Nox4-derived reactive oxygen species mediate cardiomyocyte injury in early type 1 diabetes

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Diabetic cardiomyopathy (DCM) leads to congestive heart failure and may occur in the absence of hypertension or coronary artery disease. Impaired ventricular function has been reported in human and rodent models at early stages of diabetes. Myocardial dysfunction is often accompanied by increased expression of markers of myocardial injury, including matrix proteins, α-smooth muscle actin, β-myosin heavy chain, and a shift towards an embryonic pattern of gene expression. These early markers of myocardial injury in the presence of persistent hyperglycemia and other risk factors associated with diabetes eventuate in progressive cardiomyopathy and congestive heart failure (2, 28, 35).

Hyperglycemia contributes to myocardial cell injury and plays a role in the development and progression of DCM. Oxidative stress has been implicated in the development of DCM, and hyperglycemia is associated with enhanced generation of reactive oxygen species (ROS). However, the precise sources of ROS in the myocardium in diabetes are unknown. There is evidence that nicotinamide adenine dinucleotide phosphate (NADPH) oxidases of the Nox family and particularly Nox4 contribute to oxidative stress in cardiovascular disease (29, 33). In addition, Nox4 has been implicated in the production of ROS triggered by angiotensin II and transforming growth factor-β in various cell types including cardiac cells (8). Antioxidants attenuate the effects of hyperglycemia on cardiac myocytes in vivo and in vitro (10, 12). NADPH oxidases are isoforms of the neutrophil oxidase, in which the catalytic subunits, termed Nox proteins, correspond to homologues of gp91phox (or Nox2), the catalytic moiety found in phagocytes (14, 24). In this family, Nox4 is expressed in both the heart and cultured cardiac myocytes. It has been reported that diabetes increases NADPH oxidase activity (13); however, the biological role(s) of Nox4 in the development of diabetic cardiomyopathy is not known. Song et al. (31) have demonstrated that ROS contribute to cardiac dysfunction in OVE26 diabetic mice. In transgenic animals overexpressing Nox4, aging and pressure-overload promote cardiac dysfunction, and suppression of endogenous Nox4 activity attenuates cardiac hypertrophy (1).

This study was designed to determine the role of Nox4 in mediating diabetic cardiomyopathy phenotype at early stages of type 1 diabetes. We show increased NADPH oxidase activity and Nox4 expression in cardiac myocytes exposed to high glucose (HG) and in the myocardium of type 1 diabetic rats. We also provide evidence that upregulation of Nox4 protein parallels the increase in NADPH oxidase activity in the left ventricle (LV) of type 1 diabetic rats and mediates cardiomyocyte injury. Inhibiting Nox4 expression decreased NADPH-dependent ROS generation and reversed cardiomyocyte injury phenotype in vitro and in vivo and improved cardiac function in type 1 diabetic rats.

Methods

Animals and treatments. Male Sprague-Dawley rats weighing between 200 and 225 g were divided into four groups of four animals each. Rats in group 1 were injected with sodium citrate buffer alone. Group 2 rats were injected intravenously via the tail vein with 55 mg/kg body wt streptozotocin (STZ) in sodium citrate buffer (0.01 M, pH 4.5) to induce diabetes. Rats in groups 3 and 4 were injected with STZ followed by either phosphorothioated sense (group 3) or antisense (AS) oligonucleotides for Nox4 (90 ng/g body wt−1 day−1, group 4) administered subcutaneously by an Alzet osmotic minipump (Alza, Palo Alto, CA). Oligonucleotides were administered 72 h after STZ injection for 14 days. Blood glucose concentration (LifeScan

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One Touch glucometer, Johnson & Johnson) was monitored 24 h later and periodically thereafter (17). All of the rats had unrestricted access to food and water and were maintained in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center. At day 14, hearts were rapidly excised from anesthetized rats and washed in PBS. The left ventricles accompanied by the septum were cut into base, midportions, and apex, snap-frozen, and stored at −80°C for histological and biochemical analysis. Antisense oligonucleotides were designed near the ATG start codon of rat Nox4 (5′-GAGCTCTCTCAGGACAGGCC-3′) (15, 16). Antisense and the corresponding sense oligonucleotides were synthesized as phosphorothioated oligonucleotides and purified by high-performance liquid chromatography (Advanced Nucleic Acid Core Facility, University of Texas Health Science Center at San Antonio).

Echocardiographic assessment. Rats were weighed and anesthetized using 1–2% isoflurane. Echocardiography was performed with a linear 15-MHz transducer (model CL 15-7, Philip Medical System, Best) connected to a HDI 5000 ultrasound system (ATL, Philip Medical System, Best). The anterior chest wall was shaved, rats were secured in the supine position, and normal body temperature was maintained using a controlled heating pad. M-mode and two-dimensional echocardiography images were acquired in the parasternal long- and short-axis views. LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), LV end-diastolic volume (LVEDV), and LV end-systolic volume (LVESV) were measured. The percentage of LV fractional shortening and LV ejection fraction was calculated as follows: \[(\text{LVEDD} - \text{LVESD})/\text{LVEDD} \times 100\%\] and \[(\text{LVEDV} - \text{LVESV})/\text{LVEDV} \times 100\%\].

Detection of intracellular ROS. The peroxide-sensitive fluorescent probe 2′,7′-dichlorodihydrofluorescein (DCF) diacetate (Molecular Probes) was used to assess the generation of intracellular ROS as previously described (22). Cells were grown in 12- or 24-well plates (Promocell) was used to assess the generation of intracellular ROS as previously described (22). Cells were grown in 12- or 24-well plates and serum-deprived for 24 h. Immediately before the experiments, cells were washed with Hanks’ buffered salt solution (HBSS) containing Ca\(^{2+}\) and Mg\(^{2+}\) and then loaded with 5 μmol/l DCF diacetate dissolved in HBSS for 30 min at 37°C. Cells were then incubated for 45 min with HG. DCF fluorescence was detected at excitation and emission wavelengths of 488 and 520 nm, respectively, and measured in a multiwell fluorescence plate reader (Wallac 1420 Victor2, PerkinElmer).

Statistical analysis. Results are expressed as means ± SE. Statistical significance was assessed by Student’s unpaired t-test. Significance was determined as P < 0.05.

RESULTS

Echocardiographic measurements. Pertinent characteristics and hemodynamic variables of the four groups of rats studied are shown in Table 1. Untreated diabetic rats and diabetic rats treated with either S or AS Nox4 had equivalently elevated blood glucose concentration compared with the control rats. Body weight was reduced in the diabetic rats and to a similar extent in the diabetic rats treated with either sense or antisense oligonucleotides. There was no significant change in left-
Diabetes upregulates Nox4 protein expression. To determine the effect of diabetes on Nox4 protein expression and to test whether the oligonucleotides were effectively delivered to the heart, we examined the expression of Nox4 protein in left ventricular lysates in the four groups of rats. Western blot analysis showed a predominant 64-kDa band corresponding to Nox4 that was increased in the LV of the diabetic rats compared with control rats (Fig. 2, A and B). AS Nox4 but not sense Nox4 administration significantly reduced Nox4 protein levels in the LV of the diabetic animals (Fig. 2, A and B). We next examined whether inhibition of Nox4 resulted in “compensatory” upregulation of other Nox homologues, such as Nox1 and gp91(phox)/Nox2, that are expressed in the heart. Our data show that neither Nox1 nor Nox2 protein levels were increased in the LV of the heart of the diabetic rats (Fig. 2, A, C, and D). More importantly, this experiment confirmed the specificity of the AS oligos towards Nox4, where administration of AS Nox4 had no effect on Nox1 and Nox2 protein expression (Fig. 2, A, C, and D). These data suggest that the Nox enzymes are regulated independently in the heart in diabetes.

Diabetes induces oxidative stress in the heart. Diabetes is associated with increase in ROS levels and oxidative stress. However, the effect of diabetes on NADPH oxidase including Nox4 isoform expression in the diabetic heart have not been investigated. Superoxide ($O_2^-\,*$) production in the left ventricles of the four different groups of rats was assessed by DHE staining. DHE is a fluorescent dye that specifically reacts with intracellular $O_2^-\,*$ and is converted to the red fluorescent

Table 1. Body weight, glucose level, and cardiac function of control rats, type 1 diabetic rats, and type 1 diabetic Nox4 sense- and antisense-treated rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DM1</th>
<th>DM1/S</th>
<th>DM1/AS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>284 ± 18.09</td>
<td>241.5 ± 30.84</td>
<td>245.67 ± 26.01</td>
<td>248.75 ± 6.85</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>99 ± 4.53</td>
<td>494.5 ± 66.2</td>
<td>453.67 ± 25.8</td>
<td>478.75 ± 51.7</td>
</tr>
<tr>
<td>EDD, mm</td>
<td>7.4 ± 0.23</td>
<td>7.63 ± 0.46</td>
<td>7.17 ± 0.25</td>
<td>7.62 ± 0.18</td>
</tr>
<tr>
<td>ESD, mm</td>
<td>4.01 ± 0.12</td>
<td>4.88 ± 0.31*</td>
<td>4.24 ± 0.13*</td>
<td>4.14 ± 0.17†</td>
</tr>
<tr>
<td>FS, %</td>
<td>46.48 ± 0.25</td>
<td>46.12 ± 0.82*</td>
<td>40.76 ± 1.63*</td>
<td>45.74 ± 1.48†</td>
</tr>
<tr>
<td>EDV, µl</td>
<td>339.56 ± 27.47</td>
<td>367.18 ± 49.28</td>
<td>340.56 ± 28.58</td>
<td>324.52 ± 21.28</td>
</tr>
<tr>
<td>ESV, µl</td>
<td>94.67 ± 6.74</td>
<td>141.94 ± 26.54*</td>
<td>117 ± 8.9*</td>
<td>86.48 ± 12.53†</td>
</tr>
<tr>
<td>EF, %</td>
<td>71.98 ± 2.99</td>
<td>61.49 ± 2.98*</td>
<td>65.54 ± 2.98*</td>
<td>73.44 ± 2.5†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4. Nox4, NADPH oxidase 4; DM1, type 1 diabetic rats; DM1/S, type 1 diabetic Nox4 sense-treated rats; DM1/AS, type 1 diabetic Nox4 antisense-treated rats; EDD, end-diastolic diameter; ESD, end-systolic diameter; FS, fractional shortening; EDV, end-diastolic volume; ESV, end-systolic volume; EF, ejection fraction. *P < 0.05 vs. control rats; †P < 0.05 vs. diabetic rats.
compound ethidium, which then binds irreversibly to double-stranded DNA and appears as punctuate nuclear staining. The intensity of DHE fluorescence in the LV of the diabetic rats was significantly enhanced compared with the LV of the control animals and was significantly reduced in Nox4 AS but not S-treated diabetic rats (Fig. 2, E and F). NADPH-dependent superoxide production measured by lucigenin-enhanced chemiluminescence was also significantly increased in LV homogenates of diabetic animals compared with controls (Fig. 2G). AS Nox4 but not sense Nox4 treatment reversed diabetes-induced NADPH oxidase activation in left ventricular homogenates (Fig. 2G). These data suggest that Nox4 is primarily responsible for increased ROS production in the early stages of diabetes.

Diabetes induces an increase in ROS production, NADPH oxidase activity, and Nox4 protein expression. Cardiomyocyte injury is characterized by activation of different intracellular signaling pathways and transcriptional mediators including reexpression of embryonic gene pattern and remodeling of the extracellular matrix (27). We examined the protein expression of β-MHC and α-SMA, markers of the fetal gene program and of cardiac injury, in control, diabetic, and diabetic rats treated with either S or AS-Nox4. The levels of β-MHC (Fig. 3, A and B) and α-SMA (Fig. 3, A and C) were increased in type 1 diabetic rats compared with control rats. Anti-sense treatment for Nox4 but not sense treatment significantly reduces the levels of these proteins (Fig. 3, A–C). These results indicate that Nox4-induced oxidative stress plays a role in diabetes by increasing the expression of cardiac injury markers. Accumulation of the extracellular matrix protein fibronectin is increased in response to injury. In this study, fibronectin protein expression in the LV is significantly increased in the diabetic animals compared with controls (Fig. 3, A and D). This increase was markedly reduced in the diabetic rats treated with AS- but not S-oligonucleotides for Nox4 (Fig. 3, A and D). Furthermore, the amount of interstitial cardiac fibrosis in type 1 diabetic rats, as assessed by the trichrome staining, was significantly higher than in control animals. The increase in interstitial fibrosis was prevented by AS-Nox4 treatment but not by S-Nox4 treatment (Fig. 3, E and F).

High glucose induces ROS production, NADPH oxidase activity, and Nox4 expression in cultured cardiac myocytes. To determine whether hyperglycemia in diabetes contributes to cardiomyocyte injury, cultured cardiac myocytes were exposed to either 25 mmol/l glucose (high glucose; HG) or 5 mmol/l glucose (normal glucose; NG). HG treatment results in a
relatively rapid generation of ROS as measured by DCF fluorescence, compared with cells incubated in NG or mannitol used as an osmotic control (Fig. 4, A and B). ROS generation was detected after 15 min of exposure to HG (Fig. 4A) and was sustained up to 48 h (Fig. 4B). NADPH oxidases of the Nox family are major sources of ROS in cells and tissue. The increase in ROS production, observed in cardiomyocyte treated with HG, was associated with an increase in NADPH-dependent superoxide generation (Fig. 4C). Since our in vivo data suggest that the isoform Nox4 may be involved in diabetes-induced cardiac injury, we next determined the effect of HG on the expression of the oxidase in cardiac myocytes. The increased NADPH oxidase activity was associated with upregulation of Nox4 protein expression in the cultured cardiomyocyte exposed to HG for a short- and long-term periods (Fig. 4D).

**Nox4 mediates HG-induced oxidative stress and myocyte injury in cultured cardiomyocytes.** To further assess the role of Nox4 in HG-induced oxidative stress and cardiomyocyte injury, cultured cardiomyocyte were exposed to 25 mM of glucose (HG) and infected with a dominant negative Nox4 adenovirus (Ad-DN-Nox4) encoding a truncated form of the enzyme. Twenty-four hours of HG exposure significantly increases NADPH oxidase activity (Fig. 5A) and the expression of β-MHC, α-SMA, and fibronectin as assessed by Western blot analysis (Fig. 5B). Inhibition of Nox4 using a dominant negative (Ad-DN-Nox4) adenovirus encoding a truncated form of the enzyme significantly inhibits the increase in NADPH oxidase activity induced by HG (Fig. 5A). Also, Nox4 inhibition dramatically reduces the increase in β-MHC, α-SMA, and fibronectin protein expression (Fig. 5B). These results indicate that Nox4 plays a key role in HG-mediated oxidative stress and that Nox4-derived ROS generation mediates HG-induced fetal gene induction and extracellular matrix protein fibronectin accumulation in cultured cardiac myocytes.

**DISCUSSION**

Oxidative stress has emerged as an important contributor to cardiomyocyte injury. Cumulative oxidant-mediated damage and cellular dysfunction results from imbalance in ROS generation and ROS detoxifying pathways. Increased ROS generation and impaired antioxidant defenses contribute to oxidative stress in the heart in diabetes (6). Potential sources of ROS include mitochondria and NADPH oxidases.
ases have emerged as major sources of ROS in the cardiovascular system. Recent studies have shown increased levels of NADPH oxidase subunits in the vasculature (19, 36) and kidney tissue of diabetic rodents (11, 17). NADPH oxidase isoform Nox4 plays a key role in mediating pathologic changes of diabetes in the kidney and retina (17, 25). Although Nox4 is present in the myocardium (1, 7, 18, 33), its specific role and contribution to ROS generation in the heart in diabetes are not known. In this study, we show that Nox4 is an important source of ROS in the LV of rats with type 1 diabetes; Nox4-derived ROS mediate cardiomyopathy phenotype in early diabetes and administration of antisense oligonucleotides for Nox4 in type 1 diabetic rats exerts a protective effect on cardiac phenotype.

Diabetes and oxidative stress are known to cause cardiac functional and structural changes. However, the mechanisms involved in these processes are poorly understood. Here, we show decrease in ejection fraction together with significant increase in left ventricular systolic diameter and volume, suggesting LV systolic dysfunction and impaired contractility at this early stage of diabetes. Administration of AS Nox4 but not sense oligonucleotides reversed the decrease in the ejection fraction and the fractional shortening observed in diabetic rats compared with the control animals.

We also demonstrate increased expression of Nox4 protein in the left ventricles of STZ-induced diabetic rats that is associated with an increase in NADPH-dependent ROS generation. Interestingly, Nox1 and gp91phox/Nox2 expression was unchanged in the diabetic heart and in the AS Nox4-treated rats, indicating that the decrease in ROS generation is related to inhibition of Nox4. The lack of correlation between ROS generation, Nox1, and gp91phox/Nox2 expression in the diabetic heart and in the AS Nox4-treated animals is somewhat surprising. However, unlike the requirement for Nox4 activation, activation of Nox1 and gp91phox/Nox2 is dependent on a number of cytosolic and membrane subunits that form the active enzyme complex, and these may not be readily available or not regulated by diabetes. Indeed, there is emerging evidence that, in contrast to Nox1 and gp91phox/Nox2, Nox4 is constitutively active and functions independently of the presence of the cytosolic subunits (3, 5, 26). Therefore, increase in the expression of Nox4 catalytic unit itself will be directly translated into increase in ROS generation. Elevated Nox4 levels are consistently found in other types of cardiomyopathy (32). This is consistent with the finding that the administration of AS Nox4 markedly decreased diabetes-induced NADPH oxidase activity concomitantly with the downregulation of Nox4 protein expression. The nature of the ROS (superoxide or hydrogen peroxide) produced by Nox4 in cells or tissues is controversial. It was documented that Nox4 generates mostly hydrogen peroxide in vascular smooth muscle cells or heterologous expression systems (9, 34) while other studies in renal cells or tissue detected Nox4-dependent superoxide and hydrogen peroxide production (4, 17, 30). In the present work, the fact that treatment...
with AS reduced DHE staining and NADPH oxidase activity in left ventricles of diabetic rats suggests that Nox4 produces primarily intracellular superoxide. This is in agreement with recent reports showing that transgenic mice with cardiomyocyte specific overexpression of Nox4 show enhanced superoxide production in the left ventricle (1). Moreover, it has been shown that silencing of Nox4 in the paraventricular nucleus results in inhibition of superoxide production, whereas hydrogen peroxide scavenging had no effect on DHE staining (21).

Early myocardial dysfunction in diabetes may be followed by cardiac hypertrophy, apoptosis of myocytes, and ventricular remodeling with cardiac fibrosis. Myocardial injury is also associated with changes in the molecular phenotype of the myocardium with reinduction of a fetal gene program (7). During cardiac development, α-SMA marks the onset of cardiomyocyte differentiation and as development proceeds, it is sequentially replaced by β-skeletal actin and α-cardiac actin. The same is true for the β-MHC gene predominantly expressed in late fetal life and which is progressively replaced by the α-MHC isoform. It is well known that these genes are reexpressed in cardiac injury in vivo and represent well-accepted markers of cardiomyocyte injury. In this study we show an increase in β-MHC, α-SMA, as well as an upregulation of extracellular matrix fibronectin expression in diabetic rats compared with controls. Furthermore, Masson trichrome staining shows a significant collagen accumulation. These data suggest that the deleterious effects of uncontrolled hyperglycemia including the accumulation of matrix proteins manifests at rather early stages of diabetes. AS Nox4 administration downregulates the expression of these markers known to be associated with pathological cardiac adaptation. This reduction may be a sensitive index of cardiac activity regression, which correlates with the restoration of the ejection fraction and the fractional shortening following AS treatment as evidenced by the echocardiographic findings. In recent published data, Ago et al. (1) found that upregulation of Nox4 by hypertrophic stimuli (pressure overload) and aging mediates oxidative stress, apoptosis, and LV dysfunction. Such changes have been reported in more advanced stages of diabetes in rodent models (1).

The in vivo findings, described in this study, are consistent with a key role for Nox4-derived ROS in cardiac injury. The in vitro findings obtained in cultured cardiac myocytes exposed to high glucose support the idea that hyperglycemia is responsible for the increased oxidative stress and Nox4 expression. HG concentrations (25 mmol/l) similar to those achieved in vivo elicited similar cellular responses resulting in Nox4 protein upregulation, increased superoxide anion generation, and induction of the fetal gene program, i.e., increase in β-MHC, α-SMA, and enhanced fibronectin expression in cultured cardiac myocytes. The role of Nox4 as source of ROS and mediator of cardiac injury in diabetes is supported by the in vitro observation that impairment of Nox4 expression using an adenovirus encoding a dominant negative form of the enzyme (Ad-DN-Nox4) markedly reduced high glucose-induced NADPH oxidase activation and expression of fetal genes. These data further confirm the functional link between Nox4, cardiac myocyte injury, and hyperglycemia. The Nox4-dependent alteration of cardiac myocyte phenotype is in agreement with previous studies showing that the oxidase plays a pivotal role in myofibroblast differentiation and activation resulting in renal or lung fibrosis (5, 17, 20). This reinforces the clinical relevance of the design and development of specific pharmacological Nox4 inhibitors.

Transfection of cultured cardiac myocytes with dominant negative (DN) Nox4 reduced DHE staining and NADPH oxidase activity in left ventricles of diabetic rats. Transfection of cultured cardiac myocytes with dominant negative (DN) Nox4 in the presence of high glucose (24 h) markedly reduced high glucose-induced NADPH oxidase activity (A) and protein expression of the cardiac injury markers β-MHC, α-SMA, and fibronectin (B). Ad, adenovirus. *P < 0.05 vs. NG + green fluorescent protein (GFP); #P < 0.05 vs. HG + GFP.

Fig. 5. Nox4 mediates high glucose-induced increase in the expression of the molecular markers of hypertrophy and fibrosis in cultured cardiac myocytes. Transfection of cultured cardiac myocytes with dominant negative (DN) Nox4 in the presence of high glucose (24 h) markedly reduced high glucose-induced NADPH oxidase activity (A) and protein expression of the cardiac injury markers β-MHC, α-SMA, and fibronectin (B). Ad, adenovirus. *P < 0.05 vs. NG + green fluorescent protein (GFP); #P < 0.05 vs. HG + GFP.

In conclusion, we demonstrate that oxidative stress is induced by hyperglycemia in the diabetic rat left ventricle. The present study provides the first evidence that Nox4-derived ROS contribute to oxidative stress and myocardial cell injury at early stages of diabetes. Our findings suggest that Nox4 represents a promising therapeutic target for the prevention and treatment of diabetes complication in the heart.
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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: R.M.M., A.A.E., and H.E.A. conception and design of research; R.M.M., A.A.E., and G.P.E. performed experiments; R.M.M. and A.A.E. analyzed data; R.M.M. and A.A.E. interpreted the results of the experiments; R.M.M. and A.A.E. prepared the figures; R.M.M. and A.A.E. revised and revised the manuscript; R.M.M., A.A.E., Y.C.G., K.B., S.B., and H.E.A. edited and revised the manuscript; R.M.M., A.A.E., Y.C.G., K.B., G.P.E., S.B., and H.E.A. approved the final version of the manuscript.

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