High glucose-induced Nox1-derived superoxides downregulate PKC-βII, which subsequently decreases ACE2 expression and ANG(1-7) formation in rat VSMCs

Eduard N. Lavrentyev and Kafait U. Malik

Department of Pharmacology, University of Tennessee Health Science Center, Memphis, Tennessee

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In the diabetic kidney, ACE2 expression is decreased by ~50% (32), whereas loss of ACE2 accelerates diabetes-induced kidney injury (38). Decreased ACE2 expression has been reported in rat vascular smooth muscle cells (VSMCs) treated with high glucose (HG) (18), suggesting that HG and/or glucose metabolites called glyco-toxins reduce ACE2 expression, which, in turn, could amplify the vascular damage in diabetes. ANG(1-7) contributes to the antihypertensive effects produced by inhibitors of the renin-angiotensin system, decreases the size of infarct and ischemic zones in experimental myocardial infarction, and prevents diabetes-induced cardiovascular dysfunction (2, 8, 12, 35). Indeed, in diabetic animal models, ANG(1-7) treatment reduces or prevents the development of diabetes-induced cardiovascular injury (2, 35).

Several biochemical mechanisms have been identified in the pathogenesis of hyperglycemia-induced vascular damage: 1) increased flux via the polyol pathway (22, 2) accelerated formation of advanced glycation end-products (AGEs) (6, 41); 3) NADPH oxidase-associated oxidative stress (22, 27, 29); and 4) excessive PKC activation (10).

In VSMCs, expression of the polyol pathway key enzyme aldose reductase is upregulated by HG (18), and sorbitol accumulation has been implicated in the pathogenesis of diabetic nephropathy and vascular injury (22). Simultaneously, AGEs are prevalent in the diabetic vasculature and contribute to the development of macrovascular complications (27, 41). AGEs are proteins or lipids that become nonenzymatically glycated in a Maillard reaction after an exposure to reducing sugars (6, 21). Polyol pathway activation and intracellular AGE accumulation lead to oxidative stress (22, 26, 27). In VSMCs, polyol pathway-dependent (29) and AGE-induced (26) increases of NADPH oxidase activity have been reported. ROS formed by NADPH oxidase are important signaling molecules (7). Many of the cellular perturbations initiated by AGEs are mediated by ROS (41).

In the arterial wall, NADPH oxidases are the main source of ROS (7, 37). In rodent VSMCs, two NADPH oxidase forms are present (9). Nox1-derived oxidase, localized in calveolae, consists of membrane-bound and cytosolic regulatory components. Nox4-based oxidase, found in focal adhesions and the nucleus, is without known cytosolic subunits (4, 9). AGEs and ANG II induce Nox1 expression, whereas Nox4 is downregulated by the same treatments (26, 17). In contrast to Nox1, Nox4-derived superoxides sustain VSMC

ANGIOTENSIN-CONVERTING ENZYME (ACE2), the first human homolog of ACE described, is an integral membrane protein that functions as a carboxypeptidase, cleaving a single hydrophobic/basic residue from the COOH-terminus of its substrates (33). ACE2 hydrolyzes the potent vasoconstrictor peptide ANG II to ANG(1-7). Also, ACE2 hydrolyzes dynorphin A(1-13), apelin-13, and des-Arg9 bradykinin (36). In addition to ACE2, nephrilysin has been reported to generate ANG(1-7) in vitro (25).

Address for reprint requests and other correspondence: K. U. Malik, Dept. of Pharmacology, Univ. of Tennessee Health Science Center, 874 Union Ave., Crowe Bldg. 217, Memphis, TN 38163 (e-mail: kmalik@utmem.edu).

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differentiation, indicating that the source and location of ROS production are of paramount importance in dictating the cellular response (4, 20, 28). Suppression of renal NADPH oxidase is considered a pharmacological target for treating diabetic nephropathy (34).

Hyperglycemia also increases intracellular levels of diacylglycerol (DAG), which activates PKC in various tissues associated with diabetic vascular complications, including the retina, aorta, heart, and renal glomeruli (10, 15, 30, 31). However, different PKC isoforms respond differently to hyperglycemia (30). In particular, PKC-βII activation has been reported to lead to various pathological effects that affect VSMC function (10). Clinical evaluations of selective PKC-βII inhibitors have revealed their beneficial effects for treating diabetic microvascular complications (1, 10, 31).

Therefore, we hypothesized that HG-induced alterations of the above-discussed biochemical mechanisms might participate in the HG-induced downregulation of ACE2 expression and the subsequent decrease of ANG(1-7) formation in rat VSMCs. To test this hypothesis, we investigated the effect of inhibitors of glucose transporter (GLUT)1, NADPH oxidase, and PKC-βII on HG-induced downregulation of ACE2 and ANG(1-7) formation.

MATERIALS AND METHODS

Materials

FBS, medium 199 (M199), penicillin-streptomycin, and 0.05% trypsin were obtained from Mediatech. Elastase, collagenase, and α-glucose were purchased from Sigma-Aldrich. The list of chemical inhibitors used in present study is shown in Table 1.

Rat Aortic VSMC Isolation and Culture

Experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats (Charles Rivers, Wilmington, MA), weighing 200–250 g, were anesthetized with 60 mg/kg ip pentobarbital sodium. Under aseptic conditions, the thoracic aorta was rapidly removed and incubated for 30 min in 5 mg/ml collagenase; afterward, the adventitia and intima were dissected, and the mixture was minced. Single VSMCs were separated by an incubation in 0.25 mg/ml elastase with 5 mg/ml collagenase for two 90-min periods at 37°C (Table 2). In particular, PKC-βII activation has been reported to lead to various pathological effects that affect VSMC function (10). Clinical evaluations of selective PKC-βII inhibitors have revealed their beneficial effects for treating diabetic microvascular complications (1, 10, 31).

Table 1. List of chemical inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>Source</th>
<th>Biological Effect</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochalasin B</td>
<td>Sigma-Aldrich (catalog no. C6762)</td>
<td>Glucose transporter inhibitor</td>
<td>1 μmol/l</td>
</tr>
<tr>
<td>Catalase</td>
<td>Sigma-Aldrich (catalog no. C9322)</td>
<td>ROS scavenger</td>
<td>150–200 U/ml</td>
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<tr>
<td>Apocynin</td>
<td>Sigma-Aldrich (catalog no. W508454)</td>
<td>NADPH oxidase inhibitor/general antioxidant</td>
<td>10 μmol/l</td>
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<tr>
<td>Diphenyleneiodonium</td>
<td>Sigma-Aldrich (catalog no. D2926)</td>
<td>NADPH oxidase inhibitor/general flavoprotein inhibitor</td>
<td>1 μmol/l</td>
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<tr>
<td>Aminoguanidine</td>
<td>Sigma-Aldrich (catalog no. 396494)</td>
<td>Advanced glycation end-product formation inhibitor</td>
<td>10 μmol/l</td>
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<tr>
<td>Alrestatin</td>
<td>Tocris Bioscience (catalog no. 0485)</td>
<td>Aldose reductase inhibitor</td>
<td>10 μmol/l</td>
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<tr>
<td>CG-53353</td>
<td>EMD Chemicals (catalog no. 539652)</td>
<td>PKC-βII inhibitor</td>
<td>100 nmol/l</td>
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<tr>
<td>Go-6976</td>
<td>EMD Chemicals (catalog no. 365250)</td>
<td>Conventional PKC isoform inhibitor</td>
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<tr>
<td>Calphostin C</td>
<td>BioMol (catalog no. EI198-0100)</td>
<td>Nonspecific (pan) PKC inhibitor</td>
<td>100 nmol/l</td>
</tr>
</tbody>
</table>

VSMC Transfection With Short Interfering RNA

SMARTpool short interfering RNAs (siRNAs) for silencing the expression of target genes and cyclophilin B siRNA as a negative control were purchased from Dharmacon. All transfections were carried out using the manufacturer’s protocol with DharmaFECT1 transfection reagent (Dharmacon). Briefly, VSMCs were trypsinized, counted, and plated at a density of 10⁶ cells/cm² in six-well plates (MIDSCI) in antibiotic-free M199 with 10% FBS and incubated overnight at 37°C with 5% CO₂. The next morning, when cells had reached ~40% confluence, the medium was replaced, and cells were transfected with 50, 75, or 100 nmol/l of SMARTpool siRNA or control siRNA using 6 μl of DharmaFECT1 reagent/well (Fig. 1). After 72 h, 1 ml of fresh medium was added; after 96 h, the transfection medium was replaced. Cells were washed twice with serum-free M199 and then subjected to NG or HG for 72 h as described above. The efficiency of siRNAs to silence target gene expression as determined by real-time quantitative PCR and Western blot analysis is shown in Fig. 1.

RNA Extraction and Real-Time Quantitative PCR

Cells were homogenized in 4 ml of RNA-Stat 60 (Tel-Test), and RNA isolation was carried out according to the manufacturer’s protocol. RNA samples were precipitated with 95% ethanol and dissolved in 200 μl diethylpyrocarbonate (DEPC)-water. To decrease genomic DNA contamination, samples were treated with DNase-I (Ambion) and stored at −80°C. For cDNA synthesis, we used 10 μl of DNA-free total RNA, 1 μl of random hexamers, and 1 μl of dNTPs with 1 μl of Superscript reverse transcriptase-III RNase H⁻ (Invitrogen). Total RNA samples were diluted in DEPC-water (1:100) and analyzed by real-time quantitative PCR on an iCycler (Bio-Rad Laboratories). PCR primers were designed to have ~20 nucleotides and ~50% or less of G/C content with a melting point lower than 62°C (Table 2). Amplification sequences were shorter than 75 nucleotides. Real-time quantitative PCR was performed in a total volume of 25 μl consisting of 12.5 μl of 2X FastStart SYBR Green Master Mix (Roche Diagnostic), 2.5 μl of each primer (100 nmol/l final concentration), 10 μl of cDNA, and 1 μl of DEPC-water. The thermal profile for these experiments was the following: 1 cycle at 94°C for 15 min, 40 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s. The relative quantities of the target genes were normalized to the housekeeping gene cyclophilin B, which was used as an internal control.
Fig. 1. Nox1, Nox4, PKC-βII, and glucose transporter 1 (GLUT1) short interfering (si)RNA efficiency. Subconfluent rat aortic vascular smooth muscle cells (VSMCs) were transfected in 6-well plates with siRNA for Nox1, Nox4, PKC-βII, GLUT1, or cyclophilin B (control siRNA) for 96 h. A: Nox1 expression; B: PKC-βII expression; C: Nox4 expression; D: GLUT1 expression. Data are shown as mRNA abundance and protein levels for each target gene (means ± SE; n = 4). *P < 0.05 vs. the corresponding value with control siRNA.
Preparation of Cell Lysates and Conditioned Media

Cells were harvested and lysed in 250 μl of 1× RIPA lysis buffer (Upstate) containing 1 mM Na3VO4, 1 mM PMSF, 1 mM NaF, 10 μg/ml antipain, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μg/ml captopril. The culture medium was collected in iCON concentrators (Pierce Biotechnology) and centrifuged at 3,000 rpm for 20 min. The resulting protein extract was resuspended with 250 μl of 1× RIPA buffer and is referred to as “conditioned media.” Subsequent to sonication, samples were cleared by centrifugation, and the protein concentration was determined with Bio-Rad Protein Assay Dye reagent (Bio-Rad Laboratories).

Western Blot Analysis

Equal amounts of protein per well (15 μg) were resolved by SDS-PAGE and transferred onto pure nitrocellulose membranes (0.45 mm, Bio-Rad Laboratories). Membranes were blocked with primary antibodies (1:200–1:2,000) overnight at 4°C (Table 3). The next day, membranes were exposed to their respective secondary antibodies conjugated with horseradish peroxidase (1:1,000–1:2,000), and films (Molecular Technologies) were developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology). The density of the bands was analyzed using NIH ImageJ software. For assurance of equal gel loading, membranes were stripped with Restore Western Blot Stripping Buffer (Pierce Biotechnology) and reprobed with anti-α-actin antibody.

Table 2. Primers designed for real-time quantitative PCR and mRNA sequence accession numbers in GenBank

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>GenBank Accession Number</th>
</tr>
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<tr>
<td>18S RNA</td>
<td>5'-AGGGAGCTCTGGTACAAGCTA-3'</td>
<td>5'-CCTTCTAATGATGCGCTCA-3'</td>
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<td>ACE2</td>
<td>5'-CTTACTGAGCTCTGGTACGAC-3'</td>
<td>5'-AATGCAAGAACACTCAGCAG-3'</td>
<td>NM_001012006</td>
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<tr>
<td>PKC-β</td>
<td>5'-AAAGCCTCTTGAGATGCTCTA-3'</td>
<td>5'-TGCTCTGCGCTTCGCTCTCA-3'</td>
<td>NM_012713</td>
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<tr>
<td>Nox1</td>
<td>5'-TCTTCCCCTGGACAAAGATGGA-3'</td>
<td>5'-CCAGCAAGACCTGAGCATTGG-3'</td>
<td>NM_053683</td>
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<tr>
<td>Nox4</td>
<td>5'-CAGACAACTGCTGTTAGATGAT-3'</td>
<td>5'-CGATGCGATGCGTTAAGTCT-3'</td>
<td>NM_053524</td>
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ACE, angiotensin-converting enzyme; Nox, NADPH oxidase isofrom.

Statistical Analysis

All statistical analyses, including means ± SE, were determined using GraphPad 4 software (GraphPad Software). Data were analyzed using ANOVA. The Newman-Keuls test was used for multiple comparisons. Values of P < 0.05 were considered statistically significant.

RESULTS

HG Decreased ACE2 mRNA Expression, ACE2 Protein, and ANG(1-7) Levels in Rat VSMCs

We have previously shown that in VSMCs, ACE2 expression is downregulated by HG (18). In present study, we confirmed this observation and performed experiments to elucidate the mechanism of HG-induced ACE2 downregulation. Initially, HG increased ACE2 mRNA expression by 1.71 ± 0.57-fold with a subsequent decrease after 12 h by >7-fold compared with NG (Fig. 2A). In cell lysates, ACE2 protein levels were reduced after 4 h of HG treatment and at 72 h reached 0.47 ± 0.03-fold of the value obtained in the presence of NG (Fig. 2B). In conditioned media within the first 12 h, ACE2 protein levels increased by 1.34 ± 0.21-fold and were almost undetectable thereafter (Fig. 2C). The ACE2 protein (media/cell) ratio reflects ACE2 shedding from cell surface, which was upregulated by 1.79 ± 0.33-fold within the first 12 h of HG treatment and was 0.25 ± 0.02-fold thereafter versus NG (Fig. 2D). ANG(1-7) levels were lowered by more than a half after 4 h of HG treatment and reached 0.16 ± 0.03-fold versus the value obtained in the presence of NG at 72 h (Fig. 2E). To eliminate the osmolar effect of 25 mmol/l d-glucose on ACE2 expression, mannitol, 1-

Table 3. Western blot conditions and list of antibodies used in the present study

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Source</th>
<th>Host Animal</th>
<th>Blocking Buffer</th>
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<th>Secondary Antibody Dilution</th>
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<tr>
<td>ACE2</td>
<td>Santa Cruz Biotechnology (catalog no. sc-17719)</td>
<td>Goat</td>
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<td>Nox1</td>
<td>Santa Cruz Biotechnology (catalog no. sc-5821)</td>
<td>Goat</td>
<td>7% milk</td>
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<td>1:1,500</td>
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<td>Nox4</td>
<td>Santa Cruz Biotechnology (catalog no. sc-30141)</td>
<td>Rabbit</td>
<td>7% milk</td>
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<td>PKC-βI</td>
<td>Santa Cruz Biotechnology (catalog no. sc-209)</td>
<td>Rabbit</td>
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<td>1:1,000</td>
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<td>Phospho-PKC-βII</td>
<td>Upstate (catalog no. 07-873)</td>
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<td>PKC-βIII</td>
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<tr>
<td>Phospho-EGFR</td>
<td>Cell Signaling Technology (catalog no. 2236)</td>
<td>Rabbit</td>
<td>3% BSA</td>
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<td>1:1,000</td>
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<td>EGFR</td>
<td>Santa Cruz Biotechnology (catalog no. sc-03)</td>
<td>Rabbit</td>
<td>5% milk</td>
<td>1:1,000</td>
<td>1:1,000</td>
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<tr>
<td>VSMC α-actin</td>
<td>Sigma-Aldrich (catalog no. A5228)</td>
<td>Mouse</td>
<td>1% casein (Bio-Rad)</td>
<td>1:20,000</td>
<td>1:1,000</td>
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<tr>
<td>Anti-rabbit antibody</td>
<td>Santa Cruz Biotechnology (catalog no. sc-2313)</td>
<td>Donkey</td>
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<td>Anti-goat antibody</td>
<td>Santa Cruz Biotechnology (catalog no. sc-2020)</td>
<td>Donkey</td>
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<tr>
<td>Anti-mouse antibody</td>
<td>GE Healthcare (catalog no. NXA 931)</td>
<td>Sheep</td>
<td>7% milk</td>
<td></td>
<td>1:1,000</td>
</tr>
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</table>

EGFR, EGF receptor; VSMC, vascular smooth muscle cell.
Inhibition of Glucose Uptake and/or Glycotoxin Accumulation by Rat VSMCs Diminished the Effect of HG on ACE2 Expression and ANG(1-7) Levels

In this experiment, we used 1 μmol/l cytochalasin B to inhibit glucose uptake (Fig. 3A), 10 μmol/l aminoguanidine to prevent the formation of AGEs (Fig. 3B), and 10 μmol/l alrestatin (an aldose reductase inhibitor) (Fig. 3C). All three compounds diminished the HG effect on ACE2 expression. Although cytochalasin B and aminoguanidine fully reversed the HG effect, alrestatin was only partially effective. Alrestatin minimized the effect of HG on ACE2 mRNA and ACE2 protein levels but did not prevent ANG(1-7) loss in the presence of HG (Fig. 3D). In addition to chemical inhibitors, we silenced GLUT1 expression (Fig. 1D), which diminished the effect of HG on ACE2 mRNA expression and protein levels (Fig. 4).

HG-Induced Downregulation of ACE2 Expression and ANG(1-7) Levels Was Minimized by NADPH Oxidase Inhibitors/General Antioxidants and Nox1 siRNA

The effect of HG on ACE2 and ANG(1-7) levels was fully reversed by the ROS scavenger catalase (150–200 U/ml), the antioxidant apocynin (10 μmol/l), and the flavoprotein inhibitor diphenyleneiodonium (DPI; 1 μmol/l) (Fig. 5). However, the very same treatments decreased ACE2 expression and ANG(1-7) levels in the presence of NG (Fig. 6).

ACE2 Expression Was PKC-βII Dependent: Effect of PKC Inhibitors and PKC-βII siRNA on ACE2 Expression and ANG(1-7) Levels

Biochemical mechanisms involved in hyperglycemia-induced vascular damage include impaired PKC-dependent intracellular signaling (10). Therefore, we tested whether PKC inhibitors would prevent HG-induced ACE2 and ANG(1-7) loss. In the presence of HG, 100 nmol/l calphostin C (a pan PKC inhibitor) did not affect ACE2 and ANG(1-7) levels but in the presence of NG downregulated ACE2 mRNA, ACE2 protein, and ANG(1-7) levels.

Two forms of NADPH oxidase (Nox1 based and Nox4 based) are present in rodent VSMCs (9). To evaluate the contribution of each NADPH oxidase in HG-induced ACE2 downregulation, we silenced Nox1 and Nox4 expression with siRNA (Fig. 1, A and C). Nox1 siRNA did not reduce Nox 4 mRNA levels, and Nox 4 siRNA did not alter Nox 1 mRNA levels (Fig. 7).

Nox4 silencing decreased ACE2 mRNA, ACE2 protein, and ANG(1-7) levels in the presence of NG (0.73 ± 0.08-, 0.55 ± 0.04-, and 0.62 ± 0.04-fold vs. vehicle, respectively) but had no effect on ACE2 expression in the presence of HG (Fig. 6). Unlike Nox4, Nox1 silencing diminished the effect of HG on ACE2 expression and increased ACE2 mRNA, ACE2 protein, and ANG(1-7) levels in the presence of HG (3.35 ± 0.5-, 2.35 ± 0.22-, and 3.47 ± 0.09-fold vs. vehicle, respectively) but did not affect ACE2 expression or ANG(1-7) levels in the presence of NG (Fig. 6).

ACE2 Expression and ANG(1-7) Formation in Cultured Rat VSMCs

AJP-Heart Circ Physiol • VOL 296 • JANUARY 2009 • www.ajpheart.org

Fig. 2. Effect of high glucose (HG) on angiotensin-converting enzyme (ACE2) expression and ANG(1-7) formation. A: ACE2 mRNA expression (means ± SE; n = 9). B: ACE2 protein levels in cell lysates (means ± SE; n = 11). C: ACE2 protein levels in cultured media (means ± SE; n = 4). D: ACE2 protein (media/cell) ratio (means ± SE; n = 4). E: ANG(1-7) levels in cell lysates (means ± SE; n = 3). *P < 0.05 vs. the corresponding value in the presence of normal glucose (NG). F: ACE2 protein levels in cell lysates. Mannitol, L-glucose, and 2-deoxyglucose (2-DO-glucose) were used as an osmotic controls (means ± SE; n = 3). *P < 0.05 vs. vehicle.
and ANG(1-7) levels (0.14 ± 0.03-, 0.35 ± 0.03-, and 0.35 ± 0.02-fold vs. vehicle, respectively; Fig. 8, A and C).

Similar to calphostin C, 1 μmol/l Gö-6976 (a conventional PKC isoform inhibitor) downregulated ACE2 mRNA, ACE2 protein, and ANG(1-7) levels in the presence of NG (0.29 ± 0.06-, 0.57 ± 0.01-, and 0.50 ± 0.03-fold vs. vehicle, respectively). However, in the presence of HG, Gö-6976 prevented the decrease of ACE2 expression and ANG(1-7) levels (Fig. 8, A and C).

The effect of HG on ACE2 expression and ANG(1-7) levels was fully reversed by 100 nmol/l CG-53353 (a selective PKC-βII inhibitor), whereas in the presence of NG, CG-53353 slightly downregulated ACE2 mRNA expression (Fig. 8, A–C). To confirm the selectivity of the effect of CG-53353 on ACE2 expression, PKC-βII expression was silenced with siRNA (Fig. 1B). The effects of PKC-βII siRNA and CG-53353 on ACE2 mRNA expression, ACE2 protein, and ANG(1-7) levels were contradictory. In contrast to CG-53353, PKC-βII silencing decreased ACE2 mRNA, ACE2 protein, and ANG(1-7) levels in the presence of NG (0.35 ± 0.07-, 0.55 ± 0.02-, and 0.37 ± 0.2-fold vs. vehicle, respectively) but did not affect ACE2 levels in the presence of HG (Fig. 8, A and C). Moreover, in presence of NG, the ACE2 decrease correlated with the level of PKC-βII silencing (Fig. 8D), whereas in presence of HG, when PKC-βII was already downregulated, PKC-βII silencing had no effect on ACE2 expression.

HG Induces Nox1 and Decreases Nox4 Expression; Effect of HG on Nox1 Expression Was Diminished by Cytochalasin B, Aminoguanidine, and PKC Inhibitors But Not by PKC-βII siRNA or Alrestatin

HG induced Nox1 mRNA and protein levels (1.70 ± 0.22- and 1.62 ± 0.10-fold vs. NG at 72 h, respectively) and downregulated Nox4 mRNA and protein levels (0.29 ± 0.01- and 0.43 ± 0.01-fold vs. NG at 72 h, respectively) (Fig. 9). The effect of HG on Nox1 levels was diminished by 1 μmol/l cytochalasin B and 10 μmol/l aminoguanidine, whereas 10 μmol/l alrestatin had only a partial inhibitory effect (Fig. 10A). These compounds did not affect Nox1 levels in the presence of NG.

The PKC inhibitors calphostin C, Gö-6976, and CG-53353 effectively diminished the effect of HG on Nox1 expression, whereas none of the PKC inhibitors affected Nox1 expression in the presence of NG. In contrast to CG-53353, PKC-βII silencing did not affect Nox1 expression in the presence of HG (Fig. 10B).

HG Decreases PKC-β mRNA Expression and Switches PKC-β Splicing Toward the PKC-βI Isoform; This Effect of HG Was Diminished by CG-53353

In rat VSMCs, both PKC-β splicing isoforms are expressed (29). Consequently, we found that HG decreased PKC-β mRNA abundance (0.47 ± 0.02-fold vs. NG; Fig. 11A) and
affected PKC-β mRNA splicing toward PKC-βI. Thus, in the presence of HG, PKC-βII protein levels were decreased (0.32 ± 0.02-fold vs. NG; Fig. 11C), whereas PKC-βI levels remained unchanged (Fig. 11B). These changes in PKC-β mRNA expression and PKC-βI and PKC-βII protein levels were diminished by CG-53353 (Fig. 11, D–F).

**HG-Induced PKC-βII Downregulation Was Reversed by NADPH Oxidase Inhibitors/Antioxidants, Nox1 Silencing, and by Prevention of Glycotoxin Accumulation**

Our data suggest that ACE2 expression is PKC-βII dependent (Fig. 8D) and HG downregulates PKC-βII levels (Fig. 11C). Therefore, we postulated that the inhibition of glycotoxin formation and/or NADPH oxidase-derived oxidative stress stimulated by glycotoxin accumulation should diminish the HG-induced PKC-βII downregulation, which subsequently reduces ACE2 expression, as previously observed (Figs. 3–6).

Cytochalasin B, alrestatin, or aminoguanidine reversed the effect of HG on PKC-βII expression (Fig. 12A) as well as DPI, apocynin, catalase, and Nox1 siRNA (Fig. 12B), whereas Nox4 silencing did not affect PKC-βII levels (Fig. 12B). Neither NADPH oxidase inhibitors, nor catalase, nor Nox1 siRNA affected PKC-βII expression in the presence of NG.

**DISCUSSION**

Despite great improvements in the diagnosis and treatment of diabetes, the majority of these patients still die because of the progression of diabetes-induced cardiovascular complications.

In animal models, the development of diabetic cardiovascular injury was prevented by ACE2 overexpression or by treatment with ANG(1-7), which makes ACE2 and ANG(1-7) potential pharmacological targets for the prevention/treatment of diabetes-induced cardiovascular complications (5, 8, 12, 35, 38).

ACE2 abundance/activity can be regulated via two distinct mechanisms: short term (fast), resulting from ACE2 shedding from the cell surface, and long term (slower), involving the regulation of ACE2 expression (16). In rat VSMCs, HG downregulates ACE2 expression (18). Our present results suggest that HG affects both mechanisms and results in rapid ACE2 shedding (Fig. 2, C and D) and downregulation of ACE2 mRNA expression (Fig. 2A), which subsequently decreases ANG(1-7) formation (Fig. 2E). Likewise, ACE2 expression was decreased by ~50% in diabetic renal tubules (32).

Our study was performed to elucidate the mechanism by which HG downregulates ACE2 expression in rat VSMCs. Three biochemical pathways (glycotoxin influx, NADPH oxidase-associated oxidative stress, and HG-impaired PKC signaling) were studied. The proposed mechanism of HG-induced ACE2 downregulation is shown as a diagram in Fig. 13.
HG influx and/or Glycotoxin Accumulation Initiate the Whole Cascade That Downregulates ACE2 Expression and ANG(1-7) Formation

Glycotoxin influx plays the key role in HG-induced ACE2 downregulation (Fig. 2, A and B). Silencing of GLUT1 expression (Fig. 4) or inhibition of AGEs and sorbitol formation with chemical inhibitors (Fig. 3) prevented the HG-induced changes in Nox1 (Fig. 10) and PKC-βII (Fig. 11) expression that attenuated the effect of HG on ACE2 and ANG(1-7) levels. The HG-induced decrease in ACE2 expression was not due to increased osmolarity because L-glucose, mannitol, or 2-deoxyglucose in similar concentrations as D-glucose did not alter ACE2 expression (Fig. 2F).

HG Downregulates Nox4 and Increases Nox1 Expression, and Nox1-Derived Superoxides Mediate HG-Induced ACE2 Downregulation

In our study, HG increased Nox1 and decreased Nox4 expression (Fig. 9). The HG-induced Nox1 increase was inhibited by GLUT1 siRNA, cytochalasin B, aminoguanidine, and alrestatin, suggesting the involvement of AGEs in Nox1 expression. Alrestatin was much less effective (Fig. 10A), which could be due to a posttranscriptional modification of aldose reductase, such as S-thiolation, which makes this enzyme less sensitive to inhibitors (3). Similarly to HG, ANG II (17) and AGEs (26) increase Nox1 and downregulate Nox4 expression in VSMCs, suggesting the involvement of identical pathways. Indeed, in the vascular wall, ANG II, AGEs, and HG share proinflammatory and proatherosclerotic activities (41, 29, 27). HG and glycotoxins stimulate NADPH oxidase-dependent superoxide formation in VSMCs and cause oxidative stress (6, 22, 26, 27, 41). Our finding that the general antioxidant inhibitor apocynin, the flavoprotein inhibitor DPI, and the ROS scavenger catalase blocked the HG effect on ACE2 expression and ANG(1-7) levels suggests that the effect of HG is mediated via ROS (Fig. 5). Silencing of Nox1 prevented the HG-induced decrease in ACE2 expression and ANG(1-7) formation, suggesting that Nox1-derived ROS mediate this effect of HG (Fig. 6).

In contrast to Nox1, Nox 4 silencing had no effect on the HG-induced decrease in ACE2 expression and ANG(1-7) production (Fig. 6). However, in the presence of NG, Nox4 silencing and NADPH oxidase inhibitors decreased ACE2 expression and ANG(1-7) levels, suggesting that Nox4-derived superoxides are required for maintaining ACE2 expression and ANG(1-7) formation in VSMCs (Fig. 6). In fact, Nox4-derived superoxides are required for maintaining the differentiated VSMC phenotype (4).

Contribution of HG-Impaired PKC Signaling in the Downregulation of ACE2 Expression: Distinct Effects of Different PKC Inhibitors and PKC-βII siRNA

Glycotoxin-driven Nox1 upregulation is dependent on conventional PKC isoform(s). In our study, calphostin C [a non-specific (pan) PKC inhibitor], G6-6976 (a conventional PKC isoform inhibitor), and CG-53353 (a PKC-βII inhibitor) pre-
Fig. 8. Effect of PKC inhibitors and PKC-βII siRNA on ACE2 expression and ANG(1-7) levels in the presence of NG or HG. A: ACE2 mRNA expression (means ± SE; n = 3) and protein levels (means ± SE; n = 4). *P < 0.05 vs. vehicle in the presence of HG; †P < 0.05 vs. the corresponding treatment in the presence of NG. B: ACE2 mRNA expression (means ± SE; n = 3) and protein levels (means ± SE; n = 5). *P < 0.05 vs. the corresponding time point in the presence of NG; †P < 0.05 vs. vehicle in the presence of NG. C: ANG(1-7) levels in cell lysates (means ± SE; n = 3). *P < 0.05 vs. vehicle in the presence of HG; †P < 0.05 vs. vehicle in the presence of NG; ‡P < 0.05 vs. vehicle in the presence of NG. D: effect of PKC-βII silencing on ACE2 mRNA expression and protein levels in the presence of NG or HG (means ± SE; n = 3). ‡P < 0.05 vs. vehicle in the presence of NG.

Fig. 9. Effect of HG on Nox1 and Nox4 expression. A: Nox1 mRNA abundance and protein levels (means ± SE; n = 4). B: Nox4 mRNA abundance and protein levels (means ± SE; n = 3). CT, threshold cycle. *P < 0.05 vs. the corresponding value in the presence of NG.
vented the HG-induced Nox1 mRNA increase (Fig. 10B), suggesting that conventional PKC isoform(s) is(are) responsible for increased Nox1 expression, as previously reported (17). However, PKC-βII silencing did not affect Nox1 levels in the presence of HG, suggesting that PKC-βII does not mediate HG-induced Nox1 upregulation and that the effect of CG-53353 is nonspecific (Fig. 10B).

Calphostin C, Gö-6976, and CG-533353 inhibit different PKC isoforms (Table 1). Therefore, their specificity in diminishing HG-induced Nox1 upregulation must be a subject of

Fig. 10. Effect of cytochalasin B, alrestatin, aminoguanidine, PKC inhibitors, and PKC-β siRNA on Nox1 expression. A: Nox1 mRNA abundance and protein levels (means ± SE; n = 5). B: Nox1 mRNA and protein levels (means ± SE; n = 4). *P < 0.05 vs. vehicle in the presence of HG; †P < 0.05 vs. the corresponding value in the presence of NG.

Fig. 11. CG-53353 diminished the effect of HG on PKC-β mRNA expression and PKC-β isoform splicing. A: PKC-β mRNA expression (means ± SE; n = 4). B: PKC-βI protein levels (means ± SE; n = 4). C: phospho- and total PKC-βII protein levels (means ± SE; n = 4). D: effect of CG-53353 on PKC-βI mRNA expression in the presence of HG (means ± SE; n = 7). E and F: effect of CG-53353 on PKC-βI and PKC-βII protein levels in VSMCs in the presence of HG (means ± SE; n = 4). *P < 0.05 vs. the corresponding value in the presence of NG; †P < 0.05 vs. the 0-h value.
future studies. In fact, the question of which PKC(s) is(are) responsible for NADPH oxidase activation/upregulation by HG is still an open issue (10, 15, 23, 29, 39).

HG decreases PKC-β expression and switches PKC-β isoform splicing toward PKC-βII; ACE2 expression is PKC-βII dependent. PKC-βI and PKC-βII are splice isoforms of PKC-β mRNA (24) with opposing functions (40). It has been previously reported that PKC-βII is preferentially activated in tissues susceptible to diabetes-induced injury (11, 29, 39). However, our results demonstrate that HG downregulates PKC-β mRNA expression and switches PKC-β isoforms splicing toward PKC-βI (Fig. 11, A and B), whereas phospho-PKC-βII and total PKC-βII levels are decreased by 62% (Fig. 11C). In accordance with our observation, HG-induced PKC-βII downregulation accelerates VSMC proliferation, whereas HG does not affect PKC-βI levels (23). In the diabetic kidney cortex, the abundance of membrane-associated PKC-βII (the active form) is decreased by 50.00 ± 11.68% (42).

In the presence of NG, calphostin C, Gö-6976, and CG-53335 decreased ACE2 expression and ANG(1-7) formation, suggesting that ACE2 expression is conventional PKC dependent (Fig. 8A). Furthermore, in the presence of NG, 100 nmol/l of PKC-βII siRNA produced a greater decrease in PKC-βII and ACE2 expression compared with that caused by 75 nmol/l PKC-βII siRNA (Fig. 8D), whereas 50 nmol/l PKC-βII siRNA did not affect PKC-βII expression (Fig. 1B) and subsequently did not change ACE2 expression in VSMCs (Fig. 8D). Thus, the correlation between the level of PKC-βII and ACE2 expression suggests that ACE2 expression is PKC-βII dependent (Fig. 8D).

In the presence of HG, Gö-6976 and CG-53353 diminished the ACE2 decrease, indicating that HG-induced downregulation of ACE2 expression is mediated via conventional PKC (Fig. 8A). However, PKC-βII activation/upregulation could not be responsible for HG-induced ACE2 downregulation for the following reasons: 1) PKC-βII activity/expression was downregulated in the presence of HG (Fig. 11C), and 2) PKC-βII silencing did not attenuate the HG-induced ACE2 decrease (Fig. 8D). These results suggest that the effect of the PKC-βII inhibitor CG-533353 to diminish HG-induced ACE2 downregulation is due to its nonspecific effect mediated through a non-PKC-βII-dependent pathway (Fig. 8B).

Specificity of the “selective” PKC-βII inhibitor CG-533353: protective effects of “selective” PKC-βII inhibitors could be due to their non-PKC-βII-dependent pathways. Despite the fact that, in clinical trials, PKC-βII inhibitors have been shown to improve diabetes-induced cardiovascular damage, they are known to have other nonspecific effects. In our study, CG-53353 and PKC-βII siRNA exerted opposite effects on ACE2 and Nox1 expression and ANG(1-7) levels. Thus, in the pres-
ence of HG, CG-53353 prevented the ACE2 loss and ANG(1-7) decrease, whereas PKC-βII siRNA did not (Fig. 8, B and C). In the presence of NG, CG-53353 slightly down-regulated ACE2 mRNA abundance (Fig. 8A), whereas PKC-βII siRNA decreased ACE2 expression and ANG(1-7) levels (Fig. 8D), suggesting that ACE2 expression is PKC-βII dependent, whereas the CG-53353 effect on ACE2 expression in the presence of HG is PKC-βII independent. For example, the PKC-βII inhibitor LY-333531, which is undergoing a clinical trial phase III (1, 31), forms a complex with and inhibits 3-phosphoinositide-dependent protein kinase (PDK)-1, a key kinase for the insulin signaling pathway (14). DAG-dependent PKC activation is a key factor for hyperglycemia-induced vascular damage (10, 30). Insulin decreases DAG levels and slows the progression of diabetic cardiovascular complications (30) by preferentially switching the splicing of PKC-β isozymes to PKC-βII (24). Identical to insulin, in our study, CG-533535 prevented the HG-induced PKC-βII loss (Fig. 11F).

PKC-βII inhibitors may also bind with reversed orientation to different conformations of PKA (5). Alternatively, CG-53353 could block the EGF receptor (EGFR). It is marketed by EMD Chemicals as a PKC-βII/EGFR inhibitor (IC_{50} = 0.7 and 0.41 μM, respectively). However, the EGFR in VSMCs was dephosphorylated in the presence of HG (data not shown).

Furthermore, deletion of PKC-β isoforms in vivo reduces renal hypertrophy but not albuminuria in the streptozotocin-induced diabetic mouse model (19), whereas LY-333531 treatment slows glomerulosclerosis development and decreases urinary protein excretion (13). Similar to LY-333531, ANG(1-7) or the ANG(1-7) analog AVE-0991 reduced proteinuria and the progression of diabetic kidney injury (2).

Conclusions

HG/glycotoxin influx downregulate ACE2 expression and ANG(1-7) formation in rat VSMCs. The following is the proposed sequence of a biochemical pathway leading to this effect of HG: initially, glycotoxins (through conventional PKC) upregulate Nox1-based NADPH oxidase. Nox1-derived superoxides mediate PKC-β mRNA downregulation and PKC-βII protein loss. ACE2 mRNA expression is PKC-βII dependent, and the PKC-βII protein loss would subsequently result in the lowering of ACE2 expression and ANG(1-7) formation (Fig. 13).

Further studies using molecular tools such as siRNA are required to establish the PKC isoform(s) that is(are) involved in HG-induced Nox1 upregulation. Moreover, the role of PKC-βII in the development of diabetic cardiovascular complications and the precise target of PKC-βII inhibitors to ameliorate these complications remain to be established. Finally, a better understanding of the mechanisms regulating ACE2 expression in VSMCs and of tissue ANG(1-7) formation during hyperglycemia should allow the development of novel pharmacological strategies to prevent/treat diabetic cardiovascular complications.

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