High Glucose-suppressed Endothelin-1 Ca\(^{2+}\) Signaling via NADPH Oxidase and Diacylglycerol-sensitive Protein Kinase C Isozymes in Mesangial Cells

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High glucose (HG) is the underlying factor contributing to long term complications of diabetes mellitus. The molecular mechanisms transforming the glomerular mesangial cell phenotype to cause nephropathy including diacylglycerol-sensitive protein kinase C (PKC) are still being defined. Reactive oxygen species (ROS) have been postulated as a unifying mechanism for HG-induced complications. We hypothesized that in HG an interaction between ROS generation, from NADPH oxidase, and PKC suppresses mesangial Ca\(^{2+}\) signaling in response to endothelin-1 (ET-1). In primary rat mesangial cells, growth-arrested (48 h) in 5.6 mM (NG) or 30 mM (HG) glucose, the total peak [Ca\(^{2+}\)] response to ET-1 (50 nM) was 630 \(\pm\) 102 nM in NG and was reduced to 159 \(\pm\) 15 nM in HG, measured by confocal imaging. Inhibition of PKC with phorbol ester down-regulation in HG normalized the ET-1-stimulated [Ca\(^{2+}\)] response to 541 \(\pm\) 74 nM. Conversely, an inhibitory peptide specific for PKC-\(\delta\) did not alter Ca\(^{2+}\) signaling in HG. Furthermore, overexpression of conventional PKC-\(\beta\) or novel PKC-\(\delta\) in NG diminished the [Ca\(^{2+}\)] response to ET-1, reflecting the condition observed in HG. Likewise, catalase or p47\(^{phox}\) antisense oligonucleotide normalized the [Ca\(^{2+}\)] response to ET-1 in HG to 521 \(\pm\) 58 nM and 514 \(\pm\) 48 nM, respectively. Pretreatment with carbonyl cyanide m-chlorophenylhydrazone or rotenone did not restore Ca\(^{2+}\) signaling in HG. Detection of increased intracellular ROS in HG by dichlorofluorescein was inhibited by catalase, diphenylethionidone, or p47\(^{phox}\) antisense oligonucleotide. HG increased p47\(^{phox}\) mRNA by 1.7 \(\pm\) 0.1-fold as measured by reverse transcriptase-PCR. In NG, H\(_2\)O\(_2\) increased membrane-enriched PKC-\(\beta\) and -\(\delta\), suggesting activation of these isoforms. HG-enhanced immunoreactivity of PKC-\(\delta\) visualized by confocal imaging was attenuated by diphenylethionidone chloride. Thus, mesangial cell [Ca\(^{2+}\)] signaling in response to ET-1 in HG is attenuated through an interaction mechanism between NADPH oxidase ROS production and diacylglycerol-sensitive PKC.

High glucose (HG)\(^1\) is the key factor contributing to long term complications of diabetes mellitus (1). One of the phenotypic changes observed in mesangial cells exposed to HG is altered Ca\(^{2+}\) signaling. Several groups have shown that the Ca\(^{2+}\) signal induced by vasoactive compounds, including endothelin-1 (ET-1), is markedly reduced in the presence of HG. The mechanism(s) by which HG may depress Ca\(^{2+}\) signaling is unknown. One possible candidate is the activation of protein kinase C (PKC) in HG. Mené et al. (2) have shown that HG inhibits Ca\(^{2+}\) influx through store-operated channels via a PKC-dependent mechanism. An alternative postulate is the involvement of reactive oxygen species (ROS), which have been demonstrated to modify intracellular Ca\(^{2+}\) signaling responsiveness depending on the cell type, the species of ROS, and the magnitude and duration of ROS generation. HG induces dysfunction in mesangial cells and other target cells through enhanced synthesis of autocrine growth factors such as transforming growth factor-\(\beta\), ET-1, and altered signaling via pathways such as PKC (3, 4). In the last few years, enhanced production of ROS in response to HG, identified in many target cells including mesangial cells (5, 6), has been postulated as a unifying mechanism causing diabetes complications (7–9).

Although ROS have been implicated in causing cell damage and apoptosis, they also play a physiological role in intracellular signaling pathways (10, 11). In particular, several growth factors including ET-1, angiotensin II, platelet-derived growth factor, and epidermal growth factor stimulate production of ROS as second messengers (12, 13). In several cell types, targeted ROS production is due to activation of NADPH oxidase, a multicomponent enzyme (14). In phagocytic cells, the multiple subunits of NADPH oxidase are localized in subcellular compartments. gp91\(^{phox}\), the catalytic moiety of the phagocyte oxidase, and p22\(^{phox}\) associate to form a flavocytochrome in the plasma membrane. The cytosolic components p47\(^{phox}\), p67\(^{phox}\), p40\(^{phox}\), and the small GTPase, Rac1 (or Rac2), are recruited to the membrane for assembly of a fully active oxidase (15–17). In nonphagocytic cells, most of the subunits of NADPH oxidase have been identified, although the precise mechanisms of regulation are not completely understood. A functional glomerular mesangial NADPH oxidase has been inferred through the use of diphe-

\(\text{\(^1\)The abbreviations used are: HG, high glucose; NG, normal glucose; ET-1, endothelin-1; ROS, reactive oxygen species; PKC, protein kinase C; DPI, diphenylethionidone chloride; DAG, diacylglycerol; CM-H\(_2\)DCFDA, fluo-3,5-(and 6)-chloromethyl-2,7-\[3H\]-dichlorodihydrofluorescin diacetate acetyl ester; PMA, phorbol 12-myristate 13-acetate; CCCP, carbonyl cyanide m-chlorophenylhydrazone; GFP, green fluorescent protein; DCF, carboxymethyl-H\(_2\)-dichlorofluorescin diacetate.\)
nyleeniodium chloride (DPI), an inhibitor of flavoproteins, in response to cytokine (18, 19) and serotonin stimulation (20). An earlier report identified the expression of components of human glomerular mesangial cell NADPH oxidase (21). To date, no report has described the role of NADPH oxidase in HG-induced altered mesangial cell phenotype.

We reasoned that if HG causes enhanced and sustained ROS generation in mesangial cells, Ca2+ signaling responsiveness to ET-1 may be modified through a ROS-dependent mechanism. Since previous reports (22–26), including work from our laboratory (27–30), have demonstrated enhanced PKC activity in HG, we postulated a mechanistic interaction between diacylglycerol (DAG)-dependent PKC and enhanced NADPH oxidase function.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following materials were used: Dulbecco’s modified Eagle’s medium (Invitrogen); fetal bovine serum (Winsent Inc.); fluo-3,5- (and 6)-chloromethyl-2,7’-dichlorodihydrofluorescein diacetate acetyl ester (CM-H<sub>2</sub>DCFDA) (Molecular Probes, Inc., Eugene, OR); endothelin-1, anti-PKC antibodies, DPI, catalase, phorbol 12-myristate 13-acetate (PMA), carbonyl cyanide (3-chlorophenylhydrazon) (CCCP), and rotenone (Sigma); anti-p47<sub>phox</sub> and other representative confocal images of Ca2+ responses to 12.5 nM ionomycin in NG and HG. B, graphical analysis of Ca2+ release from 30–35 cells. Bar, 25 μm.

**Fig. 1. Ca2+ signaling in response to low concentration of ionomycin is reduced in HG.** Mesangial cells were growth-arrested in 5.6 mM (NG) or 30 mM (HG) and stimulated with ionomycin (100, 50, 25, or 12.5 nM) after loading with fluo-3 (1 h). Digitized confocal images were captured every 15 s for 120 s. Pixel intensity from basal and peak responses was analyzed and converted into nM intracellular Ca2+.

**Transient Transfection of GFP-PKC and PKC-ζ Inhibition**—Mesangial cells were transiently transfected with either GFP-PKC-β or GFP-PKC-ζ using FuGENE6 (Roche Applied Science) as instructed by the manufacturer. To inhibit PKC-ζ activity, mesangial cells were pretreated with the PKC-ζ peptide inhibitor (Zi; 10 μM, 24 h) (31) in HG. Cells were loaded with fluo-3 and imaged for Ca2+ response to ionomycin or ET-1.

**Reverse Transcriptase-Polymerase Chain Reaction Expression of p47<sub>phox</sub> and β-Actin**—Total RNA was extracted using the Qiagen RNeasy kit (Qiagen) as instructed by the manufacturer’s instructions. After first-strand synthesis of DNA, 3 μl of cDNA was amplified using RevertAid H Minus First Strand cDNA Synthesis Kit (MBI Fermentas, Hanover, MD); and p47<sub>phox</sub> primers, p47<sub>phox</sub> antisense oligonucleotide, and a cell-permeant myristoylated peptide that specifically inhibits PKC-ζ activity (31) (The Centre for Applied Genomics, DNA Synthesis Facility, Hospital for Sick Children, Toronto, Canada).

**Cell Culture**—Primary rat mesangial cells were isolated from Sprague-Dawley rat kidney glomeruli and characterized as previously described (32). Passages 12–17 were used for all studies. Mesangial cells were grown in Dulbecco’s modified Eagle’s medium containing 20% fetal bovine serum to confluence and then growth-arrested in 0.5% fetal bovine serum containing either 5.6 mM glucose (NG) or 30 mM glucose (HG) for 48 h.

**Confocal Imaging of Intracellular Ca2+**—Mesangial cells were cultured on glass coverslips to subconfluence under the above conditions and then loaded with 2.5 μM fluo-3 in Dulbecco’s modified Eagle’s medium (containing CaCl<sub>2</sub>) with 0.02% Pluronic F-127 for 60 min at 37°C. The coverslip was mounted in a chamber on the stage of a Zeiss confocal microscope (LSM 410; Duesseldorf, Germany), and the cells were imaged prior to and during the response to ET-1. Digitized images from basal condition and peak response of total cell [Ca2+]i were analyzed. Intracellular Ca2+ concentration ([Ca2+]i) was calculated as follows,

\[
[Ca^{2+}]_i = \frac{K_f(R - R_{min})}{R_{max} - R} \times \frac{S_{t0}}{S_{t2}}
\]

where, \(K_f\) for fluo-3 was 320 nM (33). The \(R_{max}\) and \(R_{min}\) represent the fluorescence ratios measured with excitation at 340/380 nm under minimum free Ca2+ (bound with EGTA) and maximum Ca2+ (ionomycin plus 10 mM CaCl<sub>2</sub>). \(S_{t0}\) represents the fluorescence at saturating Ca2+ levels. \(S_{t2}\) is the fluorescence at 380-nm excitation in the absence of Ca2+. Autofluorescence, measured by quenching fluo-3 with MnCl<sub>2</sub>, was subtracted from the \(R_{max}\) and \(R_{min}\) values (32, 34).

**HG Suppressed ET-1 Ca2+ Signaling via NADPH Oxidase and PKC**

**A** 

| Bar |  |
|-----|--|
| t=0 | peak |
| t=0 | peak |

**B**

![Graphical analysis of Ca2+ release from 30–35 cells.](image)

**Table 1.** Confocal immunofluorescence of p47<sub>phox</sub> and PKC—Mesangial cells were cultured on coverslips to subconfluence and growth-arrested in 0.5% fetal bovine serum NG or HG. Cells were fixed in 3.7% formaldehyde and permeabilized with methanol. To prevent nonspecific binding of antibody, cells were blocked with goat serum containing 0.1% bovine
serum albumin and then incubated with anti-p47phox monoclonal antibody or PKC-δ antibody. The primary antibody was detected with fluorescein isothiocyanate-conjugated secondary antibody using confocal image analysis.

Transient Expression of p47phox Antisense Oligonucleotide—Mesangial cells were grown to 80% confluence on coverslips and transiently transfected with either antisense or scrambled phosphorothioate-modified oligonucleotide for p47phox using Effectene or Fugene6 transfection reagents according to the manufacturer's instructions. The sequence to the p47phox antisense oligonucleotide was derived from a published report by Bey and Cathcart (36). The sequence is as follows: TTCACCTGGCTGTCATTGG. Cells were transfected with a 5 μM concentration of the oligonucleotide for 48 h, and decreased expression of p47phox protein was ascertained by immunoreactivity of p47phox using confocal imaging.

Measurement of ROS—Generation of intracellular ROS was measured with the fluoroprobe carboxymethyl-H₂-dichlorofluorescein diacetate (CM-H₂DCFDA; Molecular Probes). CM-H₂DCF-DA is a nonpolar compound that is converted into a nonfluorescent polar derivative (CM-H₂DCF-DA) by cellular esterases after incorporation into cells. CM-H₂DCF-DA is rapidly oxidized to the fluorescent 2',7'-dichlorofluorescein in the presence of intracellular ROS. Brieﬂy, mesangial cells were incubated for 60 min at 37 °C with 10 μmol/liter CM-H₂DCF-DA in Dulbecco’s modiﬁed Eagle’s medium in NG or HG (1, 3, 24, or 48 h). Fluorescence intensity was measured by confocal microscopy (Zeiss; excitation 488 nm, emission 513 nm). Average intensity for each experimental group of cells was determined using Scion Image Analysis software, and values were expressed as above control. As a positive control, cells were stimulated with 100 μM H₂O₂.

Membrane-enriched Cellular Fraction—To obtain membrane fractions, mesangial cells grown on 10-cm plates were lysed in ice-cold buffer A containing 50 mmol/liter Tris-HCl, pH 7.5, 10 mmol/liter EGTA, 2 mmol/liter EDTA, 1 mmol/liter benzamidine, 1 mmol/liter NaF, vanadate, 1 mM phenylmethylsulfonyl fluoride, and 25 μg/ml leupeptin. Cells were disrupted by passage through a 26-gauge needle and centrifuged at 100,000 g for 30 min at 4°C (TL-100; Beckman Instruments Canada, Mississauga, Canada). The pellet was resuspended in buffer A plus 1% Triton X-100 and centrifuged at 100,000 × g for 30 min. The supernatant was collected as the plasma membrane enriched fraction.

Statistical Analysis—All results are expressed as means ± S.E. Statistical analysis was performed using InStat 2.01 statistics software (Graph Pad, Sacramento, CA). The means of three or more groups were compared by one-way analysis of variance. If significance of p < 0.05 was obtained in the analysis of variance, the Tukey multiple comparison post-test was applied. Differences described as significant are p < 0.05 unless otherwise stated.

**FIG. 2.** Ca²⁺ signaling in response to ET-1 is suppressed in HG and reversed by chronic PMA. Mesangial cells were growth-arrested on glass coverslips in 5.6 mM (NG) glucose or 30 mM (HG) glucose for 48 h and stimulated with ET-1 (50 nM) after loading with fluo-3 (1 h). Digitized confocal images were captured every 15 s for 120 s. Pixel intensity from basal and peak responses was analyzed and converted into nm intracellular Ca²⁺, [Ca²⁺]; A–D, basal conditions of cells in NG, HG, HG + PMA (48 h), and HG + PKC-δ myristoylated peptide inhibitor (Zi; 10 μM, 24 h), respectively. E–H, representative of the peak responses. I, graphical analysis of Ca²⁺ release from total cell for 30–35 cells and 70 cells from HG + Zi. *p < 0.001 versus NG peak; **, p < 0.01 versus HG peak; #, p < 0.01 versus NG peak. Bar, 25 μm.
RESULTS

Ca\textsuperscript{2+} Signaling in NG and HG in Response to Ionomycin—To study Ca\textsuperscript{2+} signaling, mesangial cells were grown on glass coverslips and growth-arrested in 5.6 mM (NG) glucose or 30 mM (HG) glucose for 48 h and stimulated with the calcium ionophore, ionomycin. Ca\textsuperscript{2+} release was determined by converting the mean pixel intensity of the entire cell into values [Ca\textsuperscript{2+}]\textsubscript{i} expressed in nM using the calibration formula (32).

There was no difference between the Ca\textsuperscript{2+} response in NG and HG to ionomycin at 100 nM (data not shown). However, at a low concentration of ionomycin (12.5 nM), the peak total cell [Ca\textsuperscript{2+}]\textsubscript{i} response in NG was 657 ± 86 nM, and in HG, the response was reduced to 346 ± 22 nM (Fig. 1).

DAG-sensitive PKC Inhibition Reverses HG-suppressed Ca\textsuperscript{2+} Release in Response to ET-1—To study the Ca\textsuperscript{2+} response of mesangial cells to the vasoactive peptide ET-1, cells were grown on glass coverslips and growth-arrested in NG or HG glucose for 48 h and stimulated with ET-1 (50 nM). In all experiments, values from total cell [Ca\textsuperscript{2+}]\textsubscript{i} release from basal and peak response are reported. In NG, the total cell peak [Ca\textsuperscript{2+}]\textsubscript{i} response to ET-1 (50 nM) was 630 ± 102 nM, which was attenuated to 159 ± 15 nM in HG (p < 0.001 versus NG peak) (Fig. 2). To determine whether PKC was involved in decreased Ca\textsuperscript{2+} signaling in HG, we down-regulated DAG-sensitive PKC isozymes by chronic treatment of PMA (100 nM) in HG for 48 h. Inhibition of PKC reversed the effect seen in HG. The peak total cell [Ca\textsuperscript{2+}]\textsubscript{i} response was elevated to 541 ± 74 nM (p < 0.01 versus HG peak) (Fig. 2).

Since chronic PMA down-regulates only DAG-sensitive PKC isozymes, the role of PKC-ζ in Ca\textsuperscript{2+} signaling was ascertained using a cell-permeant myristoylated peptide inhibitor, previously demonstrated to be specific for this PKC isozyme (31). Mesangial cells were growth-arrested in HG with or without the PKC-ζ inhibitor (Zi; 10 μM, 24 h). Pretreatment with the PKC-ζ inhibitor in HG did not alter Ca\textsuperscript{2+} signaling in response to ET-1 (268 ± 31 nM, p < 0.01 versus NG peak) (Fig. 2).

To further support a role of DAG-sensitive PKC isozymes in Ca\textsuperscript{2+} release in response to ET-1, mesangial cells were transiently transfected with green fluorescent protein (GFP)-PKC-ζ or GFP-PKC-δ to represent both the conventional and novel class of PKC isozymes. Mesangial cells expressed functional GFP-PKC-δ and -ζ in these experiments as they responded to PMA (1 μM, 10 min) stimulation by translocating.
to the membrane, the hallmark of PKC activation (Fig. 3). In fluo-3-loaded cells, expression of the GFP-PKC constructs was clearly distinguishable from the surrounding cells (Fig. 4). When stimulated with ET-1, cells transfected with either GFP-PKC-β-H9252 or GFP-PKC-δ-H9254 displayed a blunted Ca\(^{2+}\)/H11001 response (Fig. 4A). This was particularly evident in the nucleus. Conversely, when cells were stimulated with ionomycin (25 μM), all cells responded, including the transfected ones, with a robust intranuclear release of Ca\(^{2+}\) (Fig. 4B). Thus, the transfection procedure did not affect the loading of fluo-3.

**Increased ROS in Mesangial Cells in HG Is Inhibited by Catalase or DPI**—To examine the effect of HG on ROS production, mesangial cells cultured on coverslips were growth-arrested in 30 mM glucose for various time points, loaded with carboxymethyl-H2-dichlorofluorescein diacetate (DCF), and imaged with confocal microscopy. HG caused increased ROS generation as measured by DCF intensity as early as 1 h (Fig. 5). The effect was sustained in long term incubation with HG up to 48 h (Fig. 5). When cells were pretreated with catalase (100 units, 1 h) or DPI (1 μM, 1 h), HG-induced ROS generation was significantly reduced (control: 41 ± 1 S.E. mean pixel intensity/cell, n = 73 cells; HG: 150 ± 5, n = 90 cells, p < 0.001 versus NG; HG + catalase: 16 ± 2, n = 122 cells, p < 0.001 versus HG; and HG + DPI: 37 ± 3, n = 83 cells, p < 0.001 versus HG) (Fig. 6).

**Protein Expression of p47phox and Modified Expression by Antisense Oligonucleotide**—To investigate a role of NADPH oxidase, particularly the p47phox subunit, in HG-induced production of ROS, we first determined the expression of p47phox in mesangial cells. Confocal immunofluorescence imaging showed that mesangial cells indeed express p47phox, which is localized in a perinuclear pattern (Fig. 7). HG caused increased intensity of the staining. Semiquantitative analysis indicates that the mean pixel intensity per cell in NG was 73 ± 2 S.E., whereas in HG, the value was 110 ± 2 S.E. (100 cells analyzed, p < 0.01) (Fig. 7).

To address the precise function of p47phox, mesangial cells were transiently transfected with p47phox antisense oligonucleotides. Fig. 7 shows that this method was sufficient to reduce expression of p47phox protein, normally found in a perinuclear distribution.

Since both catalase and DPI prevented HG-induced ROS production, the function of NADPH oxidase was further investigated. Fig. 8 shows that the HG-enhanced DCF fluorescence (144 ± 5, pixel intensity/cell, n = 119 cells) was inhibited when mesangial cells were transfected with p47phox antisense oligonucleotide (45 ± 2, p < 0.001 versus HG, n = 126 cells). Transfection of the scrambled version of p47phox antisense oligonucleotide did not reduce DCF fluorescence (166 ± 6, p < 0.001 versus NG, n = 34 cells).

**Inhibition of ROS Formation Reverses the Effect of HG on Ca\(^{2+}\)/H11001 Signaling in Response to ET-1**—To determine the role of ROS on the observed reduced Ca\(^{2+}\)/H11001 signaling, mesangial cells were growth-arrested in HG and then treated with the addition of catalase (100 units, 1 h). In these experiments, the peak total cell [Ca\(^{2+}\)/H11001] response in NG to ET-1 (50 nm) was 446 ± 79 nm. In HG, the effect was reduced to 146 ± 13 nm. Catalase significantly rescued the ET-1-induced Ca\(^{2+}\)/H11001 response to ET-1 (50 nm) in HG (Fig. 8).

**Fig. 5.** High glucose increases intracellular ROS. Mesangial cells were growth-arrested on coverslips and loaded with DCF (10 μM, 1 h) and imaged with confocal microscopy. A, depicts cells in NG. B, cells in NG were stimulated with H2O2 (100 mM, 1–3 min). C–F, cells were incubated in HG (1, 3, 24, or 48 h). G, graphical analysis of pixel intensity using Scion Image analysis. Bar, 25 μm.
response in HG to 521 ± 58 nM (p < 0.001 versus HG peak without catalase) (Fig. 9).

To further elucidate the role of ROS in Ca \(^{2+}\) signaling, particularly to determine whether the p47\(^{phox}\) subunit of NADPH oxidase is necessary for the effect of HG on Ca \(^{2+}\) release, mesangial cells were transiently transfected with p47\(^{phox}\) antisense oligonucleotides in HG and stimulated with ET-1. In NG, the peak total cell [Ca\(^{2+}\)] response was 426 ± 34 nM. In HG, the peak was reduced to 177 ± 17 nM. Inhibition of p47\(^{phox}\) by down-regulation with antisense oligonucleotides reversed the effect of HG to a level that was not significantly different than the response seen in NG (514 ± 48 nM, p < 0.001 versus HG peak) (Fig. 10). Transfection of the scrambled (SCR) version of p47\(^{phox}\) antisense oligonucleotide did not reverse the decreased [Ca\(^{2+}\)], observed in HG (144 ± 16 nM, p < 0.001 versus peak NG + ET-1) (Fig. 10). Thus, ROS derived from NADPH oxidase plays a pivotal role in the reduced Ca\(^{2+}\) response in HG.

Since several reports demonstrated the importance of ROS derived from a mitochondrial source in the diabetic milieu, we tested the effects of CCCP, an uncoupler of oxidative phosphorylation, and rotenone on Ca\(^{2+}\) signaling in response to ET-1. These experiments were performed using mesangial cells in passages 19–21. In these experiments, the peak total cell [Ca\(^{2+}\)] response was 173 ± 10 nM, which was significantly attenuated to 88 ± 10 nM (p < 0.05 versus peak NG + ET-1) in HG (Fig. 11). CCCP (100 nM, 1 h) did not significantly alter the peak total cell [Ca\(^{2+}\)], in NG (187 ± 14 nM, p > 0.05 versus peak NG + ET-1) or in HG (103 ± 17 nM, p > 0.05 versus peak HG + ET-1) (Fig. 11). Pretreatment with rotenone (1 \(\mu\)g/ml, 1 h) decreased the basal and peak total cell [Ca\(^{2+}\)], in NG and was therefore not used in HG.

**Effect of PKC Down-regulation on p47\(^{phox}\) mRNA Expression and ROS on PKC Activation**—To determine the effect of HG on p47\(^{phox}\) expression, mesangial cells were growth-arrested in NG or HG, and p47\(^{phox}\) mRNA was measured by reverse transcriptase-PCR. HG increased p47\(^{phox}\) mRNA by 1.7 ± 0.1-fold over control (Fig. 12). To determine whether PKC plays a role in HG-increased p47\(^{phox}\) mRNA expression, mesangial cells were pretreated with chronic PMA exposure (100 nM, 48 h). Fig.
12 shows that down-regulation of mesangial cell PKC did not alter the effect of HG regulation on p47phox mRNA (1.9 ± 0.4-fold over control). Since PKC inhibition did not affect p47phox mRNA expression, we postulated that ROS act upstream of PKC. As previously shown in our laboratory, the immunoreactivity of
PKC-δ is enhanced in HG (29), depicted in the confocal micrographs (Fig. 12). Pretreatment of mesangial cells with DPI prevented the increase in immunofluorescence. Mesangial cells were also stimulated with H₂O₂, and membrane-enriched cell fractions were immunoblotted for various PKC isozymes. Interestingly, a low concentration of H₂O₂ (10 μM, 10 min) increased PKC-δ in the cell membrane, whereas a slightly longer exposure time (30 min) or higher H₂O₂ concentration (100 μM) was required for PKC-β to be isolated in the membrane (Fig. 12). The enrichment of PKCs in the cell membrane fraction is in agreement with our previous report showing activation of PKC in HG (30).

DISCUSSION

In this study, we have demonstrated that Ca²⁺ signaling in response to vasoactive peptides such as ET-1 is suppressed in HG through a mechanism requiring both NADPH oxidase ROS and DAG-sensitive PKC isozymes. We have verified that mesangial cell generation of ROS in HG is inhibited by catalase or DPI. Furthermore, transient transfection of p47⁷phox antisense oligonucleotide, at a concentration that significantly reduced expression of p47⁷phox protein prevented the increased generation of ROS in HG. Likewise, transient transfection of p47⁷phox antisense oligonucleotide was sufficient to reverse the effect of HG-induced depressed Ca²⁺ signaling in response to ET-1. Treatment with catalase also normalized the ET-1-induced Ca²⁺ response in HG. DAG-sensitive PKC isozymes, known to be activated in HG, are necessary for suppression of Ca²⁺ signaling to ET-1 in HG, since down-regulation of DAG-sensitive PKC isozymes by chronic exposure to PMA in HG restored the Ca²⁺ response, and a PKC-ζ peptide inhibitor failed to normalize the Ca²⁺ signaling in response to ET-1 in HG. Moreover, overexpression of a conventional (GFP-PKC-β) or novel (GFP-PKC-δ) PKC isozyme, mimicking their increased activity observed in HG, attenuated Ca²⁺ signaling in response to ET-1. These data conclusively reveal the functional relationship between enhanced ROS generation in HG through NADPH oxidase and DAG-sensitive PKC-dependent suppression of Ca²⁺ signaling.

Several reports indicate that HG attenuates Ca²⁺ signaling in response to vasoactive peptides in certain cell types, although the hypothesized mechanism(s) for the attenuated Ca²⁺ signaling appear to be multifaceted. In mesangial cells, Nutt and O’Neil (37) recently reported that both acute and chronic exposure to HG depressed ET-1-stimulated Ca²⁺ signaling that is probably mediated by receptor-operated calcium influx. In transformed mouse mesangial cells, transforming growth factor-β1 was shown to decrease the expression of inositol 1,4,5-trisphosphate receptors, leading to a reduced Ca²⁺ response (38). We have observed that ET-1-stimulated Ca²⁺ signaling measured by confocal fluorescence imaging probably originates predominantly from endoplasmic reticulum stores, since treatment with thapsigargin abolishes the Ca²⁺ response.² Mené et al. (2) showed that HG inhibited store-operated capacitance Ca²⁺ influx in mesangial cells by a PKC-dependent mechanism. Our findings are similar, since we also observed that reduced Ca²⁺

² H. Hua, S. Munk, H. Goldberg, I. G. Fantus, and C. I. Whiteside, unpublished observations.
signaling in HG is PKC-dependent. Ours is the first study to show that only DAG-sensitive PKC isozymes are involved, since inhibition of PKC-δ did not affect Ca$^{2+}$/ release in response to ET-1. We are the first to demonstrate that overexpression of individual GFP-PKC isozymes is sufficient to attenuate Ca$^{2+}$/ signaling.

A rapidly emerging area of research suggests that ROS play a major role in several aspects of signaling (39, 40). In bovine aortic endothelial cells, acute exposure to HG was shown to abolish Ca$^{2+}$/ oscillation in response to ATP (41). The authors stipulated that glucose-derived superoxide anion diminished Ca$^{2+}$/ oscillation by accelerating Ca$^{2+}$/ leak from intracellular stores and impairing Ca$^{2+}$/ release-activated Ca$^{2+}$/ entry. In human aortic endothelial cells, it was shown that NADPH oxidase-dependent ROS are critical to the generation of histamine-induced Ca$^{2+}$/ oscillations, perhaps by altering the sensitivity of the endoplasmic reticulum to inositol 1,4,5-trisphosphate (42). Here we have established that in HG, NADPH-generated ROS significantly diminished Ca$^{2+}$/ release stimulated by ET-1 in mesangial cells as catalase, and antisense oligonucleotide to p47$^{phox}$/ restored Ca$^{2+}$/ signaling in HG, similar to that seen in NG. In order to address the role of mitochondrial ROS, we used two reagents that inhibit the mitochondrial electron transport chain as reported by others (43). In our experiments, CCCP failed to reverse HG-attenuated Ca$^{2+}$/ signaling. This finding suggests that mitochondria production of ROS is not involved in reducing the Ca$^{2+}$/ response to ET-1 in HG. Rotenone reduced Ca$^{2+}$/ response to ET-1 in NG and therefore could not be used in HG.

Although ROS derived from a mitochondrial source was shown to be predominant in endothelial cells in HG (7, 8), certainly other enzymes play pivotal roles. In Otsuka Long-Evans Tokushima Fatty rats, a model of type 2 diabetes, the aorta showed increased superoxide production compared with the control LETO rats, and the effect was inhibited by DPI (44). Furthermore, expression of p22$^{phox}$/ mRNA (44) as well as gp91$^{phox}$/ (45) was up-regulated. Likewise, smooth muscle cell-
or endothelial cell-increased free radical production in HG was
inhibited by DPI (46). In streptozotocin-induced diabetic rats,
immunoreactivity of p47phox was increased in the kidney (47).
Furthermore, increased generation of ROS in monocytes from
diabetic humans was prevented by p47phox antisense oligonu-
cleotide (48). We showed that HG-induced ROS generation in
rat mesangial cells was most likely through enhanced NADPH
oxidase activity. Both catalase and DPI significantly reduced
DCF fluorescence in HG. In addition, transient transfection of
p47phox antisense oligonucleotide also prevented ROS stimula-
tion by HG in rat mesangial cells.

Although the NADPH oxidase is a multicomponent complex,
several reports suggest that down-regulation of a single sub-
unit is sufficient to inhibit enzymatic activity. Inhibition of p22phox by stable transfection of antisense p22phox cDNA into
vascular smooth muscle cells resulted in significantly reduced
angiotensin II-stimulated NADPH-dependent superoxide pro-
duction (49). Coronary microvascular endothelial cells isolated
from p47phox−/− mice lost ROS formation in response to tumor
t necrosis factor-α, but the effect was restored when full-length
p47phox cDNA was transfected into the cells (50). Smooth mus-
cle cells from p47phox null mice also showed diminished super-
oxide production (51) and decreased the proliferative response
to growth factors, suggesting that superoxide from NADPH
oxidase plays a prerequisite role in atherosclerosis (52). In the
same manner, we have shown that inhibition of NADPH oxid-
ase with p47phox antisense oligonucleotide was sufficient to
prevent HG-induced ROS production, leading to an altered
Ca2+ response to ET-1.

In neutrophils, PKCs may regulate NADPH oxidase, partic-
ularly p47phox, by direct phosphorylation (53). In nonphagocytic
cells, the role of PKC in NADPH oxidase activation is less well
known. Inoguchi et al. (46) showed that in aortic smooth muscle
cells and endothelial cells, HG and palmitate stimulate ROS
through PKC-dependent activation of NADPH oxidase by the
use of DPI. In our study, we asked whether HG activation of
PKC could regulate p47phox. We found that down-regulation
of PKC isozymes with chronic PMA was insufficient to prevent
HG-induced increase of p47phox mRNA. Conversely, pretreat-
ment with DPI decreased the “activation pattern” visualized by
confocal immunofluorescence imaging of PKC-δ in HG. Like-
wise, H2O2 caused increased accumulation of PKC-δ and -β in
the membrane-enriched cellular fraction, indicating that ROS
probably stimulate these mesangial cell DAG-sensitive PKC
isozymes.

Activation of PKC is an essential mechanism for HG-induced
cellular dysfunction, since it represents a critical downstream
event in the pathogenesis of diabetic complications (3, 24, 25,
54). In this study, the importance of DAG-sensitive PKC
isozymes is further elucidated in the response of mesangial
cells to HG. Our data reflect a sequence of events whereby HG suppressed Ca\(^{2+}\) signaling in response to ET-1 is dependent on DAG-sensitive PKC and NADPH oxidase ROS.

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FIG. 12. Effect of chronic PMA on p47\(^{phox}\) mRNA expression and ROS on PKC activation. Mesangial cells were growth-arrested in NG, HG, or HG plus PMA (48 h), and total RNA was collected and reverse transcribed under “Experimental Procedures.” A, representative of the PCR product using the p47\(^{phox}\) and actin primers. B–E, micrographs depicting the immunoreactivity of PKC-\(\beta\), total PKC, and p47\(^{phox}\) in NG and HG with and without DPI (1 \(\mu\)M, 1 h). Bar, 25 \(\mu\)m. F, micrograph depicting representative immunoblots of membrane-enriched fractions of mesangial cells following 10 and 30 min HG treatment. G, representative of the PCR products for PKC-\(\alpha\), total PKC, and p47\(^{phox}\) in HG and HG plus DPI.
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