2 is in the form of a positive feedback loop. The finding that inhibition of iNOS, which we have shown previously to prevent the diabetes-induced increase in RhoA expression and ROCK activity, also prevented the activation of PKCβ2 in the heart provides additional evidence in support of the loop. This could not be detected in cardiomyocytes isolated from control hearts, indicating that this feedback mechanism occurs only under pathophysiological conditions.

There is compelling evidence linking increased oxidative stress, in particular ROS production, to the development of diabetic cardiovascular complications (6, 39). As noted in the introduction, oxidative stress contributes to the activation of PKCβ2 in the heart as in other tissues (19, 57), whereas increased PKCβ2 activity has been shown to lead to increased production of ROS in diabetes (30, 34, 40, 42, 44, 63). Similarly, oxidative stress appears to positively regulate induction of iNOS (61), whereas uncoupling of both endothelial nitric oxide synthase and iNOS in diabetes leads to the generation of ROS (43, 49). Consistent with this, direct inhibition of both PKCβ2 and iNOS decreased ROS production in diabetic cardiomyocytes in the present study. In addition, however, we found that inhibition of both RhoA and ROCK also markedly attenuated ROS production. ROS production was reduced to the same extent by long-term incubation with each of the inhibitors, a finding that is consistent with the presence of the loop and that demonstrates that its disruption at any point reduces oxidative stress. It should also be noted that short-term exposure to inhibitors of the RhoA/ROCK pathway produced more profound decreases in ROS production than did direct inhibition of PKCβ2 or iNOS. This may indicate that, in addition to increasing oxidative stress by promoting the activity of the loop, RhoA/ROCK may promote production of ROS through other mechanisms.

The production of ROS has been implicated in the phenomenon known as “metabolic memory,” the persistence of diabetic complications despite normalization of glucose levels (7, 21). Interestingly, we found that ROS production remained elevated for at least 8 h after cardiomyocytes isolated from diabetic hearts were cultured in low glucose, and this was
associated with continued activation of ROCK and PKCβ2. Although it is not clear how long this sustained activation continues, the possible role of the positive feedback loop in metabolic memory in diabetic hearts warrants further investigation.

The results of the present study suggest that an intact actin cytoskeleton is required to sustain the activity of the positive feedback loop and its production of ROS. The RhoA/ROCK pathway is a well-known regulator of the actin cytoskeleton (8, 54), and we have shown previously that actin polymerization is increased in cardiomyocytes from diabetic rats as a result of RhoA/ROCK activation (32). On the other hand, an intact cytoskeleton was shown to be required for RhoA activation in response to leptin in neonatal cardiomyocytes (60). An important role for the actin cytoskeleton in the activation of PKCβ2 has also been reported. For instance, a number of researchers have demonstrated that PKCβ2 associates with the actin cytoskeleton in different cell types (4, 14, 18, 25, 46). Blobe et al. (4) reported that PKCβ2, but not PKCβ1, binds to F-actin and this enhances its autophosphorylation and activation. Moreover, Pascale et al. (46) found that an intact actin cytoskeleton was essential for the translocation of PKCβ2 to the plasma membrane of astrocytes upon activation. Thus it is possible that the actin depolymerization produced by cytochalasin D in the present study interfered with the activity of the loop at multiple sites, including activation of both RhoA and PKCβ2.

A number of researchers have reported cross talk between RhoA/ROCK and PKC under high glucose conditions or in diabetes, where ROCK was reported to be either upstream (31, 41) or downstream (23, 58, 59) of PKC. Our results demonstrating that RhoA/ROCK and PKCβ2 form part of a positive feedback loop help to reconcile those reports. Interestingly, Xie et al. (59) found that, although high glucose-induced CPI-17 phosphorylation in cultured vascular smooth muscle cells was abolished by short-term RhoA or ROCK inhibition for 30 min, it was not inhibited by PKC inhibition for the same length of time. However, if the PKC inhibitor treatment was extended for 48 h, the high glucose-induced CPI-17 phosphorylation was attenuated, and this was associated with a decrease in RhoA and ROCK activity. These results are in agreement with our suggested positive feedback loop in that PKCβ2 does not activate RhoA and ROCK until iNOS and consequently RhoA

Fig. 7. Effect of cytochalasin D (CD, 2 μM) treatment for 8 h on Thr641 phosphorylation of PKCβ2 (A), PKCβ2 membrane translocation (B), ROCK activity (C), iNOS expression (D), RhoA expression (E), and Ser188 phosphorylation (F) in cardiomyocytes isolated from diabetic rat hearts. G: representative Western blot of the effect of C3 exoenzyme or cytochalasin D treatment for 24 h on the F-to-G-actin ratio in isolated adult rat cardiomyocytes and photomicrographs of isolated adult cardiomyocytes treated for 24 h with C3 exoenzyme or cytochalasin D. *P < 0.05 compared with the other groups (n = 3–4).
expression is increased, a process that may take hours to occur. On the other hand, our data demonstrate that the effect of ROCK inhibition on PKCβ phosphorylation and translocation was much more rapid, occurring within 30 min.

In the present study, inhibition of ROCK with Y-27632 decreased iNOS expression in cardiomyocytes from diabetic rat hearts. Similarly, infection of cardiomyocytes incubated in high glucose with the dominant-negative RhoA mutant Ad-RhoAN19 also significantly reduced iNOS expression. On the other hand, in control cells, the use of C3 exoenzyme or RhoA N19 increased iNOS expression, and, in the case of RhoA N19, this increase was statistically significant. This is consistent with previous reports indicating that inhibition of Rho by statins or C3 exoenzyme augments cytokine-induced iNOS induction in cells incubated in low glucose media (17, 37, 48). Our data provide a possible explanation for the opposite effects of RhoA inhibition on iNOS in diabetic vs. nondiabetic conditions. While activation of RhoA appears to suppress iNOS induction under nondiabetic conditions, the diabetes-induced positive feedback loop enables RhoA to upregulate iNOS, and the latter mechanism overrides the former, resulting in a net decrease in iNOS expression on inhibition of RhoA. Interestingly, Iwasaki et al. (24) showed that ROCK inhibition decreased the transcriptional activity of nuclear factor-κB (NF-κB), the main transcription factor for iNOS, only under high glucose conditions. Additionally, we previously reported

Fig. 9. Schematic diagram summarizing the main findings of the present study. In diabetes, hyperglycemia leads to activation of a positive feedback loop that sustains the activation of RhoA/ROCK and PKCβ2 in cardiomyocytes. This loop requires an intact actin cytoskeleton for its operation and contributes to the elevation of oxidative stress, eventually resulting in cardiomyocyte damage and diabetic cardiomyopathy.
that PKCβ2 inhibition decreased Ser536 phosphorylation and activation of the p65 subunit of NF-κB only under high glucose conditions (38).

Adult ventricular cardiomyocytes were used in the present study, rather than neonatal cardiomyocytes or H9c2 cardiomyoblasts, since they have the closest resemblance to adult human cardiac cells with respect to morphology, function, and cellular signaling (20). However, a limitation of these cells, particularly those from diabetic hearts, is that, within 24 h after isolation, viability drops substantially or cells begin to dedifferentiate. This limits the use of genetic approaches that require longer times to knock down target proteins such as PKCβ2 and ROCK that have a relatively long half-life and slow turnover (5). However, we were able to use alternative approaches to support the results obtained with chemical inhibitors, including Tat-β1V5 and Ad-RhoA N19. The results obtained by these approaches are in complete agreement with those obtained with the small molecule inhibitors, providing additional support for our findings.

In conclusion, the results of this study suggest that, in the diabetic heart, the RhoA/ROCK pathway contributes to contractile dysfunction at least in part by sustaining PKCβ2 activation, iNOS induction, and ROS production via a positive feedback loop that requires an intact cytoskeleton (Fig. 9). The significance of this loop is that it links proteins that have been shown to play a pivotal role in the pathogenesis of diabetic cardiomyopathy, since inhibition of ROCK, PKCβ2, or iNOS has been shown by us or others to substantially improve cardiac function in the diabetic state (11, 32, 52, 62).

Author Contributions

Author contributions: H.S., G.L., and K.M.M. conception and design of research; H.S., A.G., Y.-H.L., and G.B. performed experiments; H.S., A.G., and Y.-H.L. analyzed data; H.S. and K.M.M. interpreted results of experiments; H.S. prepared figures; H.S. drafted manuscript; H.S., G.L., G.B., and K.M.M. approved final version of manuscript.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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References


27. Kalyanaraman B, Darley-Usmar V, Davies JK, Denner PA, Forman HH, Grisham MB, Mann GE, Moore K, Roberts LJ, 2nd, Ischiropou-


Park KH, Han DJ, Rhee YH, Jeong SJ, Kim SH, Park YG. Protein kinase C betaII and delta/theta play critical roles in bone morphogenic protein-4-stimulated osteoblastic differentiation of MC3T3-E1 cells. Biochem Biophys Res Commun 403: 7–12, 2010.


