Glycated Proteins Stimulate Reactive Oxygen Species Production in Cardiac Myocytes

Involvement of Nox2 (gp91phox)-Containing NADPH Oxidase

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Background—Nonenzymatic glycation that results in the production of early-glycation Amadori-modified proteins and advanced-glycation end products may be important in the pathogenesis of diabetic complications. However, the effects of early-glycated proteins, such as glycated serum albumin (Gly-BSA), are poorly defined. In this study, we investigated the effects of Gly-BSA on reactive oxygen species (ROS) production by cardiomyocytes.

Methods and Results—Cultured neonatal rat cardiomyocytes were incubated with Gly-BSA or vehicle (bovine serum albumin [BSA]) for up to 48 hours. Gly-BSA dose-dependently increased in situ ROS production (whole-cell dichlorodihydrofluorescein fluorescence), with an optimum effect at 400 μg/mL after 24-hour incubation (152 ± 100% versus BSA 100%; P < 0.01). Treatment with the NADPH oxidase inhibitor apocynin, a Nox2 (gp91phox) antisense oligonucleotide (Nox2 AS), or the peptide gp91ds-tat significantly reduced Gly-BSA–induced ROS production at 24 hours (68.5 ± 2.2%, 61.4 ± 8.3%, and 53.2 ± 5.4% reduction, respectively). NADPH-dependent activity in cell homogenates was also significantly increased by Gly-BSA at 24 hours (161 ± 8% versus BSA) and was inhibited by diphenyleneiodonium, apocynin, NOX2AS, and the protein kinase C inhibitor bisindolylmaleimide I but not by a nitric oxide synthase inhibitor or mitochondrial inhibitors. Furthermore, bisindolylmaleimide I prevented Gly-BSA–stimulated Rac1 translocation, an essential step for NADPH oxidase activation. Gly-BSA–induced increases in ROS were associated with apocynin-inhibitable nuclear translocation of nuclear factor-κB and an increase in atrial natriuretic factor mRNA expression.

Conclusions—Gly-BSA stimulates cardiomyocyte ROS production through a protein kinase C–dependent activation of a Nox2-containing NADPH oxidase, which results in nuclear factor-κB activation and upregulation of atrial natriuretic factor mRNA. These findings suggest that early-glycated Amadori products may play a role in the development of diabetic heart disease. (Circulation. 2006;113:1235-1243.)

Key Words: glycation ■ NADPH oxidase ■ free radicals ■ glucose ■ diabetes mellitus

Diabetes mellitus is an established risk factor for cardiovascular events, especially the development of diabetic heart disease. Although ischemic heart disease (IHD) is common in diabetic patients, a cardiomyopathy independent of IHD is well recognized and is associated with left ventricular hypertrophy, cardiac fibrosis, and diastolic dysfunction.1,2 Several studies have shown that hyperglycemia, as an independent risk factor, directly causes cardiac damage, which can lead to diabetic cardiomyopathy.3,4 However, mechanisms for the pathogenesis remain unclear.1–3 One of several hypotheses proposed to explain the link between hyperglycemia and the development of diabetic cardiomyopathy is the accumulation of advanced-glycation end products (AGEs).5,6 AGEs are formed by the process of nonenzymatic glycation, in which reducing sugars such as glucose react nonenzymatically with amino groups of proteins and other macromolecules. This leads to the formation of intermediary Schiff bases and Amadori products and finally to irreversible AGE.7

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The Amadori adducts or early-glycated products, most of which are hemoglobin A1C (HBA1C) and fructosamine, constitute the overwhelming majority of circulating glycated proteins in vivo, and their levels, driven by the ambient glucose concentration, are significantly increased in diabetes mellitus.8 In type 1 diabetic patients, Amadori albumin was increased approximately 2-fold and was correlated with markers of endothelial vascular dysfunction, as well as with early nephropathy and with retinopathy status.8 Furthermore, previous studies have demonstrated

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that antiglycated albumin therapy prevented nephropathy and retinopathy in diabetic mice.\textsuperscript{9} In vascular smooth muscle cells, glycated serum albumin resulted in a significant stimulation of proliferation and migration, as well as nuclear factor (NF)-κB activation, which led to the induction of monocyte chemoattractant factor and interleukin-6.\textsuperscript{10} These studies suggest that Amadori products may play a role in the pathogenesis of diabetic microvascular complications.

A key player in the development of diabetic complications is oxidant stress.\textsuperscript{11} Previous studies in vascular cells have linked generation of reactive oxygen species (ROS) and enhanced oxidant stress with the interaction of AGE with its receptors.\textsuperscript{12,13} Although multiple ROS sources are implicated in diabetes, a major source that is important in diabetic vascular disease is NADPH oxidase. The latter is a multisubunit complex that consists of a membrane-bound cytochrome \( b_{558} \) (made up of the subunits \( p22^{phox} \) and 1 of several NADPH oxidase [Nox] isoforms). Of the 5 Nox isoforms identified to date,\textsuperscript{14} the major isoforms expressed in cardiomyocytes are gp91phox (Nox2) and Nox4.\textsuperscript{15,16} Whereas Nox2 is regulated by 4 cytosolic subunits (\( p47^{phox} \), \( p67^{phox} \), \( p40^{phox} \), and Rac1 or Rac2), which translocate to the membrane on enzyme activation, current evidence suggests that Nox4 is constitutively active and may not require cytosolic subunits for increased activity.\textsuperscript{14,17} In human endothelial cells, incubation with AGEs induced intracellular generation of hydrogen peroxide, which was inhibited by the flavoprotein inhibitor diphenylethenonidium (DPI) but not by inhibitors of nitric oxide, consistent with a role for NADPH oxidase in this process.\textsuperscript{13} Furthermore, macrophages derived from mice deficient in the Nox2 subunit of NADPH oxidase failed to display enhanced levels of tissue factor on stimulation with AGE.\textsuperscript{13}

Although the above studies suggest a relationship between AGEs and increased oxidant stress, the potential relevance to cardiac disease remains unclear. Furthermore, most studies investigating diabetic complications have focused on the roles of AGEs, whereas the biological effects of early-glycation Amadori-modified proteins remain poorly defined. Interestingly, recent studies indicate an important role for ROS production, particularly that derived from NADPH oxidase. The latter is a multisubunit NOX (NADPH oxidase) complex that consists of a membrane-bound cytochrome \( b_{558} \) made up of the subunits \( p22^{phox} \), \( p67^{phox} \), \( p40^{phox} \), and Rac1 or Rac2, which translocate to the membrane on enzyme activation, current evidence suggests that Nox4 is constitutively active and may not require cytosolic subunits for increased activity.\textsuperscript{14,17} In human endothelial cells, incubation with AGEs induced intracellular generation of hydrogen peroxide, which was inhibited by the flavoprotein inhibitor diphenylethenonidium (DPI) but not by inhibitors of nitric oxide, consistent with a role for NADPH oxidase in this process.\textsuperscript{13} Furthermore, macrophages derived from mice deficient in the Nox2 subunit of NADPH oxidase failed to display enhanced levels of tissue factor on stimulation with AGE.\textsuperscript{13}

Although the above studies suggest a relationship between AGEs and increased oxidant stress, the potential relevance to cardiac disease remains unclear. Furthermore, most studies investigating diabetic complications have focused on the roles of AGEs, whereas the biological effects of early-glycation Amadori-modified proteins remain poorly defined. Interestingly, recent studies indicate an important role for ROS production, particularly that derived from NADPH oxidase, in both angiotensin II–induced and pressure overload–induced cardiac hypertrophy.\textsuperscript{15,18}

The aim of the present study was to investigate the effects of glycated serum albumin on ROS production in cardiomyocytes, to identify the potential sources of ROS production, and to assess the downstream effects of ROS generation.

**Methods**

**Reagents**

Bovine serum albumin (BSA; nonglycated and glycated) was obtained from Sigma Chemical Co. The procedure (4 to 5 days’ incubation in 28 mmol/L glucose at 25°C) used to synthesize glycated albumin (Gly-BSA) generates Amadori glucose adducts with little or no oxidative degradation products or formation of AGE.\textsuperscript{10} Gly-BSA contained 2.7 mol of fructosamine per 1 mol of albumin. AGEs (20 mg/mL) were prepared by incubating BSA with glucose for 12 weeks at 37°C.\textsuperscript{19} We confirmed the absence of endotoxin (measured as \(<0.015 \text{endotoxin U/mL}\) using the limulus amoebocyte lysate assay (Charles River Laboratories). Culture medium, fetal calf serum (FCS), and antibiotics were purchased from Gibco BRL. Apocynin was purchased from Acros Organics. Collagenype type II was purchased from Worthington, and all other products were purchased from Sigma.

**Preparation of Cultured Neonatal Rat Ventricular Myocytes**

Primary cultures of ventricular myocytes were isolated from 1- to 2-day-old neonatal Sprague-Dawley rats as described previously.\textsuperscript{20} Briefly, hearts were excised, minced, and enzymatically digested at 37°C with ADs buffer (116 mmol/L NaCl, 20 mmol/L HEPES, 0.8 mmol/L NaH\( \text{PO}_4 \), 5.6 mmol/L glucose, 5.4 mmol/L KCl, 0.8 mmol/L MgSO\(_4\) containing collagenase (5.75 U/mL) and pancreatin (1.5 mg/mL). The suspension was preincubated to remove contaminating cells, before culture at a density of \(2\times10^3 \) cells/mL. Cells were allowed to adhere for 24 hours before serum starvation for an additional 24 hours. Myocytes were then exposed to Gly-BSA, AGE, or high glucose (HG; 25 mmol/L) for up to 48 hours. Nonglycated BSA was used as a control such that each control well was exposed to an albumin concentration equivalent to that of the treated wells. Subsets of myocytes were also exposed to the antioxidants N-acetylcysteine (NAC; 10 mmol/L) or butylated hydroxyanisole (BHA; 50 \( \mu \text{mol/L} \)), the NADPH oxidase inhibitor apocynin (500 \( \mu \text{mol/L} \)), the protein kinase C (PKC) inhibitor bisindolylmaleimide I (Bis I; 5 \( \mu \text{mol/L} \)), a rabbit anti-human anti-RAGE (receptor for AGE) antibody (75 \( \mu \text{g/mL} \)), or appropriate vehicle for 1 hour before BSA, Gly-BSA, or AGE treatment.

**Antisense Nox2 and Nox4 Oligonucleotides**

Nox2 and Nox4 antisense morpholino oligonucleotides (AS; Gene Tools, USA) were designed on the basis of the published rat sequences of Nox2 cDNA (5’-CCTATTCACAGCCGATGTC-3’) and Nox4 cDNA (5’-CAGCTCCTAGGACAGCGCATC-3’) and correspond to the region near the 5’ translational start site of the Nox2/4 open reading frame. A random, scrambled morpholino oligonucleotide was used as control. The morpholino oligonucleotides were mixed with ethoxyethyl polyethyleneimine (EPEI; Gene Tools) and delivered into cells according to the manufacturer’s protocol.\textsuperscript{21} After 3 hours of incubation, the delivery solution was replaced with serum-containing medium for 1 hour before incubation with serum starvation for another 24 hours before Gly-BSA treatment. Nox2 protein expression was assessed by Western blot analysis.

**Gp91ds-tat**

Gp91ds-tat is a peptide from cytosolic domain B of Nox2, which interferes with the binding of the cytosolic oxidase subunit \( p22^{phox} \), with the membrane-bound Nox isoform.\textsuperscript{22} The peptide is linked to a 9-amino acid peptide contained in human immunodeficiency virus (HIV) viral coats (HIV-tat), which is internalized by all cells. A scrambled 9–amino acid Nox2 sequence (scramb-tat) was used as a control. Gp91ds-tat and scramb-tat were dissolved in 0.01 mmol/L acetic acid in saline. Myocytes were incubated with gp91ds-tat (20 \( \mu \text{mol/L} \)) or scramb-tat for 1 hour before either Gly-BSA or AGE incubation.

**Assessment of Intracellular ROS Production**

Intracellular ROS production was measured with 2’,7’-dichlorodihydrofluorescein diacetate (DCF-DA) (Molecular Probes). Myocytes were incubated with DCF-DA (5 \( \mu \text{mol/L} \)) in serum-free medium at 37°C for 30 minutes and then washed with PBS. DCF fluorescence was excited at 488 nm, and emission at 530 nm was recorded on a Nikon inverted epifluorescence microscope (TE300) from between 15 and 20 randomly selected separate fields per chamber well with Felix software (Photo Technology International). Whole-cell fluorescence was measured. A field of myocytes (10 to 20 myocytes) was selected and excited for <5 seconds, and fluorescence was recorded from the entire field. No bleaching of DCF occurs under these conditions. This experiment was repeated at least 3 times with independent cardiomyocyte digestions. Fluorescence intensity values were presented as the percentage of the control value, after subtraction of background fluorescence.
Lucigenin Chemiluminescence
Superoxide (O2•−) production in cell homogenates was measured with lucigenin-enhanced chemiluminescence in a microplate luminometer (Anthos Lucy). Briefly, myocytes were detached, washed in PBS, and resuspended in 400 μL of buffer (50 mmol/L KH2PO4, 1 mmol/L EGTA, 150 mmol/L sucrose, pH 7.0) with a protease inhibitor cocktail (2 μL). Cells were homogenized and distributed in triplicate (10 μg/well) onto a 96-well microplate. NADPH (300 μmol/L) and dark-adapted lucigenin (5 μmol/L) were added to wells just before they were read. O2•− production was expressed as arbitrary light units over 20 minutes. The following agents were used to assess potential sources of O2•− production: DPI, a flavoprotein inhibitor (10 μmol/L); apocynin, an NADPH oxidase inhibitor (500 μmol/L); the cell-permeable superoxide scavenger Tiron (4,5-dihydroxy-1,3-benzene disulfonic acid; 2 mmol/L); the nitric oxide synthase inhibitor L-NAME (N-nitro-l-arginine methyl ester; 100 μmol/L); the xanthine oxidase inhibitor allopurinol (100 μmol/L); the complex I mitochondrial electron chain inhibitor rotenone (2 μmol/L); and the complex II mitochondrial electron chain inhibitor thenoyltriﬂuoroaceton (10 μmol/L); and the mitochondrial uncoupler, carbonyl cyanide m-chlorophenylhydrazone (0.5 μmol/L).

Real-Time Reverse Transcription–Polymerase Chain Reaction
SYBR Green real-time reverse-polymerase chain reaction (PCR) was performed with an ABI PRISM 7700 machine (Applied Biosystems). All quantitative assays used the universal thermal cycling parameters (initial steps: 50°C for 2 minutes, 95°C for 10 minutes; and 95°C for 15 seconds, 60°C for 1 minute, for 40 cycles). Quantification was accomplished by measuring threshold cycle (CT) using β-actin as the internal control. The oligonucleotide primers were as follows: atrial natriuretic factor (ANF): forward primer 5′-CCAGGCGCATGATGGAGCAGA-3′, reverse primer 5′-GCCAGTTTCTGAATCCATCAG-3′; β-actin: forward primer 5′-CGTGAAAGATGACCCAGATCA-3′, reverse primer 5′-TGGTACGACACAGGACCATCAG-3′.

Membrane/Nuclear Protein Preparation and Western Blotting
Adherent neonatal cardiomyocytes were scraped into a hypotonic buffer (10 mmol/L HEPES, 10 mmol/L KCl, 1 mmol/L MgCl2, 1 mmol/L NaF, 1 mmol/L Na3VO4, and protease inhibitors) on ice and homogenized. Cell lysates were centrifuged (4°C, 5 minutes, 10,000 rpm) to remove intact cells and nuclei. The supernatant was further centrifuged at 13,000 rpm for 40 minutes at 4°C, and the sedimented crude plasma membrane was dissolved in 1× reducing sample buffer. Nuclear protein extractions were performed according to standard protocols.

Protein samples (15 μg) were subjected to electrophoresis and transferred to nitrocellulose. The membranes were probed with an anti-Rac1 mouse monoclonal antibody (Upstate Technology), anti-p22phox (internal loading control), or anti-NF-κB p65 rabbit polyclonal antibodies (Chemicon International) and horseradish peroxidase–conjugated secondary antibodies. The bound antibodies were visualized by chemiluminescence detection, and protein levels were quantified by scanning densitometry. Rac protein levels were normalized by the p22phox protein level and values expressed in arbitrary units.

Cell Staining
Cells were seeded onto chamber cover slides and cultured as described above. Cells were fixed and exposed to anti-Rac1 mouse monoclonal antibody or rabbit anti-NF-κB p65 polyclonal antibody, Alexa-568–conjugated anti-mouse antibody or biotinyl-labeled anti-rabbit secondary antibody was applied for 60 minutes. NF-κB staining was visualized by DAB substrate (Vector Laboratories).

Statistical Analysis
All data are shown as mean±SEM of at least 3 different experiments. Significance was determined with 1-way ANOVA with Tukey post hoc analysis. P<0.05 was considered significant.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

In Situ ROS Production
In situ ROS production, assessed by DCF fluorescence, was significantly increased after 24 hours of Gly-BSA treatment in a concentration-dependent manner (Figure 1A). The optimum response was achieved at 400 μg/mL of Gly-BSA (152±10% compared with BSA [100%]; P<0.01; n=3). No further increase in ROS production was observed when the concentration of Gly-BSA was doubled from 400 to 800 μg/mL. No acute stimulation of ROS by Gly-BSA was observed, with the earliest significant increase in ROS production apparent only after 4 hours of incubation (128±1%) and reaching a plateau at 24 hours (152±10%), remaining stable thereafter (Figure 1B). For all future experiments, myocytes were treated with 400 μg/mL Gly-BSA for 24 hours.

Because glycated products accumulate under hyperglycemic conditions, we investigated whether HG can amplify the Gly-BSA response. After 24 hours of serum-free culture, myocytes were incubated in normal glucose or HG (25.5 mmol/L) and Gly-BSA (100 and 400 μg/mL) or BSA (100 and 400 μg/mL) for a further 24 hours. As shown in Figure 1C, HG alone increased intracellular ROS production by 24.5±3.9% compared with BSA (100%; P<0.01; n=3). However, no amplification of the Gly-BSA response was seen in HG, ie, no synergism between the HG and Gly-BSA response was apparent. As observed for Gly-BSA, no acute stimulation of ROS production was observed with HG (0.5 hour, 100.2±1.5%; 1 hour, 102±1.5%; 2 hours, 101.5±1.0%; 4 hours, 100.3±2.6% versus control 100%).

Role of RAGE in Gly-BSA–Induced ROS Production
To determine whether the Gly-BSA response was mediated via RAGE, myocytes were cultured in the presence or absence of an antibody to RAGE. No effect of anti-RAGE on the Gly-BSA response was observed after 24 hours of stimulation (Figure 2). However, real-time reverse transcriptase (RT)–PCR experiments confirmed expression of RAGE mRNA in cardiac myocytes (data not shown), and anti-RAGE significantly decreased an AGE-mediated increase in ROS production (Figure 2). Hence, RAGE is present in cultured neonatal rat cardiomyocytes, and there is a functional downstream signaling pathway that can facilitate an increase in ROS production in response to AGE. However, RAGE does not appear to be involved in the Gly-BSA–mediated response.

Gly-BSA–Induced ROS Production Occurs via a PKC-Dependent Activation of NADPH Oxidase
Apocynin was used to determine the involvement of NADPH oxidase in Gly-BSA–induced ROS production. Apocynin prevents the association of the cytosolic subunit p47phox with the membrane-bound catalytic subunit of NADPH oxidase. Apocynin significantly attenuated Gly-BSA–induced ROS production (assessed by DCF fluorescence) by 68.5±2.2%
oxidase, the effect of Gly-BSA on NADPH oxidase activity was specifically assessed by lucigenin-enhanced chemiluminescence with NADPH as a substrate in cardiomyocyte homogenates. In this assay, no activity is seen in the absence of NADPH. Figure 3B clearly demonstrates that Gly-BSA significantly increased NADPH oxidase activity by 61±8% (P<0.01; n=5) and that this increase was completely blocked by the flavoprotein inhibitor DPI, the cell-permeable superoxide scavenger Tiron, and the PKC inhibitor Bis I (5 μmol/L). However, although DPI and Tiron completely abolished oxidase activity, Bis I only reduced activity to baseline levels. Further experiments demonstrated that Bis I alone had no significant effect on NADPH oxidase activity (data not shown). Importantly, ROS production was unaffected by a range of mitochondrial inhibitors, a nitric oxide synthase inhibitor, or a xanthine oxidase inhibitor.

Further information regarding the mechanism by which PKC activates NADPH oxidase is provided by immunofluorescence experiments demonstrating a clearly increased membrane translocation of Rac1 in cardiomyocytes pretreated for 24 hours with Gly-BSA (Figure 4A). This increase in membrane translocation of Rac1 is completely lost in the presence of Bis I. Confirmation of these results was provided by Western blot analysis of membrane fractions isolated from cardiomyocytes (Figure 4B). A significantly increased membrane expression of Rac1 was observed in the presence of Gly-BSA that was prevented by Bis I. These results suggest that PKC is required for the translocation of Rac1 to the plasma membrane in the presence of Gly-BSA.

**Role of a Nox2-Containing NADPH Oxidase in Gly-BSA–Induced ROS Production**

Nox2 is one of the major Nox isoforms of NADPH oxidase expressed in cardiomyocytes. To investigate the role of Nox2 in Gly-BSA–induced ROS production, myocytes were pretreated with an antisense morpholino oligonucleotide that specifically targeted the Nox2 subunit (Nox2 AS). Treatment of neonatal myocytes with the Nox2 AS markedly decreased Nox2 protein expression 48 hours after treatment (Figure 5A). In line with this, the Nox2 AS also significantly

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**Figure 1.** A, Relationship between Gly-BSA concentration and ROS production. Cardiomyocytes were exposed for 24 hours to Gly-BSA (10 to 800 μg/mL) or BSA. B, Temporal effect of Gly-BSA on ROS generation. Cardiomyocytes were treated with 400 μg/mL Gly-BSA for 0.5 to 48 hours. C, Additive effects of Gly-BSA and HG on ROS release. Cardiomyocytes in normal or HG (25.5 mmol/L, 24 hours) media were exposed for 24 hours to Gly-BSA (100 or 400 μg/mL). *P<0.05, **P<0.01 vs BSA. NS indicates no significance vs 24 hours. n=3 per group.

(P<0.01; n=4), which suggests an involvement of the oxidase in this process (Figure 3A). Pretreatment of cardiomyocytes with the antioxidants BHA (50 μmol/L) or NAC (10 mmol/L) similarly attenuated ROS production (P<0.01).

DCF fluorescence is an in situ measure of total ROS production and hence quantifies ROS from all cellular sources. Thus, to further clarify the involvement of the
attenuated Gly-BSA–induced in situ ROS production in cardiomyocytes by 61.4±8.3% (P<0.01; n=3; Figure 5B). Importantly, neither the delivery reagent EPEI nor the scrambled control oligonucleotide had any effect (Figure 5B). A Nox4 AS also had no effect on Gly-BSA–induced ROS production (Gly-BSA 158±6% versus Gly-BSA plus Nox4 AS 160±3%).

For further corroboration of the Nox2 AS results, neonatal myocytes were treated with the NADPH oxidase inhibitor gp91ds-tat. In line with the effect of the Nox2 AS, gp91ds-tat (20 μmol/L) significantly attenuated Gly-BSA–induced ROS production by 53.2±5.4% (P<0.01; n=3). Scrmb-tat had no effect (Figure 5C).

Lucigenin-enhanced chemiluminescence was used to specifically assess the effect of the oxidase inhibitors on Gly-BSA–induced NADPH oxidase activity. Preincubation with either apocynin or Nox2 AS completely abolished Gly-BSA–induced stimulation of NADPH oxidase activity (Figure 5D). Thus, the incomplete inhibition of the Gly-BSA–induced increase in DCF fluorescence by apocynin and Nox2 AS may be because only 50% to 60% of this ROS originates from a Nox2-containing oxidase. These experiments collectively strongly support the concept that a Nox2-containing NADPH oxidase is a major source of Gly-BSA–induced ROS production.

Activation of NF-κB and ANF mRNA Expression
To determine the downstream consequences of Gly-BSA–induced ROS production, we investigated whether Gly-BSA activated the redox-sensitive nuclear transcription factor NF-κB. In its inactive state, NF-κB is maintained as a latent form present in the cytoplasm that masks the nuclear localization signal. Exposure of serum-starved rat neonatal myocytes to 400 μg/mL Gly-BSA for 24 hours resulted in NF-κB activation, as depicted by enhanced translocation of NF-κB to the nucleus (Figure 6A). Western blot analysis of nuclear protein with anti-NF-κB antibody revealed a consistently increased level of nuclear NF-κB expression after Gly-BSA incubation for 24 hours (2.30±0.42-fold increase versus BSA control; P<0.01; Figure 6B). Apocynin significantly inhibited NF-κB nuclear translocation. To further confirm these data, we additionally analyzed NF-κB activity using an NF-κB–dependent promoter luciferase reporter assay. A significant increase in activity was seen after Gly-BSA treatment (124±5%) compared with control (100%; P<0.05) that was completely inhibited by either apocynin (95±9%) or gp91ds-tat (98±3%).

Associated with NF-κB activation was a significant increase in mRNA expression of the hypertrophic marker ANF (2.76±0.48-fold increase versus BSA controls; Figure 6C). Both NAC and apocynin significantly inhibited this increase, which suggests that Gly-BSA–stimulated ANF expression was redox-sensitive and occurred via NADPH oxidase activation.

Discussion
The major novel finding of this study is that a Nox2-containing NADPH oxidase is pivotally involved in the...
oxidative response of isolated cardiomyocytes to glycated albumin. Importantly, the Gly-BSA–induced increase in ROS production is linked to activation of the transcription factor NF-κB and to increased ANF mRNA expression, which suggests that Gly-BSA may play a role in altering the cardiomyocyte phenotype through activation of a Nox2-containing NADPH oxidase. These findings could be relevant to diabetic heart disease.

Growing evidence suggests that overproduction of ROS may be the key initiating event that leads to long-term development of diabetic complications.11 However, the specific mechanisms linking hyperglycemia with oxidative stress and cardiomyopathy are poorly understood. The present study provides strong evidence that an early-glycated protein stimulates ROS production in cardiomyocytes largely via a PKC-mediated activation of a Nox2-containing NADPH oxidase. AGEs have been shown to mediate many of their effects via a family of specific cellular receptors, the best characterized of which is the receptor for AGE (RAGE).25,26 However, Figure 4. A, Translocation of Rac by Gly-BSA. Cardiomyocytes were exposed to (1) BSA, (2) BSA+Bis I (5 μmol/L), (3) Gly-BSA (400 μg/mL), and (4) Gly-BSA+Bis I for 24 hours. Cells were stained with anti-Rac1 monoclonal antibody; arrows indicate Rac translocation to the plasma membrane. B, Western blot analysis of membrane Rac1 protein expression. **P<0.01 vs BSA control; ††P<0.01 vs Gly-BSA. n=4/group. p22phox was used as an internal loading control.

Figure 5. A Nox2-containing NADPH oxidase is involved in Gly-BSA–induced ROS production. A, Effect of Nox2 AS on Nox2 protein expression by immunoblotting. BSA indicates control; EPEI, oligo delivery reagent; SC, scrambled control oligo; AS, antisense Nox2 morpholino oligonucleotide; and Nox2⁺⁺⁺⁺, protein from Nox2⁻⁻⁻⁻ mouse as negative control. B, Effect of Nox2 AS on Gly-BSA (400 μg/mL, 24 hours)–induced ROS production. C, Effect of gp91 ds-tat on Gly-BSA (400 μg/mL, 24 hours)–induced ROS production. Cardiomyocytes were treated with gp91 docking sequence (gp91 ds-tat, 20 μmol/L) and scrambled control sequence (scrmb-tat) for 1 hour before Gly-BSA treatment. D, Effects of apocynin (Apo; 500 μmol/L) and antisense Nox2 on Gly-BSA–induced NADPH oxidase activity. **P<0.01 vs control; ††P<0.01 vs Gly-BSA. NS indicates no significance vs Gly-BSA. n=4/group.
little information is available on the role of RAGE in cardiomyocytes. We have demonstrated that an anti-RAGE antibody, despite inhibiting an AGE-mediated increase in ROS production, was unable to modify the Gly-BSA–mediated response at 24 hours. Similarly, Hattori et al also demonstrated in vascular smooth muscle cells that soluble RAGE was able to inhibit the increases in NF-κB and AP-1 activity in response to AGE but not Gly-BSA. Clinically relevant concentrations of both Gly-BSA and AGE were used in the present study. Interestingly, at these concentrations, comparative stimulation of ROS production was considerably greater in response to Gly-BSA (400 μg/mL) than to AGE-BSA (200 μg/mL). At 400 μg/mL, the AGE response (155±6%) was approximately equivalent to the maximal Gly-BSA response (400 μg/mL, 153±10% or 800 μg/mL, 155±6%). The slow time course of the Gly-BSA response in cardiomyocytes could be consistent with an increase in gene expression of oxidase subunits; however, real-time RT-PCR experiments did not reveal any increase in expression of Nox2, Nox4, p47phox, or p22phox after Gly-BSA treatment (data not shown). Further studies will be required to delineate both the relevant receptor and downstream signaling pathways that mediate the Gly-BSA response in cardiomyocytes.

Nox2 is a major Nox isoform within the cardiovascular system. Our previous results demonstrated a pivotal role for a Nox2-containing NADPH oxidase in angiotensin II–induced cardiac hypertrophy and in the response of the macrophage to AGE. To specifically investigate the role of the Nox2 subunit in Gly-BSA–induced ROS production, myocytes were pretreated with either an antisense morpholino oligonucleotide that specifically targeted Nox2 or the NADPH oxidase inhibitor gp91ds-tat. Gp91ds-tat has been shown to completely block angiotensin II–induced aortic NADPH oxidase activity both in vivo and in vitro, an effect thought to be mediated via the Nox2 subunit. However, it failed to attenuate either potassium superoxide– or xanthine oxidase–generated superoxide, which indicates specificity for the NADPH oxidase. No significant effect of the Nox2 AS or gp91ds-tat on ROS production was observed under baseline conditions (data not shown), but both the Nox2 AS and gp91ds-tat significantly attenuated in situ Gly-BSA–induced ROS production in cardiomyocytes. In contrast, treatment with a Nox4 AS had no effect on Gly-BSA–induced ROS production, consistent with the idea that this isoform does not require cytosolic subunits for its activation.

Interestingly, neither inhibitor completely prevented the Gly-BSA–induced ROS production. To define whether this was due to incomplete inhibition of the oxidase by the 2 inhibitors or because only a percentage of the Gly-BSA response originated from the oxidase, the ability of the Nox2 AS and apocynin to inhibit the Gly-BSA–induced increase in NADPH-dependent O₂⁻ production was examined. Both apocynin and Nox2 AS completely abolished Gly-BSA–induced stimulation of NADPH-dependent superoxide production, which suggests complete inhibition of Nox2 activity. Thus, these experiments suggest that the inability of apocynin and Nox2 AS to completely inhibit Gly-BSA–induced increases in DCF fluorescence (total cellular ROS production) is because a proportion of this ROS originates from other sources.

Activation of NADPH oxidase occurs via multiple signaling pathways; however, several studies have demonstrated important roles for both PKC and Rac. In the present study, the nonspecific PKC inhibitor Bis I, DPI, and Tiron prevented the Gly-BSA stimulation of NADPH oxidase activity. Activity was unaffected by L-NAME, allopurinol, or any of the mitochondrial inhibitors. Interestingly, Bis I only reduced oxidase activity to baseline levels, whereas both DPI
and Tiron completely abolished activity. This suggests that PKC does not tonically stimulate NADPH oxidase activity. Another important activator of the NADPH oxidase is Rac.9,14,17 In the present study, we have demonstrated that Bis completely prevented the Gly-BSA–induced membrane translocation of Rac1, which suggests that PKC lies upstream of Rac and oxidative activation in the signaling pathway that leads to Gly-BSA–induced ROS production. Activation of the transcription factor NF-κB is important in the regulation of genes in response to cellular stress.10,33 NF-κB is recognized as an important redox-sensitive transcription factor and has been implicated in the development of cardiomyocyte hypertrophy.33,34 We have demonstrated that Gly-BSA stimulates translocation of NF-κB to the nucleus and that apocynin can almost completely inhibit this translocation. Furthermore, apocynin or gp91ds-tat completely inhibited the increase in NF-κB activity induced by Gly-BSA. Collectively, these data strongly suggest that Nox2-derived ROS plays an important role in Gly-BSA–induced NF-κB activation. In line with an important role for NF-κB in the development of cardiac hypertrophy,33,34 ANF mRNA expression was stimulated to a similar degree by Gly-BSA, and this increase was inhibited by both apocynin and NAC.

Hyperglycemia has been shown to stimulate ROS production in a range of cell types6,32,35 from both mitochondrial sources35 and NADPH oxidase.32 However, the latter study used the nonspecific flavoprotein inhibitor DPI to implicate the oxidase.32 We sought to determine whether Gly-BSA–induced ROS production was increased if administered in conjunction with HG and whether the oxidase was involved in this response. However, no amplification of the Gly-BSA response was seen in the presence of hyperglycemia; rather, the 2 responses appeared additive. Nishikawa et al35 demonstrated that hyperglycemia induced a 200% increase in ROS production in cultured bovine aortic endothelial cells after only 2 hours of incubation, which was completely inhibited by a mitochondrial complex II inhibitor but not by a complex I inhibitor. However, in cardiomyocytes, the present data demonstrate that the effect of HG is more modest (25% increase) and of a similarly slow time course to the Gly-BSA response; that is, it required longer than 4 hours to develop. An involvement of the oxidase was supported by a significant stimulation of NADPH oxidase activity at 24 hours and by the fact that apocynin reduced intracellular ROS production after HG treatment by ≈50% (data not shown). Stimulation of NADPH-dependent activity by hyperglycemia was unaffected by 3 different mitochondrial inhibitors but completely prevented by DPI and Tiron (data not shown). Thus, these results suggest that in cardiomyocytes, HG stimulates ROS production modestly and slowly and that a significant portion of this response is attributable to NADPH oxidase. These results are in line with those of Basta et al,36 who recently reported that AGE-induced ROS generation in endothelial cells originates from both NADPH oxidase and mitochondria.

In conclusion, these results suggest that Gly-BSA, an early-glycated intermediate product, may contribute to the development of diabetic heart disease via the production of ROS and subsequent downstream activation of NF-κB and redox-sensitive genes. These effects involve the specific activation of a Nox2-containing NADPH oxidase. Further studies are warranted to assess whether targeting this pathway might be therapeutically beneficial.

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Disclosures
None.

References
CLINICAL PERSPECTIVE

Hyperglycemia is now recognized as a major causative factor in the development of diabetic cardiomyopathy. Acutely hyperglycemia promotes the production of reactive oxygen species (ROS) and reactive nitrogen species, and growing evidence implicates oxidative stress as a key initiating factor in diabetic vascular complications. The predominant sources of ROS in cardiac and vascular cells are the mitochondrial electron transport chain, dysfunctional nitric oxide synthases, NADPH oxidases, and xanthine oxidase. The relative importance of these sources in hyperglycemia-induced oxidative stress remains to be clearly defined. Furthermore, the mechanisms linking hyperglycemia with oxidative stress and cardiomyopathy are poorly understood. Chronically, hyperglycemia promotes the formation of glycated proteins. The importance of advanced-glycated end products in diabetes is well established; however, early-glycated products such as hemoglobin A1c and fructosamine constitute the overwhelming majority of circulating glycated products in vivo. Despite this, their biological effects remain poorly defined. The major novel finding of this study is that an early-glycated protein stimulates ROS production in cardiac myocytes largely via protein kinase C–mediated activation of a gp91phox (Nox2)-containing NADPH oxidase. Importantly, the increase in ROS production was linked to activation of the transcription factor nuclear factor-κB and to increased atrial natriuretic factor mRNA expression. Nuclear factor-κB activation and increases in atrial natriuretic factor mRNA expression were prevented by the NADPH oxidase inhibitor apocynin. These results suggest that early-glycated proteins may play a role in altering cardiomyocyte phenotype through activation of a Nox2-containing NADPH oxidase. Further studies are warranted to assess whether targeting this pathway might be therapeutically beneficial in treating the cardiovascular complications associated with diabetes.
Glycated Proteins Stimulate Reactive Oxygen Species Production in Cardiac Myocytes. Involvement of Nox2 (gp91phox)-Containing NADPH Oxidase
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