Rac1 Is Required for Cardiomyocyte Apoptosis During Hyperglycemia

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OBJECTIVE—Hyperglycemia induces reactive oxygen species (ROS) and apoptosis in cardiomyocytes, which contributes to diabetic cardiomyopathy. The present study was to investigate the role of Rac1 in ROS production and cardiomyocyte apoptosis during hyperglycemia.

RESEARCH DESIGN AND METHODS—Mice with cardiomyocyte-specific Rac1 knockout (Rac1-ko) were generated. Hyperglycemia was induced in Rac1-ko mice and their wild-type littermates by injection of streptozotocin (STZ). In cultured adult rat cardiomyocytes, apoptosis was induced by high glucose.

RESULTS—The results showed a mouse model of STZ-induced diabetes, 7 days of hyperglycemia-upregulated Rac1 and NADPH oxidase activation, elevated ROS production, and induced apoptosis in the heart. These effects of hyperglycemia were significantly decreased in Rac1-ko mice or wild-type mice treated with apocynin. Interestingly, deficiency of Rac1 or apocynin treatment significantly reduced hyperglycemia-induced mitochondrial ROS production in the heart. Deficiency of Rac1 also attenuated myocardial dysfunction after 2 months of STZ injection. In cultured cardiomyocytes, high glucose upregulated Rac1 and NADPH oxidase activity and induced apoptotic cell death, which were blocked by overexpression of a dominant negative mutant of Rac1, knockdown of gp91-phox or p47-phox, or NADPH oxidase inhibitor. In type 2 diabetic db/db mice, administration of Rac1 inhibitor, NSC23766, significantly inhibited NADPH oxidase activity and apoptosis and slightly improved myocardial function.

CONCLUSIONS—Rac1 is pivotal in hyperglycemia-induced apoptosis in cardiomyocytes. The role of Rac1 is mediated through NADPH oxidase activation and associated with mitochondrial ROS generation. Our study suggests that Rac1 may serve as a potential therapeutic target for cardiac complications of diabetes. Diabetes 58:2386–2395, 2009

Diabetic cardiomyopathy has been defined as ventricular dysfunction that occurs in the absence of changes in blood pressure and coronary artery disease (1,2). Cell death by apoptosis is the predominant damage in diabetic cardiomyopathy (3,4). Diabetes increases cardiac apoptosis in animals and patients (3–7). Cardiomyocyte death causes a loss of contractile tissue, which initiates a cardiac remodeling (8). Loss of cardiomyocytes and hypertrophy of the remaining cells characterize the diabetic cardiomyopathy (9,10). Thus, suppression of cardiomyocyte apoptosis results in a significant prevention of the development of diabetic cardiomyopathy (4). However, the underlying mechanisms by which diabetes induce apoptosis remain not fully understood.

All forms of diabetes are characterized by chronic hyperglycemia. Hyperglycemia induces reactive oxygen species (ROS) production in cardiomyocytes (6,11), which plays a crucial role in cardiomyocyte apoptosis in diabetes because the administration of antioxidant agents are able to rescue hyperglycemia-induced cardiomyocytes (4,6). The mechanisms activated by hyperglycemia, leading to myocardial oxidative stress and apoptosis, are not completely clarified.

Although multiple sources of ROS have been demonstrated, NADPH oxidase is a critical determinant of the redox state of the myocardium (12–15). Higher myocardial NADPH oxidase activity has been detected in diabetes (16,17); more importantly, NADPH oxidase activity is markedly increased by high glucose levels (18). The NADPH oxidase is a multicomponent enzyme complex that consists of the membrane-bound cytochrome b558, which contains gp91-phox and p22-phox, the cytosolic regulatory subunits p47-phox and p67-phox, and the small guanosine triphosphate-binding protein Rac. An important step for the assembly and function of this multicomponent NADPH oxidase complex is the heterodimerization of gp91-phox with p67-phox, which is mediated by Rac (19). Three isoforms of Rac (Rac1, Rac2, and Rac3) have been identified (20), and Rac1 is the predominant isoform expressed in cardiomyocytes (21). Thus, Rac1 activation may lead to myocardial oxidative stress and apoptosis during hyperglycemia. A recent study showed that Rac1 contributes to vascular injury in diabetes (22). However, no direct evidence is available on Rac1 and NADPH oxidase activation in cardiomyocyte apoptosis in diabetes.

In this study, we generated cardiomyocyte-specific Rac1 knockout (Rac1-ko) mice; analyzed the impact of Rac1 on NADPH oxidase activation, mitochondrial ROS generation, and intracellular ROS production; and investigated the role of Rac1 and NADPH oxidase activation in cardiomyocyte apoptosis during hyperglycemia.
RESEARCH DESIGN AND METHODS

Animals and adult rat cardiomyocyte culture. This investigation conforms with the guide for the care and use of laboratory animals published by the U.S. National Institutes of Health (NIH Publication No. 85–23). All experimental procedures were approved by the Animal Use Subcommittee at the University of Western Ontario, Canada. Breeding pairs of C57BL/6 mice, mice bearing the modified Raci gene containing loxP sites, and db/db mice were purchased from the Jackson Laboratory. Transgenic mice with cardiomyocyte-specific expression of Cre recombinase (Cre) under the control of α-mycosin heavy chain (α-MHC) were generously provided by Dr. Dale Evan Abel (University of Utah, UT) (23). A breeding program for mice was implemented at our animal care facilities.

Adenoviral infection of cultured ARVC. Cardiomyocytes were infected with adenoviral vectors containing a dominant-negative mutant Rac1 (Ad–RacN17, Vector Biolabs) or β-gal (Ad-gal, Vector Biolabs) as a control at a multiplicity of infection (MOI) of 100 PFU/cell. Adenovirus-mediated gene expression was determined as we previously described (24).

Streptozotocin hyperglycemic mice. Adult male mice (2 months old) were intraperitoneally injected with a single dose of streptozotocin (STZ) at 150 mg/kg body weight, dissolved in 10 mmol/l sodium citrate buffer (pH 4.5). On day 3 after STZ treatment, whole blood was obtained from the mouse tail vein and random glucose levels were measured using OneTouch Ultra II blood glucose monitoring system (LifeScan, Mountainview, CA). Blood glucose content of 20 mmol/l or greater was chosen as hyperglycemia for the present study, whereas citrate buffer-treated mice were used as normoglycemia controls.

Heart function assessment. Heart function was measured using an in situ apoptosis detection kit (Roche Biochemicals) as described previously (26).

Analysis of POL2 mRNA. POL2 mRNA expression was measured by RT-qPCR in adipose tissue samples from control and STZ-treated mice. POL2 expression in control mice was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression in the same samples.

Results

Uregulation of Rac1 activity by hyperglycemia. As shown in Fig. 1, hyperglycemia significantly increased Rac1 activity in STZ-treated compared with citrate buffer-treated hearts (Fig. 1A). In cultured ARVC exposed to normal (5.5 mmol/l) or high glucose (33 mmol/l) for 1, 6, and 24 h, Rac1 activity was significantly upregulated by high glucose compared with normal glucose (Fig. 1B).

Generation of cardiomyocyte-specific Raci-ko mice. Mice with cardiomyocyte-specific Raci-ko were generated by crossing the floxed Raci gene with mice overexpressing Cre under the control of α-MHC as we recently described (29). PCR analysis identified mice carrying both the floxed Raci gene and the α-MHC-Cre transgene (Fig. 1C). Lanes 2 and 3 show heterozygous and homozygous floxed Raci gene, respectively. Lanes 4: Expressions of Cre, Raci, and GAPDH mRNAs were determined in the heart and lung by RT-PCR. Cre mRNA was detected in Raci-ko heart only as shown in left lane. The levels of Rac1 mRNA were significantly reduced in Raci-ko heart. The low level of Rac1 mRNA in Raci-ko mouse is most likely because of vascular Rac1. In contrast, the Rac1 mRNA was not decreased in Raci-ko lung. E: Adult cardiomyocytes were isolated from Raci-ko and wild-type mice. Rac1 protein expression was determined by Western blot analysis. The levels of Rac1 protein were significantly decreased in Raci-ko cardiomyocytes. F: Rac1 activity was determined in Raci-ko and wild-type hearts after 7 days hyperglycemia. A representative Western blot from three different hearts in each group shows downregulation of Rac1 activity in Raci-ko hearts. WT, wild type; KO, knockout.
that cardiomyocyte-specific deletion of Rac1 does not affect basal myocardial function and morphology of the heart, thus excluding the possible effects of Cre expression on the heart of Rac1-ko mice.

Role of Rac1 in NADPH oxidase activation and ROS production in the hyperglycemic heart. Hyperglycemia was induced in Rac1-ko mice and their corresponding wild-type littermates including wild-type, floxed Rac1, and Cre transgenic (Cre+) mice by STZ. The levels of blood glucose were monitored on day 0, 3, and 9 after STZ injection. To eliminate possible confounding effects of STZ, insulin was immediately given using a long-term insulin preparation (Lantus Insulin glargine injection; sanofi-aventis Canada, Laval, Canada) at a dose of 5–15 units per mouse per day to maintain the blood glucose levels between 5–12 mmol/l when hyperglycemia was greater than 20 mmol/l on day 3 after STZ treatment in wild-type mice. In sham- or STZ-injected mice, blood glucose was not different between Rac1-ko mice and their corresponding wild-type littermates including wild-type, Rac1 floxed, and Cre+ mice (data not shown).

We first determined Rac1 activity in the heart. There was no difference of Rac1 activity in between Rac1-ko corresponding wild-type littermates under basal or diabetic conditions. Therefore, Rac1 floxed mice were presented as littermate wild-type controls for Rac1-ko mice. Rac1 activity was much lower in Rac1-ko compared with wild-type hearts (Fig. 2). We then examined the membrane translocation of Rac1 and p67phox, as well as NADPH oxidase activation. As shown in Fig. 2A, the protein levels of Rac1 and p67phox in plasma membranes were significantly upregulated in hyperglycemic compared with normoglycemic wild-type hearts, which were correlated with the increases in NADPH oxidase activity and ROS production in hyperglycemic hearts (Fig. 2B and C). However, myocardial ROS production was not increased in insulin-supplemented mice (data not shown). There were no changes in protein levels of NADPH oxidase subunits, gp91phox, p67phox, and Rac1 in hyperglycemic hearts (data not shown). Deletion of Rac1 decreased membrane Rac1 and p67phox, NADPH oxidase activation, and ROS production in the hyperglycemic heart (Fig. 2A–C). Finally, we analyzed the effect of NADPH oxidase activation on ROS production during hyperglycemia. Adult wild-type mice were injected with STZ. Two days later, apocynin (30 mg · kg⁻¹ · day⁻¹) or vehicle was given in drinking water for 7 days. The levels of blood glucose were similar between apocynin- and vehicle-treated mice on day 9 (data not shown). Administration of apocynin blocked NADPH oxidase activity and ROS production in hyperglycemic hearts (Fig. 2B and C). These results demonstrate that Rac1 is required for NADPH oxidase activation and ROS production during hyperglycemia.

Decreased apoptosis in the hyperglycemic Rac1-ko heart. Myocardial caspase-3 activity was not different between Rac1-ko corresponding wild-type littermates including wild-type, Rac1 floxed, and Cre+ mice under either normal or diabetic conditions (data not shown). Therefore, Rac1 floxed mice were chosen as littermate wild-type controls for Rac1-ko mice in the following experiments. Compared with citrate buffer–treated mice, hyperglycemia significantly increased myocardial caspase-3 activity (Fig. 3A) and the number of TUNEL-positive cardiomyocytes in STZ-treated wild-type mice (Fig. 3B and C), whereas myocardial caspase-3 activity was not increased in insulin-supplemented mice (data not shown). These results exclude the direct possible effects of STZ on myocardial apoptosis, which is in agreement with a recent report (4). Deficiency of Rac1 decreased caspase-3 activity and the number of TUNEL-positive cells in the hyperglycemic heart (Fig. 3). Similarly, inhibition of NADPH oxidase by apocynin blocked caspase-3 activation and reduced the number of TUNEL-positive cells in hyperglycemic hearts (Fig. 3C). These data demonstrate that Rac1-NADPH oxidase activation induces apoptosis in the hyperglycemic heart.

Role of Rac1 and NADPH oxidase in mitochondrial ROS production. Mitochondria are known to constitutively generate superoxide radicals as a by-product of electron transport and are also important sources for intracellular ROS production in hyperglycemia (30,31). Inhibition of mitochondrial ROS production has been shown to prevent the development of diabetic cardiomyopathy (32,33). We explored the impact of Rac1-NADPH oxidase on mitochondria in producing ROS. Myocardial...
mitochondria were isolated from Rac1-ko mice, wild-type littermates, and apocynin-treated mice on day 9 after STZ treatment. We first determined superoxide generation in the isolated frozen-thawed mitochondria. In the normoglycemic hearts, addition of NADH but not NADPH slightly increased superoxide production and there was no difference between Rac1-ko mice and wild-type littermates. However, in the hyperglycemic hearts, mitochondria superoxide production was significantly increased (Fig. 4A). Deficiency of Rac1 or apocynin administration decreased mitochondrial superoxide production in the hyperglycemic heart (Fig. 4A). Addition of succinate did not elicit superoxide production in mitochondrial preparations (data not shown). This suggests that mitochondrial complex I and/or III but not complex II is involved in superoxide generation.

To further confirm the role of Rac1 and NADPH oxidase in mitochondrial ROS production, we analyzed the release of ROS from mitochondria by using DCF-DA as an indicator. Similarly, ROS production was increased in intact mitochondria from the hyperglycemic compared with the normoglycemic heart on addition of pyruvate/malate (5/5 mmol/l). Deletion of Rac1 or apocynin administration blocked the increase of ROS production in mitochondria (Fig. 4B). Thus, these data demonstrate that Rac1 and NADPH oxidase contribute to mitochondrial ROS generation in the hyperglycemic heart.

Effects of Rac1 and NADPH oxidase inhibition on apoptosis in cardiomyocytes. To determine whether Rac1 activation directly contributes to apoptosis in cultured ARVC exposed to high glucose, we used adenoviral vector Ad-RacN17 expressing a dominant negative mutant of Rac1, which specifically blocks Rac1 activation (22,34). ARVC were infected with Ad-RacN17 or Ad-gal and then incubated with normal (5.5 mmol/l) or high glucose (33 mmol/l) for 48 h. High glucose significantly induced NADPH oxidase activity in ARVC (Fig. 5A). In contrast, equal amount of mannitol did not affect NADPH oxidase activity (data not shown). Infection of Ad-RacN17 blocked NADPH oxidase activity in high glucose–treated ARVC (Fig. 5A). Similarly, high glucose significantly increased caspase-3 activity compared with normal glucose, whereas, as an osmotic control, equal amount of mannitol did not induce caspase-3 activity in ARVC (data not shown).

FIG. 3. Role of Rac1 in apoptosis in the heart. Hyperglycemia was induced in Rac1-ko mice and wild-type littermates. A and D: Myocardial caspase-3 activity was increased in hyperglycemic wild-type littermates. Deficiency of Rac1 or apocynin treatment reduced caspase-3 activity in hyperglycemic hearts. B and E: Quantification of TUNEL staining–positive cardiomyocytes. Deficiency of Rac1 or apocynin treatment decreased the number of TUNEL-staining cells. Data are means ± SD, n = 4–6; *P < 0.05 versus sham or wild type and †P < 0.05 versus STZ + wild-type littermates (floxed Rac1) or vehicle (Veh) in STZ. C: Representative of TUNEL staining in cardiomyocytes (yellow–blown signal, arrows showing nuclear localization) from hyperglycemic wild-type hearts (a) and hyperglycemic Rac1-ko hearts (b). Magnification ×40; scale bar: 10 μm. WT, wild type; KO, knockout. (A high-quality digital representation of this figure is available in the online issue.)

FIG. 4. Impact of Rac1 and NADPH oxidase on ROS generation in isolated mitochondria. Myocardial mitochondria were isolated from wild-type and Rac1-ko mice under normoglycemia or hyperglycemia and were assayed for superoxide generation and ROS production (see details in RESEARCH DESIGN AND METHODS). Hyperglycemia upregulated both superoxide generation (A) and ROS production (B). Deficiency of Rac1 or apocynin (Apo) treatment significantly decreased superoxide generation and ROS production in isolated mitochondria. Data are means ± SD, n = 5–6. *P < 0.05 versus sham, †P < 0.05 versus STZ + Veh and †P < 0.05 versus STZ + wild-type littermates (floxed). KO, knockout; Veh, vehicle.
shown). Ad-RacN17 blocked high glucose–induced caspase-3 activity (Fig. 5B). In association with upregulation of caspase-3 activity, high glucose increased the percentage of annexin V positive cells, which was significantly reduced by infection of Ad-RacN17 (Fig. 5C and E). The increased percentage of annexin V positive cells was also significantly reduced by a selective caspase-3 inhibitor, AC-DEVD carbohydrate (up to 90% reduction). These results suggest that Rac1 is important in high glucose–induced apoptosis. Cardiomyocyte death was also measured by phosphatidylinositol nuclear staining. Compared with normal glucose, high glucose increased the percentage of phosphatidylinositol positive cells, which was significantly decreased by Ad-RacN17 (Fig. 5D and F). Although phosphatidylinositol is a viability exclusion dye and does not enter cells with intact membrane, in the late
stage of apoptosis there is also a loss of membrane integrity as the cell dies (35). As such, the population of phosphatidylinositol-positive cells is a mixture of necrotic and apoptotic cells.

To examine if NADPH oxidase activation mediated apoptosis, we first incubated ARVC with normal or high glucose in the presence of apocynin (100 µmol/L), NAC (2 mmol/L), or vehicle. Twenty-four hours later, coinubation with apocynin or NAC inhibited caspase-3 activity and decreased the percentage of annexin-V positive and phosphatidylinositol-positive cells in high glucose–induced ARVC (Fig. 6A–C). To clarify the contribution of gp91^phox^, NADPH oxidase, we transfected ARVC with gp91^phox^–specific siRNA and then incubated these cells with normal or high glucose for another 24 h. Two different scramble siRNAs (Con1 and Con2) were used as controls. Transfection with gp91^phox^ siRNA significantly decreased gp91^phox^ protein compared with either Con1 or Con2 siRNA in ARVC (Fig. 6D). To confirm the specific knockdown by gp91^phox^ siRNA, we also measured Nox4 expression because our recent study has shown that cardiomyocytes express both Nox2 and Nox4 under physiological conditions (25). Gp91^phox^ siRNA did not alter Nox4 expression in ARVC (data not shown). In response to high glucose, gp91^phox^ sRNA blocked caspase-3 activation and reduced annexin V–staining positive cells in ARVC (Fig. 6E and F), compared with either Con1 or Con2 scramble siRNA. These results demonstrate that gp91^phox^–NADPH oxidase activation induces apoptosis in high glucose–stimulated ARVC. The role of NADPH oxidase in high glucose–induced apoptosis was further demonstrated by using p47^phox^ siRNA. Transfection of p47^phox^ siRNA blocked NADPH oxidase activation in high glucose–stimulated ARVC (data not shown). Similarly, caspase-3 activation and annexin V–staining positive cells were significantly decreased in p47^phox^ siRNA–transfected ARVC (Fig. 6G and H).

**Contribution of Rac1 to myocardial dysfunction in STZ-induced type 1 diabetic mice.** To investigate the functional significance of Rac1, we assessed myocardial function in Rac1-ko and wild-type mice after 8 weeks of STZ injection. Although there was no change in heart rate, rate of contraction/relaxation was significantly reduced in diabetic wild-type mice compared with sham animals (Fig. 7). Lack of Rac1 restored the rate of contraction and relaxation without affecting heart rate in diabetic Rac1-ko mice (Fig. 7). These results demonstrate that Rac1 contributes to myocardial dysfunction in diabetic mice.

**Role of Rac1 in cardiac apoptosis in db/db mice.** To further demonstrate the role of Rac1 in cardiac apoptosis in diabetes, we used type 2 diabetic model of db/db mice. A highly selective Rac1 inhibitor NSC23766 (36) was given to db/db mice starting at age of 12 weeks (2.5 mg · kg^-1 · day^-1 i.p.) for 2 weeks. Rac1 activity, NADPH oxidase activity, caspase-3 activity, and TUNEL-positive cells were significantly increased in the heart of db/db mice compared with wild-type mice (Fig. 8A–D). Administration of NSC23766 inhibited NADPH oxidase activation, blocked caspase-3 activity, and reduced TUNEL-positive cells in db/db mice (Fig. 8B–D), whereas blood glucose levels were similar between NSC23766 and vehicle-treated db/db mice (20–28 mmol/L). Finally, myocardial dysfunction in db/db mice was slightly but not significantly (P = 0.1418 and 0.0648 for the rate of contraction and relaxation, respectively) attenuated by NSC23766 treatment (Fig. 8E and F).

**DISCUSSION**

The present study generated cardiomyocyte-specific Rac1-ko mice and investigated the role of Rac1 in ROS production and apoptosis in the hyperglycemic hearts. We demonstrated for the first time that Rac1, through gp91^phox^–NADPH oxidase activation and ROS production, induces apoptosis in both hyperglycemic hearts and high glucose–stimulated cardiomyocytes. Rac1 also plays a role in cardiac apoptosis in type 2 diabetic db/db mice. Furthermore, Rac1 and NADPH oxidase activation is associated with mitochondrial ROS production in the hyperglycemic hearts. Finally, Rac1 contributes to myocardial dysfunction in STZ-induced type 1 diabetes. Thus, Rac1/NADPH oxidase/mitochondrion axis is important in ROS production and cardiomyocyte apoptosis in hyperglycemia, which may contribute to the development of diabetic cardiomyopathy.

Accumulating evidence suggests that hyperglycemia induces ROS (6,11) and overproduction of ROS is associated with apoptosis in the diabetic heart (4,6). However, the pathophysiologically relevant source of increased myocardial ROS production in diabetes remains to be further characterized. Recent studies have demonstrated that myocardial NADPH oxidase activity is increased in diabetes (16,17). Hyperglycemia has been shown to activate NADPH oxidase in cardiomyocytes (18). These studies suggest the pathophysiologic relevance of NADPH oxidase activation for cardiomyocyte apoptosis in hyperglycemia. An important step for the assembly of active NADPH oxidase is the interaction between cytosolic subunits (p67^phox^, p47^phox^, and Rac1) and membrane subunits (gp91^phox^ and p22^phox^) (19). Rac1 is required to anchor cytosolic p67^phox^ to the membrane for the assembly of active NADPH oxidase, leading to superoxide generation (21). In the present study, deficiency of Rac1 inhibited the relocation of NADPH oxidase subunits to membrane and diminished NADPH oxidase activation and ROS production in the hyperglycemic heart. Either Rac1 deletion or inhibition of NADPH oxidase prevented myocardial apoptosis in hyperglycemia. These findings suggest that Rac1 contributes to apoptosis through NADPH oxidase activation in the hyperglycemic heart. To characterize whether the role of Rac1 in apoptosis could be reproduced by high glucose levels, we extended our analyses to cardiomyocytes. Direct exposure of RAVC to high glucose, through Rac1, promoted NADPH oxidase activation and induced apoptosis. Selective inhibition of Rac1, knockdown of gp91^phox^ or p47^phox^, or scavenging ROS prevented apoptosis in high glucose–stimulated ARVC. These data support the notion that Rac1 is critical for gp91^phox^–NADPH oxidase activation, which is the major source of ROS production responsible for apoptosis in cardiomyocytes under hyperglycemic conditions. Rac1 was also reported to induce NADPH oxidase activation and ROS production in angiotensin-II stimulated cardiomyocytes (21). Thus, the results reported herein also suggest that Rac1 may be a universal mechanism for NADPH oxidase activation in the heart under various stresses, which may have potential therapeutic implications.

Mitochondrion is another important source of ROS production contributing to apoptosis (37,38). Increased mitochondrial ROS generation has been shown in diabetic hearts (4,33,39). However, relatively few studies have directly measured mitochondrial ROS production in the isolated mitochondria from diabetic hearts, and the cross
FIG. 6. Roles of gp91phox–NADPH oxidase in cardiomyocyte apoptosis. Cultured cardiomyocytes were incubated with normal or high glucose in the presence of apocynin (Apo), NAC, or vehicle for 24 h. Caspase-3 activity and cell death were determined. Caspase-3 activity (A), the percentages of annexin V (B) and phosphatidylinositol staining–positive cells (C) were significantly inhibited by incubation with apocynin or NAC in high glucose–stimulated cardiomyocytes. D–F: Cardiomyocytes were transfected with gp91phox (Nox2) siRNA, p47phox siRNA, or scramble siRNAs (Con1 or Con2) as control. Twenty-four hours later, the cells were incubated with normal or high glucose for another 24 h. D: Representative Western blot for Nox2 protein in Nox2 siRNA and Con1- or Con2-siRNA–transfected cardiomyocytes. E and G: Caspase-3 activity. F and H: The percentages of annexin V. Data are means ± SD from at least three different cell cultures. *P < 0.05 versus Veh in normal glucose or Con1 in normal glucose, #P < 0.05 versus Veh in high glucose, Con1 in high glucose, or Con2 in high glucose. NG, normal glucose; HG, high glucose; Veh, vehicle.
talk of other ROS sources to mitochondrion has not been fully elucidated. In the present study, we measured ROS production directly from the isolated mitochondria and analyzed the role of Rac1-NADPH oxidase in mitochondrial ROS production. In agreement with a recent report of Boudina et al. (39), our data showed that ROS production was increased in the isolated mitochondria from the hyperglycemic hearts. The increased mitochondrial ROS was achieved by using mitochondrial complex I substrates, NADH or pyruvate/malate, but not succinate, a complex II substrate; this is different from the result of Boudina et al. (39), which demonstrated that mitochondrial ROS is induced on addition of succinate in the isolated mitochondria from the diabetic heart. The cause of this discrepancy is unknown but may result from different diabetic models used. Interestingly, cardiomyocyte-specific deletion of Rac1 or inhibition of NADPH oxidase significantly attenuated ROS production in isolated mitochondria from the hyperglycemic heart, suggesting that Rac1 and NADPH oxidase activation functions upstream of mitochondrion in generating mitochondrial ROS in cardiomyocytes exposed to hyperglycemia. This finding is supported by a recent observation demonstrating that NADPH oxidase–derived superoxide anion (O₂⁻) directly activates the mitoK_ATP channels, presumably through a direct action on the sulfhydryl groups of this channel, or reacts with nitric oxide to form peroxynitrite in vascular endothelial cells in response to angiotensin-II (40). Both the increase in K⁺ influx through opening of the mitoK_ATP channels and the peroxynitrite formation can damage respiratory complexes, leading to mitochondrial ROS production (41). Alternatively, one-electron transfer and/or rapid dismutation of superoxide result in substantial H₂O₂ generation. H₂O₂ is a quantitatively important signaling species (42), which may mediate mitochondrial ROS production. Indeed, direct addition of H₂O₂ to isolated mitochondria significantly increased superoxide generation (41). Alternatively, one-electron transfer and/or rapid dismutation of superoxide result in substantial H₂O₂ generation. H₂O₂ is a quantitatively important signaling species (42), which may mediate mitochondrial ROS production. Indeed, direct addition of H₂O₂ to isolated mitochondria significantly increased superoxide generation (41). Alternatively, one-electron transfer and/or rapid dismutation of superoxide result in substantial H₂O₂ generation. H₂O₂ is a quantitatively important signaling species (42), which may mediate mitochondrial ROS production.

FIG. 7. Cardiac function in Rac1-ko and wild-type mice after 8 weeks of STZ injection. Mouse hearts were isolated and perfused in a Langendorff system. Contractile function of heart was determined. Changes in the rate of contraction (+dF/dt_max, A) and relaxation (−dF/dt_min, B) are presented. Data are means ± SD, n = 5–12 per group. *P < 0.05 versus wild type in sham; #P < 0.05 versus wild type in STZ. WT, wild type.
regulation of Rac1 provides a better protection in diabetic hearts. However, complete deletion of Rac1 may have adverse effects since recent studies have suggested that Rac1 is also required for cardiovascular development and physiological functions (49,50).

In conclusion, Rac1 plays a critical role in hyperglycemia-induced apoptosis in cardiomyocytes through NADPH oxidase activation and ROS production, leading to myocardial dysfunction in diabetes. Furthermore, Rac1 and NADPH oxidase activation contributes to mitochondrial ROS production in the hyperglycemic heart. Given excessive ROS and cardiac apoptosis significantly contributes to diabetic cardiomyopathy (1–3,32,33,35), these findings suggest that myocardial Rac1 may serve as a potential target for the treatment of cardiac complications in diabetes.

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The present study also found that deficiency of Rac1 attenuated myocardial dysfunction in diabetic mice, suggesting a pathophysiological significance of Rac1 activation in diabetic hearts. This protection may result from inhibition of cardiomyocyte apoptosis by Rac1 blockade. Rac1-mediated ROS has been shown to induce cardiac hypertrophy in response to angiotensin-II stimulation (21). A recent study also indicated that Rac1 may be associated with cardiac inflammatory responses in hyperglycemia (47). Both cardiac hypertrophy and inflammation are important in the development of cardiac complications of diabetes. Thus, it is possible that Rac1 via NADPH oxidase activation and ROS production contributes to the diabetic cardiac hypertrophy, inflammatory responses, mitochondrial dysfunction, in addition to apoptosis, leading to myocardial dysfunction in diabetes (Fig. 9), which requires further investigations. However, Rac1 may also play a role in cardiac hypertrophy in diabetes through NADPH oxidase–independent mechanisms (48).

It is worthwhile to mention that Rac1 activity was decreased by about 40% in the whole heart of Rac1-ko mice compared with their wild-type littermates. The exact deletion of Rac1 in cardiomyocytes from Rac1-ko mice is currently unknown. A recent study showed that the CreloxP system could achieve ~70% deletion of Rac1 in cardiomyocytes in transgenic mice (21). Thus, the deletion of Rac1 in cardiomyocytes from our Rac1-ko mice is estimated to be around 40–70%, which may lead to some limitations of the interpretation of results from the present study. For example, it is unclear whether further down-
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