High Glucose Enhances Thrombin Responses via Protease-Activated Receptor-4 in Human Vascular Smooth Muscle Cells

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Objective—Diabetes is associated with vascular remodeling and increased thrombin generation. Thrombin promotes vascular smooth muscle cell (SMC) mitogenesis and migration via protease-activated receptors (PAR)-1, PAR-3, and PAR-4. We investigated the effect of high glucose on expression and function of vascular thrombin receptors.

Methods and Results—In human vascular SMCs, high glucose (25 versus 5.5 mmol/L) induced a rapid and sustained increase in PAR-4 mRNA, protein, and cell surface expression. PAR-1 and PAR-3 expression were not changed. High glucose pretreatment (48 hours) enhanced thrombin or PAR-4–activating peptide but not PAR-1–activating peptide evoked intracellular calcium mobilization, migration, and tumor necrosis factor α gene expression. This enhancement of thrombin-stimulated migration and gene expression by high glucose was abolished by endogenous PAR-4 knockdown. PAR-4 regulation was prevented by inhibition of protein kinase (PKC)-β and -δ isoforms or nuclear factor (NF)κB. Nuclear translocation of NFκB in high glucose–stimulated SMCs led to PKC-dependent NFκB binding to the PAR-4 promoter in a chromatin immunoprecipitation assay. Furthermore, in situ hybridization and immunohistochemistry confirmed high abundance of PAR-4 in human diabetic vessels as compared with nondiabetic vessels.

Conclusion—High glucose enhances SMC responsiveness to thrombin through transcriptional upregulation of PAR-4, mediated via PKC-β, -δ, and NFκB. This may play an important role in the vascular complications of diabetes. (Arterioscler Thromb Vasc Biol. 2011;31:624-633.)

Key Words: diabetes mellitus ■ thrombin ■ vascular muscle ■ protease-activated receptors

Chronically elevated plasma glucose levels in diabetes are associated with cardiovascular complications such as vascular remodeling and poor outcome after revascularization.1,2 At the cellular level, hyperglycemia is a potent stimulus for the proliferation and migration of vascular smooth muscle cells (SMCs),3 which are major factors contributing to vascular remodeling and accelerated atherosclerosis.4 In addition, diabetes represents a hypercoagulable state associated with enhanced thrombin generation and increased risk of thrombotic complications.5 Thrombin is the central component of the coagulation cascade that becomes activated when vascular injury allows contact between blood-borne components and tissue factor–expressing cells such as fibroblasts and SMCs.6 However, the overwhelming majority of total thrombin generated is released by the thrombus after clotting is completed,6,7 indicating functions beyond coagulation.

Thrombin can exert direct coagulation-independent actions such as SMC migration and proliferation through activation of a unique family of G protein–coupled receptors, the protease-activated receptors (PARs).8 PARs are activated through proteolytic cleavage of the extracellular N terminus, which unmasks a new N terminus that acts as a tethered ligand to autoactivate the receptor.8 Synthetic hexapeptides mimicking this tethered ligand can elicit most of the biological actions of thrombin independently of receptor cleavage. Of the 4 PARs identified to date, PAR-1, PAR-3, and PAR-4 are activated by thrombin, whereas another receptor, PAR-2, is activated by other proteases such as activated factor X.9

PAR-1 is the prototypical receptor to which most of the cellular and platelet actions of thrombin are attributed,9 and its role in vascular remodeling and atherosclerosis is well defined.10,11 The predominant role of PAR-3 and PAR-4 is in platelet activation9,12; however, both receptors are expressed in human vascular SMCs and contribute to mitogenic signaling.13,14 The net response to thrombin in SMCs is therefore likely to involve the cooperation of multiple PARs. Like other G protein–coupled receptors, activated PARs are rapidly uncoupled from signaling and internalized.9 The reappear-
ance at the cell surface is partly dependent on de novo synthesis. Thus, the vascular actions of thrombin are controlled in part through transcriptional regulation of PARs. The potential impact of elevated glucose levels on the expression and function of these receptors is not known. The present study therefore investigates whether high glucose influences thrombin receptor expression in human vascular SMCs and tries to explore the transcriptional mechanisms involved.

Methods

Cell Culture and Incubations

Human saphenous vein SMCs were cultured as described and maintained in DMEM containing 5.5 mmol/L D-glucose and 15% FCS (GibcoBRL, Rockville, MD). Subconfluent (~90%) SMCs at passages 5 to 11 were serum-deprived for 48 hours before stimulation with high glucose (25 mmol/L; Calbiochem, San Diego, CA) and stimulated with study drugs. Mannitol (19.5 mmol/L in 5.5 mmol/L D-glucose; Calbiochem, San Diego, CA) served as osmolar control. Activating peptides for PAR-1 (PAR-1AP, TFFLRN) and PAR-4 (PAR-4AP, AYPGKF) were synthesized by Biosyntan (Berlin, Germany). Bovine α-thrombin was a generous gift from the late Dr J. Stürzebecher (Zentrum für Vaskuläre Biologie und Medizin, Jena, Germany). Protein kinase (PKC-β inhibitor and staurosporine were from Calbiochem (San Diego, CA).

Quantitative Real-Time PCR

Target gene expression levels were determined by quantitative real-time PCR, relative to GAPDH, as described.

Immunoblotting

Total protein expression was detected in cell lysates by immunoblotting as described using goat polyclonal anti-human PAR-4 primary antibody (sc-8464, Santa Cruz Biotechnology) or mouse monoclonal anti-human PAR-4 antibody (no. WH0009002M1, Sigma-Aldrich, München, Germany), mouse monoclonal anti-human β-actin (Sigma, Schnelldorf, Germany) and horseradish peroxidase–coupled secondary antibodies (Santa Cruz Biotechnology, Heidelberg, Germany).

Immunocytochemistry

Subconfluent human vascular SMCs seeded on 10-mm glass coverslips were stimulated with high glucose or mannitol for the indicated times, then fixed in paraformaldehyde (4%) for 20 minutes at room temperature (RT) and permeabilized in 0.1% Triton X-100 for 5 minutes at RT. This was followed by incubation with primary goat anti-PAR-4 antibody (sc-8464, 1:100) overnight at 4°C and then with FITC-conjugated secondary antibody (both from Santa Cruz Biotechnology, Heidelberg, Germany) for 1 hour at RT in the dark. Nuclear staining was performed with Hoechst-33342 (Invitrogen, Karlsruhe, Germany). Fluorescence images were captured immediately with a Colorview-II camera and Soft Imaging System connected to an Olympus BX50 microscope (Hamburg, Germany).

Flow Cytometry

Surface expression of PAR-4 was detected by flow cytometry as described using FITC-conjugated anti–PAR-4 extracellular N-terminal antibody (APR-034-F, Alamone Labs, Jerusalem, Israel).

Small Interfering RNA–Induced Gene Knockdown

Subconfluent SMCs were transfected with 40 nmol/L of following small interfering (si)RNA control (sc-37007), PKC-δ (sc-36253), PAR-4 (sc-72068, all from Santa Cruz Biotechnology), or Signal silence nuclear factor (NFκB-p65 (Cell Signaling Technology, Frankfurt, Germany) using Lipofectamine transfection reagent (Invitrogen) according to the instructions of the manufacturer. Gene knockdown was validated by immunoblotting or real-time PCR (online Data Supplement, available at http://atvb.ahajournals.org). Cells were used for study 48 hours after transfection.

NFκB Activation

SMCs were stimulated with high glucose for indicated times before extraction of cytosolic and nuclear fractions as described. After adjusting to equal protein concentrations, immunoblotting was performed using NFκB-p65 (Cell Signaling Technology), phospho-IκBα (391431, sc-52943), and total IκBα (C-21, sc-371, both from Santa Cruz Biotechnology) antibodies.

Chromatin Immunoprecipitation Assay

The specific binding of NFκB to the PAR-4 promoter was investigated in SMCs stimulated for 3 hours with high glucose with or without staurosporine (1 μmol/L) by a modified chromatin immunoprecipitation (ChIP) assay as described. This time point was chosen based on the maximal nuclear translocation of NFκB at 3 hours. Immunoprecipitation was performed with NFκB-p65 antibody (C-20, sc-372; Santa Cruz Biotechnology). Final PCR analysis used the following primers (Invitrogen): (1) 5′-GAGAACAG-TGGCTGCAGATG-3′ (forward), 5′-GGAGGACTGAGTGTTGGGT-3′ (reverse) to amplify a 212-bp region of human PAR-4 promoter containing the NFκB-binding site; (2) 5′-ATGGTT-GCCACTGGGGATCT-3′ (forward), 5′-TGGCCAAGCCTAGGGGAAAGA-3′ (reverse) to amplify a 174-bp region of genomic DNA between the GAPDH and CNA1 genes. Cyclical conditions were 5 cycles of 94°C/30 seconds, 72°C/60 seconds; 5 cycles of 94°C/30 seconds, 70°C/30 seconds, 72°C/60 seconds; then 32 cycles 94°C/30 seconds, 57°C/30 seconds, 72°C/ 60 seconds; and then 72°C/15 minutes. PCR products were resolved on a 1.8% agarose/ethidium bromide gel.

Agonist-Stimulated [Ca2+]i Release From Intracellular Stores

Release of intracellular calcium ([Ca2+]i), was measured as described with minor modification. Subconfluent (50% to 60%) SMCs seeded on 10-mm glass coverslips were pretreated with normal or high glucose for 48 hours. After washing with HEPEs buffer (in mmol/L: HEPES 10, pH 7.4, NaCl 145, Na2HPO4 0.5, glucose 5.5, MgSO4 1, and CaCl2 1.5), cells were loaded with the calcium-sensitive fluorescent dye Fluo-4 acetoxyethyl ester (10 μmol/L, Invitrogen) for 30 minutes at RT. Cells were again washed and kept immersed in HEPEs buffer. Transient [Ca2+]i release induced by addition of thrombin or PAR-activating peptide was recorded on 1 Hz with back-illuminated EMCCD camera (iXon3897, Andor Technology, South Windsor, CT), attached to an inverted fluorescence microscope (Olympus IX-70). Fluo-4 was excited with a 488-nm laser beam from an argon/krypton ion laser (StabiliTE 2017, Newport Spectra Physics), and emitted light was passed through a dichroic mirror and a long pass filter (505DRLPXR and 500ALP, Omega Filters, Brattleboro, VT) before detection. Image sequences were analyzed using ImageJ software (http://rsweb.nih.gov/ij; NIH). Changes in calcium signal in individual cells elicited by agonist application were normalized to the respective prestimulation basal level recorded for 60 seconds before stimulation. The mean of all recordings was obtained from 5 individual experiments.

Migration Assay

SMC migration was studied by “wound-scratch” assay. Subconfluent SMCs seeded on 24-well plates were pretreated with high (25 mmol/L) glucose or the osmolar control mannitol (19.5 mmol/L in 5.5 mmol/L glucose) for 48 hours. A 1-mm cleft was scratched into cell monolayer with a sterile pipette tip and medium was replaced with serum-free DMEM containing hydroxy-urea (5 mmol/L) to prevent cell proliferation. Cells were then stimulated with various stimuli for 72 hours, fixed with ice-cold methanol for 5 minutes at RT. Images were captured with a Colorview-II camera and Soft Imaging System attached to microscope (IX50, Olympus) at multiple sites along the cleft. Data were analyzed using ImageJ.

In Situ Hybridization

To construct riboprobes, human PAR-4 cDNA was cloned into pcDNA3, and PCR fragments covering the T7 promoter (T7 up-
stream primer: 5′-CGCTAACGTGGATTTTGGCTGACCC-3′, PAR-4 downstream primer: 5′-CCAGGGCGACGAGGCTCA-3′) or the SP6 promoter (PAR-4 upstream primer: 5′-GCTGAGGCTGACGCGAGTGG-3′, SP6 downstream primer: 5′-CCAGCTGATCCTATGGCTTCTCC-3′) were synthesized. Digoxigenin-labeled PAR-4 riboprobes were transcribed as instructed by manufacturer using SP6 or T7 RNA polymerase (DIG RNA labeling kit, Roche), resulting in SP6 promoted PAR-4 antisense runoff transcript with 380-bp homology and T7 promoted sense runoff transcript with 409-bp homology.

Five diabetic and 7 nondiabetic human saphenous vein specimens were obtained directly from the operating room and cryopreserved in liquid nitrogen. Cryosections (10 μm) were thaw-mounted onto SuperFrostPlus slides, dried, and stored at -80°C until used. For the experiment, sections were thawed and dried for 30 minutes at RT, fixed for 10 minutes in freshly made 4% paraformaldehyde, digested for 30 minutes at 37°C with proteinase-K (1 μg/mL), and incubated for 10 minutes in acetylation solution (3.3 mL triethanolamine, 438 μL of diethyl-pyrocatecol–treated water) with PBS washing after each step. Sections were postfixed with 4% paraformaldehyde, washed with PBS, and then prehybridized for 2 hours at RT in hybridization solution (50% deionized formamide, 5× SSC, 5× Denhardt solution, 250 μg/mL baker’s yeast tRNA and 500 μg/mL salmon sperm DNA; all from Sigma). Hybridization was performed overnight at 62°C with 600 ng/mL digoxigenin-labeled PAR-4 antisense and sense probes in 150 μL of hybridization buffer. Slides were covered with Næcosfilm (Roth GmbH, Karlsruhe, Germany) to prevent drying. Nescofilm strips were removed by soaking in 2× SSC at 62°C and, followed by washing in 2× SSC at 62°C for 2 hours, then at RT for 5 minutes. For immunohistochemical detection of the hybridized mRNA, sections were washed with TBS (pH 7.4) for 5 minutes at RT, blocked with 10% heat-inactivated FCS in TBS at RT for 1 hour and incubated overnight at 4°C with alkaline phosphatase–conjugated mouse antidigoxigenin Fab-fragment (1:2000 diluted in TBS/1% heat-inactivated FCS, Roche). Following washing with TBS and equilibration with buffer-2 (200 mL: Tris-HCI 100 [pH 9.5], MgCl2 50, and NaCl 100), the color reaction was performed with 45 μL of NBT, 35 μL of BCIP (Roche), and 2.4 mg of tetramisole (Sigma) in 10 mL of buffer-2 in the dark for 3 to 12 hours at RT. The slides were then washed with Tris-EDTA buffer (pH 8) and dH2O and mounted with Aquatex (Merck). Parallel sections were immunostained with mouse monoclonal anti-human αo-actin antibody (1:200, A-556, Abcam), alkaline phosphatase–conjugated secondary antibody (1:40, Abcam), and Fast red detection system (Thermo Scientific, Dreieich, Germany).

**Immunohistochemistry**

PAR-4 immunohistochemistry was performed on paraffin-embedded sections of 5 diabetic and 3 nondiabetic human carotid plaques, as well as 4 artery and vein specimens (from 2 healthy organ donor subjects) obtained from the Department of Cardiac Surgery at the University Hospital Düsseldorf, with approval of the institutional ethics committee and informed consent of donors. Tissue sections of 5 diabetic and 3 nondiabetic human carotid plaques, as well as 4 artery and vein specimens (from 2 healthy organ donor subjects) obtained from the Department of Cardiac Surgery at the University Hospital Düsseldorf, with approval of the institutional ethics committee and informed consent of donors. Tissue sections (3 μm) were deparaffinized in xylene, rehydrated in ethanol, and washed with PBS. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol for 20 minutes. Antigen retrieval was performed in 0.1 mol/L sodium citrate/0.1 mol/L citric acid (pH 6) at 98°C for 20 minutes. Sections were blocked with 10% FCS/1% BSA for 1 hour at RT, PAR-4 antibody (sc-8464, Santa Cruz Biotechnology) was applied overnight at 4°C and visualized by horseradish peroxidase–conjugated secondary antibodies (Santa Cruz Biotechnology) and the DAB Substrate Kit (Zytomed Systems, Berlin, Germany). Actin staining used primary mouse monoclonal α-actin antibody (Dako) and alkaline phosphatase–conjugated secondary antibody (Abcam) with final detection by Fast red kit (Thermo Scientific). Nuclei were stained with Mayer’s hemalum solution (Merck). Nonspecific isotype-matched IgG with secondary antibodies were used as control. Images were taken with a Colorview-II camera and Soft Imaging System connected to an Olympus BX50 microscope.

**Results**

**High Glucose Selectively Upregulates PAR-4 Expression in Human SMCs**

In human saphenous vein SMCs, the constitutive expression of PAR-1 and PAR-3 mRNA was not significantly influenced over 96 hours of exposure to high glucose (both n=7; Figure 1A). PAR-4 mRNA however was rapidly and significantly induced to almost 3-fold within 3 hours, and this increase was sustained over 96 hours (n=7, P<0.05; Figure 1A). A similar pattern was seen in human coronary artery SMCs (n=5, Figure 1B). The osmolar control mannitol did not influence PAR mRNA expression in saphenous vein SMCs (n=3, data not shown). In these cells, high glucose also elicited time-dependent increases in PAR-4 immunofluorescence, whereas mannitol was without effect (n=3; Figure 1C). Similarly, PAR-4 total protein expression as determined by immunoblotting was significantly increased over 48 to 96 hours (n=5, P<0.05; Figure 1D). PAR-1 and PAR-3 total protein expression was unaltered (n=3, data not shown). Accordingly, flow cytometry analysis showed significant increases in PAR-4 cell surface expression at 48 to 72 hours (n=4, P<0.05; Figure 1E).

**High Glucose Enhances Thrombin-Evoked Ca2+ Signaling in Human SMCs**

Cellular signaling of thrombin receptors was determined by measuring changes in [Ca2+]i. The application of thrombin (3 U/mL) evoked a rapid calcium mobilization, whereas no response by repeated application confirmed the complete tachyphylaxis (Figure 2A). The sequential addition of PAR-4AP (200 μmol/L) then PAR-1AP (20 μmol/L) also elicited a transient elevation of [Ca2+]i, (Figure 2B). A small thrombin-induced [Ca2+]i transient was retained after previous application of PAR-4AP and PAR-1AP (Figure 2B) indicates a modest contribution of PAR-3 to the net [Ca2+]i response to thrombin. SMC pretreatment with high glucose (48 hours) significantly enhanced the peak [Ca2+]i, signal evoked by thrombin or PAR-4AP, but did not alter the response to PAR-1AP (all n=5, P<0.05; Figure 2C and 2D).

**High Glucose Enhances Thrombin-Induced SMC Migration and Tumor Necrosis Factor α Gene Expression**

The functional significance of High glucose–regulated PAR expression was assessed by wound-scratch migration assay and tumor necrosis factor (TNF)α expression. In SMCs maintained in normal glucose conditions, both PAR-1AP and PAR-4AP elicited only modest migratory responses, whereas thrombin significantly enhanced migration of SMCs (n=4, P<0.05; Figure 3A). Pretreatment with high glucose for 48 hours significantly enhanced the number of migrated cells in response to PAR-4AP and thrombin, but not to PAR-1AP. To assess whether the enhanced responsiveness to thrombin can be attributed to increased expression of PAR-4, SMCs were transfected.
with PAR-4 siRNA to knock down endogenous PAR-4 mRNA (>75%, n=3, P<0.05; online Data Supplement). PAR-4 siRNA suppressed the migratory response to thrombin, and high glucose did not augment responsiveness (n=3; Figure 3B) as compared with cells transfected with control siRNA.

In addition, the influence of high glucose on thrombin-stimulated gene expression was also examined. High glucose pretreatment (48 hours) significantly enhanced thrombin-induced TNFα mRNA expression (to 2.6±0.3 fold control, P<0.05, n=4; Figure 3C), which was completely abolished in SMCs transfected with PAR-4 siRNA.

**PAR-4 Regulation Is Mediated Through PKC Isoforms-β and -δ**

The stimulatory action of high glucose on PAR-4 mRNA (at 6 hours, n=4; Figure 4A) and PAR-4 protein expression (at...
48 hours, n=4; Figure 4B) was completely prevented by PKC-β inhibitor (50 nmol/L). Knockdown of endogenous PKC-δ with siRNA, by almost 50% (n=4, P<0.05; online Data Supplement), also abolished the stimulatory effect of high glucose on PAR-4 mRNA and total protein expression (at 6 hours and 48 hours respectively, n=4; Figure 4C and 4D). Comparable effects were observed with the nonspecific PKC inhibitors staurosporine (1 μmol/L), calphostin-C (200 nmol/L), or the PKC-δ inhibitor rottlerin (1 μmol/L, all n=4, data not shown).

Transcriptional Control of PAR-4 via NFκB

Sequence analysis of the human PAR-4 promoter using Transfac (http://www.gene-regulation.com,) and Genomatix databases (http://www.genomatix.de) identified a potential NFκB recognition motif (GGGACCCCCC) at position 543 upstream of the start codon, whereas neither PAR-1 nor PAR-3 promoter possesses such a site. High glucose promoted the nuclear translocation of NFκB, determined by immunoblotting of the NFκB-p65 subunit in the nuclear fractions. Degradation of NFκB, total IκBα, and accumula-
tion of phospho-IkBα in cytosol were also observed in parallel. These observations, consistent with NFκB activation, were time-dependent, with maximal changes at 3 hours (n=3; Figure 5A). This time point was chosen to assess binding of NFκB to the PAR-4 promoter in a modified ChIP assay. PCR with primers amplifying the region encoding the potential NFκB-binding site demonstrated enhanced NFκB-p65:PAR-4 DNA binding in SMCs exposed to high glucose (n=3; Figure 5B). This was prevented by the nonspecific PKC inhibitor staurosporine (1 μmol/L). The absence of GAPDH signal in all samples indicated the absence of nonspecific contaminating DNA. Suppression of endogenous NFκB-p65 protein with siRNA to 40% (P<0.05; online Data Supplement) prevented the stimulatory effect of high glucose on PAR-4 mRNA and protein expression (Figure 5C and 5D; n=4, P<0.05). Similar loss of PAR-4 regulation was seen with the NFκB activation inhibitor (100 nmol/L, data not shown).

Evidence of PAR-4 Upregulation in Human Diabetic Vessels

In Situ Hybridization

Diabetic and nondiabetic saphenous veins were examined by in situ hybridization (Figure 6A). Strong positive hybridization for PAR-4 was observed in diabetic vein sections, whereas nondiabetic veins showed minimal hybridization (Figure 6A, b and e; ×10 objective). Human smooth muscle actin immunostaining on sequential sections confirmed that positive PAR-4 hybridization was predominantly in the vessel media, rich in SMCs (Figure 6A, c and f). No hybridization was seen in control slides probed with PAR-4 sense riboprobe (Figure 6A, a and d).

Immunohistochemistry

The abundance of PAR-4 in human diabetic and nondiabetic carotid plaques (Figure 6B) as well as in healthy vessels was investigated by immunohistochemistry. Tissue sections from the diabetic carotid plaques showed positive immunoreactivity for PAR-4 in comparison with nondiabetic sections (Figure 6B, b and c; ×10 objective) or sections treated with control IgG (Figure 6B, a and d). Actin immunostaining in parallel sections corresponded well to the regions showing positive PAR-4 immunoreactivity (Figure 6B, c and f). Immunostaining on healthy vessel sections failed to show any positive PAR-4 immunostaining (online Data Supplement).

Discussion

This study provides the first evidence of a direct effect of high glucose on the cellular actions of thrombin in human vascular SMCs through transcriptional regulation of PAR. Exposure of human vascular SMCs to high glucose selectively induces PAR-4 expression, with no influence on other thrombin receptors PAR-1 or PAR-3. As a consequence, this enhances thrombin and PAR-4–mediated SMC signaling and migration and TNFα expression. Transcriptional regulation of PAR-4 involves the PKC-β and -δ isoforms and the downstream effector NFκB and is independent of osmolar effects.

Diabetes is strongly associated with vascular complications and increased thrombin generation, leading to atherothrombosis.1,5 The relative contribution of PAR-type thrombin receptors has not been defined. Most cellular actions of thrombin are attributed to the prototypical receptor PAR-1,9 and recently developed PAR-1 antagonists represent new concept to prevent thrombotic and restenotic complications.10 We previously reported functional expression of both PAR-3 and PAR-4 in human vascular SMCs,13,14 but their precise role remains to be defined. Vascular PAR-3 is thought to act predominantly as a cofactor for PAR-1 in endothelium,18 whereas PAR-4 is reportedly involved in myocardial reper-
fusion injury\textsuperscript{19} and in the endothelial response to inflammatory challenge.\textsuperscript{20,21}

In the present study in human saphenous vein SMCs, elevated glucose led to a rapid and sustained induction of PAR-4 mRNA, whereas neither PAR-1 nor PAR-3 was significantly influenced. A similar pattern was observed in SMCs from human coronary artery. Our finding reflects reports in endothelial cells that PARs are subject to differen-

\textbf{Figure 4.} A and B, High glucose–stimulated PAR-4 mRNA (at 6 hours) (A) and PAR-4 total protein expression (at 48 hours) (B) in human saphenous vein SMCs in the absence and presence of the PKC-\(\beta\) inhibitor (50 nmol/L) (\(n=4\)). C and D, High glucose-stimulated PAR-4 mRNA (at 6 hours) (C) and PAR-4 total protein expression (at 48 hours) (D) in SMCs transfected with control or PKC-\(\delta\) siRNA (all \(n=4\)). Means\(\pm\)SEM. \(*P<0.05\) vs control.

\textbf{Figure 5.} A, High glucose–induced translocation of NF\(\kappa\)B-p65, cytosolic accumulation of phosphorylated-I\(\kappa\)B\(\alpha\), and degradation of total I\(\kappa\)B\(\alpha\) in venous SMCs (representative of \(n=3\)). B, ChIP assay showing NF\(\kappa\)B:PAR-4 DNA binding in SMC stimulated with high glucose with or without staurosporine (1 \(\mu\)mol/L, 3 hours) before chromatin IP with NF\(\kappa\)B-p65 antibody. Input samples served as positive controls. PCR performed with primers amplified the PAR-4 promoter region encoding the NF\(\kappa\)B-binding site (PAR-4 PCR) or GAPDH. Representative of \(n=3\). C and D, High glucose–stimulated PAR-4 mRNA (at 6 hours) (C) and PAR-4 total protein expression (at 48 hours) (D) in SMC transfected with control or NF\(\kappa\)B-p65 siRNA (all \(n=4\)). Means\(\pm\)SEM. \(*P<0.05\) vs control.
tial regulation\textsuperscript{20, 21} and raises the interesting possibility that individual PARs play distinct roles in various pathological conditions. The increase in PAR-4 mRNA by high glucose was sustained over the 96 hours of incubation and accompanied by increased PAR-4 protein and cell surface expression. Accordingly, the responsiveness of SMCs to thrombin and a selective PAR-4–activating peptide were enhanced. Calcium mobilization is an early signaling event on PAR activation. Under resting conditions, all PARs expressed at the cell surface of SMCs are available for stimulus, but on activation, these receptors would no longer be available for repeated application of the same stimulus because of rapid uncoupling of the receptor from signaling or other desensitization mechanisms.\textsuperscript{9} We found that thrombin, PAR-4AP, and PAR-1AP induced a similar pattern of transient $[\text{Ca}^{2+}]_i$ increases in SMCs when applied in sequence (PAR-4AP followed by PAR-1AP and then thrombin). Repeated application of the same agonist no longer induced any calcium signaling, consistent with rapid desensitization of PARs. The ability of thrombin to induce a residual $[\text{Ca}^{2+}]_i$ elevation when applied after PAR-4AP and PAR-1AP can possibly be explained by the activation of PAR-3. Although the contribution of this receptor to thrombin-evoked net calcium signaling was negligible, we have earlier identified that PAR-3 is functionally active on vascular SMCs.\textsuperscript{14} High glucose pretreatment (48 hours) enhanced thrombin or PAR-4AP–evoked $[\text{Ca}^{2+}]_i$ transients, with no change in the response to PAR-1AP. This reflects the selective increase in PAR-4 and highlights the potential importance of this receptor in hyperglycemic settings.

As a further measure of cellular responsiveness, the impact of high glucose on the migratory behavior of SMCs and TNF$\alpha$ gene expression was determined. SMC migration is a central event in the tissue repair process subsequent to injury, but excessive migration facilitates neointima formation and lumen narrowing.\textsuperscript{22} In a wound-scratch migration assay, we observed modest migratory effects PAR-4AP or PAR-1AP alone. Pretreatment of vascular SMCs with high glucose (48 hours), however, strongly enhanced the migratory responses to PAR-4AP or thrombin, whereas migration induced by PAR-1AP was not affected. To confirm that the enhanced cellular responses to thrombin were attributable to increased expression of PAR-4, endogenous PAR-4 was suppressed with specific siRNA. As observed in nontransfected cells, in SMCs transfected with control siRNA, thrombin was found to stimulate the number of migrating cells. This effect was significantly enhanced by 48 hours pretreatment with high glucose. In SMCs transfected with PAR-4 siRNA, however, the stimulatory action of thrombin on migration was abolished and responsiveness was not influenced by high glucose.

Beside migration, inflammation is another key factor in atherosclerosis and vascular remodeling.\textsuperscript{23} Diabetes is associated with chronic inflammation and increased plasma cytokine levels.\textsuperscript{24} In present study, thrombin-induced expression of proinflammatory gene TNF$\alpha$ was significantly enhanced in presence of high glucose, which was completely sup-

**Figure 6.** PAR-4 abundance in diabetic (D) or nondiabetic (ND) vessels. A, PAR-4 in situ hybridization: sense (a and d), antisense (dark purple) (b and e), and actin immunostaining (red) (c and f) on human saphenous vein. B, PAR-4 immunostaining on human carotid atherosclerotic plaques: IgG control (a and d); PAR-4, brown, arrows (b and e); and actin, red (c and f).
pressed by knocking down PAR-4 in SMCs. These findings clearly indicate that PAR-4, as a regulated thrombin receptor, may participate in the cardiovascular remodeling, accelerated atherosclerosis, and inflammation that are typical for diabetic patients.25

Support for this hypothesis comes from the high abundance of PAR-4 particularly in vessels obtained from diabetic patients in comparison with nondiabetic patients. Positive PAR-4 in situ hybridization on human saphenous vein sections, as well as PAR-4 immunostaining on human carotid plaques, was strongly enhanced particularly in the media of vessels and the near intimal regions of the plaque rich in smooth muscle cells. This clearly shows that regulation of PAR-4 is evident in clinical settings of diabetes and foresees the importance of this thrombin receptor in diabetes related vascular pathologies. We observed minimal PAR-4 in situ hybridization in nondiabetic veins, as was expected from real-time PCR data, but we could not detect PAR-4 immunostaining in nondiabetic atherosclerotic plaques indicating the importance of PAR-4 upregulation particularly in diabetic settings. PAR-4 potentially has a role in vascular consequences of diabetes, although its contribution in atherosclerosis in nondiabetic settings may be minimal.26

Unlike high glucose, the osmolar control mannitol did not influence the expression or migratory actions of PAR-4. Thus the regulatory actions of high glucose on this receptor are likely to involve specific signaling events leading to transcriptional changes in PAR-4. Extensive data support a central role of PKC in vascular complications of diabetes and elevated glucose-stimulated gene expression.3,27,28 Human saphenous vein SMCs express conventional (α, β), novel (δ, ε, μ), and atypical (λ, ξ) PKC isozymes.27 Of these, the PKC-β and -δ isozyme appear to be preferentially activated by high glucose.28 In this study, the ability of high glucose to regulate PAR-4 mRNA and protein expression was prevented by nonspecific PKC inhibitors staurosporine and calphostin-C. A selective inhibitor of PKC-β and siRNA against PKC-δ also abolished the induction of PAR-4 by high glucose, implicating these PKC isoforms in the transcriptional control of PAR-4.

A major target downstream of PKC in SMCs is the transcription factor NFκB.28 Activated NFκB has been detected in atherosclerotic vessels,29 suggesting an important role in atherosclerosis. A recognition motif for NFκB could be identified in the promoter for PAR-4, but not for PAR-1 or PAR-3; hence, NFκB could represent a critical transcriptional regulator responsible for the selective control of PAR-4 expression by high glucose. NFκB is normally sequestered in the cytoplasm of resting cells as an inactive trimeric complex (IκB/p65/p50) but rapidly translocates to nucleus on inflammatory challenge. Activation of NFκB involves IκBα/β phosphorylation by IκB kinase complex and subsequent proteosomal degradation. This allows the NFκB dimer (p65/p50) to enter the nucleus and to bind to cognate DNA sequence to regulate gene transcription.30 Dominant negative expression of IκB kinase has been reported to blunt cytokine-induced PAR-4 mRNA expression in human endothelial cells, although this induction could not be confirmed at the level of increased protein expression or functional responsiveness.20 In the human SMCs used in the present study, high glucose induced time-dependent accumulation of phospho-IκBα in the cytosol and the translocation of the free NFκB-p65 to the nucleus. NFκB shuttling was maximal at 3 hours and associated with increased binding of free NFκB-p65 subunit to the NFκB-binding site in the human PAR-4 promoter, as demonstrated by ChIP analysis. This interaction was suppressed by PKC inhibitor staurosporine, further highlighting the central role of PKC as a critical regulator of PAR-4 upstream of the NFκB. Accordingly, siRNA-mediated knockdown of NFκB prevented high glucose–induced PAR-4 expression.

In conclusion, we demonstrate that high glucose activates PKC-δ and NFκB to induce selective PAR-4 expression, signaling and function in human vascular SMCs and thereby enhancing vascular responses to thrombin. These findings in addition to strong upregulation of PAR-4 expression in human vessels highlight a unique role of PAR-4 in diabetic settings, which is likely to contribute to the exaggerated cardiovascular complications of diabetes and warrants further attention.

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Disclosures
None.

References
10. Ahn HS, Chackalamannil S, Boykow G, Graziano MP, Foster C. Development of proteinase-activated receptor 1 antagonists as therapeutic


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High glucose enhances thrombin responses via protease-activated receptor-4 in human vascular smooth muscle cells

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METHODS

Luciferase reporter assay
Promoter analysis of human PAR-1 and PAR-4 with 2 different databases (Transfac and Genomatix) reported transcription factor binding sites predominantly in the 1000 upstream of the ATG. This portion of the promoters was incorporated into the luciferase reporter vectors and confirmed to be sufficient to drive basal and stimulated PAR-promoter activities. The NFκB binding site (CTGGGACCCCCCC) in the PAR-4 promoter is located at position 544-556 of the forward strand. The human PAR-1 promoter vector has been described previously.14 For generation of a PAR-4 promoter vector, the human PAR-4 genomic clone (pBeloBAC11) was obtained from (RZPD, Heidelberg, Germany). Competent DNA was isolated using the big BAC DNA Isolation Kit (Princeton Separations, Philadelphia, PA). A 14096 bp fragment containing the PAR-4 promoter was cloned into a pSK-Bluescript vector, and used to transform K12 E.coli (DH10B strain). DNA from transformed E.coli was isolated using Midi-Prep Kits (Qiagen, Hilden, Germany) and correct clones were identified by restriction enzyme digestion and a final 2617 bp Smal fragment was ligated into the pGL3basic luciferase reporter vector. This construct was transfected into VSMC seeded in 24-well plates using Lipofectamine2000® (Invitrogen, Karlsruhe, Germany) as per manufacturer’s instructions. Stimuli were added 24h post-transfection for indicated times and cell lysates were collected to measure luciferase reporter activity using the Luciferase Assay System (Promega, Mannheim, Germany) and protein content using Bradford Reagent as detailed by the manufacturer (Bio-Rad Laboratories, München, Germany). The mean of at least 3 replicates per treatment group was taken for each experiment.

siRNA-induced gene knock-down
siRNA mediated gene knockdown was performed as described in the methods and validated by western blotting for PKC-δ or NFκB using primary rabbit polyclonal and monoclonal antibodies respectively (Cell Signalling Technology, Danvers, MA) or by real time PCR for PAR-4.

Migration Assay
SMC migration was observed in wound scratch migration assay as described in methods.

Immunohistochemistry
PAR-4 abundance in human carotid plaques was detected by immunostaining for PAR-4 as described in methods section.

Statistical analysis
Data are expressed as mean± SEM and normalized to untreated controls. Statistical analysis utilized one-way analysis of variance (ANOVA) with Bonferroni’s post-hoc multiple comparison procedure applied as appropriate. Analysis of two groups was done using student-t test. P<0.05 was accepted as significant.
FIGURE LEGENDS

Figure I. (A) Luciferase reporter assay showing normal and high glucose (24h)-driven promoter activities of human PAR-1 and PAR-4 in human saphenous vein SMC. (B) Time-course of high glucose-driven PAR-4 promoter activity. All n=4. Mean±SEM, *p<0.05 vs control.

Figure II. siRNA mediated knock-down of endogenous (A) PAR-4 mRNA expression by real-time PCR (n=3), (B) PKC-δ and (C) NFκB-p65 protein expression by western blotting in human saphenous vein smooth muscle cells (n=4). Mean±SEM, *P<0.05 vs control.

Figure III. Representative images of (A) SMC migration induced by activating peptides (AP) for PAR-1 (200 µmol/L) or PAR-4 (400 µmol/L), or thrombin (3 U/mL) after normal or high glucose pretreatment (48h) in wound-scratch assay (n=4) and (B) thrombin (3 U/mL)-stimulated migration of SMC transfected with control or PAR-4 siRNA, after pretreatment (48h) with normal or high glucose (n=3 individual experiments).

Figure IV. Representative images of PAR-4 immunostaining in diabetic (D), non-diabetic (ND) human carotid atherosclerotic plaques or healthy (H) artery sections- IgG control: (a, d, g), PAR-4: brown, arrows (b, e, h) and actin- red (c, f, i). (n= 5 diabetic, 3 non-diabetic human carotid atherosclerotic plaque specimens and 4 healthy vessels).
**A**

PAR-4 mRNA (fold control)

control | PAR-4 siRNA

0.0 | 1.0

**B**

PKC-δ

control | PKC-δ siRNA

0.0 | 1.0

NFkB-p65

control | NFkB-p65 siRNA

0.0 | 1.0

**C**

PKC-δ protein (fold control)

control | PKC-δ siRNA

0.0 | 1.0

NFkB-p65 protein (fold control)

control | NFkB-p65 siRNA

0.0 | 1.0
A

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