Vascular Medicine

Thrombin Receptor Protease-Activated Receptor 4 Is a Key Regulator of Exaggerated Intimal Thickening in Diabetes Mellitus

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Background—Diabetes mellitus predisposes to thrombotic and proliferative vascular remodeling, to which thrombin contributes via activation of protease-activated receptor (PAR) 1. However, the use of PAR-1 inhibitors to suppress remodeling may be limited by severe bleeding. We recently reported upregulation of an additional thrombin receptor, PAR-4, in human vascular smooth muscle cells exposed to high glucose and have now examined PAR-4 as a novel mediator linking hyperglycemia, hypercoagulation, and vascular remodeling in diabetes mellitus.

Methods and Results—PAR-4 expression was increased in carotid atherectomies and saphenous vein specimens from diabetic versus nondiabetic patients and in aorta and carotid arteries from streptozotocin-diabetic versus nondiabetic C57BL/6 mice. Vascular PAR-1 mRNA was not increased in diabetic mice. Ligated carotid arteries from diabetic mice developed more extensive neointimal hyperplasia and showed greater proliferation than arteries from nondiabetic mice. The augmented remodeling response was absent in diabetic mice deficient in PAR-4. At the cellular level, PAR-4 expression was controlled via the mRNA stabilizing actions of human antigen R, which accounted for the stimulatory actions of high glucose, angiotensin II, and H2O2 on PAR-4 expression, whereas cicaprost via protein kinase A activation counteracted this effect.

Conclusions—PAR-4 appears to play a hitherto unsuspected role in diabetic vasculopathy. The development of PAR-4 inhibitors might serve to limit mainly proliferative processes in restenosis-prone diabetic patients, particularly those patients in whom severe bleeding attributed to selective PAR-1 blockade or complete thrombin inhibition must be avoided or those who do not require anticoagulation. (Circulation. 2014;130:1700-1711.)

Key Words: diabetes mellitus ■ prostaglandins ■ remodeling ■ thrombin

The coagulant protease thrombin is a key player in thrombotic vessel occlusion and vascular remodeling, events that critically limit the coronary lumen gain subsequent to percutaneous transluminal angioplasty or bypass grafting. Diabetic patients in particular are highly prone to thrombotic and proliferative lumen narrowing.1–2 Several antithrombotic agents have been examined for their potential antistenotic potential, but success was limited and occurred at the expense of increased bleeding, including life-threatening and intracerebral bleeds.3,4 An emerging concept is direct blockade of the prototypic thrombin receptor protease-activated receptor (PAR) 1.5,6 PAR-1 is the predominant effector of thrombin-stimulated platelet activation and of vascular smooth muscle cell (SMC) proliferation, migration, matrix synthesis, and inflammatory gene expression.7,8 PAR-1 antagonists have shown promising results in animal models of injury-induced vascular remodeling9–11; however, the 2 new PAR-1 blockers, vorapaxar (SCH530348) and atopaxar (E5555), were associated in clinical trials with severe bleeding and, in the case of atopaxar, liver toxicity,12–14 and, thus, do not meet the original expectations. Nevertheless, the concept of controlling thrombin-induced cellular responses via modulation of the thrombin receptor remains attractive.

Clinical Perspective on p 1711

In addition to PAR-1, 2 additional PAR-type receptors are activated by thrombin, PAR-3, which is not thought to signal autonomously,15 and PAR-4, a low-affinity thrombin receptor with distinct on-off kinetics, essential for the sustained human

Received November 18, 2013; accepted September 11, 2014.
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The online-only Data Supplement is available with this article at http://circ.ahajournals.orglookup/suppl/doi:10.1161/CIRCULATIONAHA.113.007590/-/DC1.

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Circulation is available at http://circ.ahajournals.org

DOI: 10.1161/CIRCULATIONAHA.113.007590

1700

113.007590/-/DC1
platelet response to thrombin. PAR-4 is the predominant thrombin receptor in rodent platelets but is also expressed in human vascular SMCs. Unlike other PARs, PAR-4 is upregulated in high-glucose–stimulated SMCs. Enhanced PAR-4 sensitivity was also reported in platelets from type 1 diabetic mice, associated with increased susceptibility to arterial thrombosis. Whether PAR-4 also contributes to proliferative vessel remodeling, particularly in settings of diabetes mellitus, is not known. PAR-4 upregulation is partially mediated via protein kinase C isoforms and the transcription factor nuclear factor-κB. Posttranscriptional modification of PAR-4 has not yet been described. A candidate regulator is the mRNA stabilizing factor HuR (human antigen R, ELAV), which is activated by protein kinase C-δ and angiotensin II, and is highly abundant in human vessels with neointimal hyperplasia, atherosclerosis, or restenosis.

We now report increased PAR-4 expression in aorta and carotid arteries of streptozotocin (STZ)-diabetic C57BL/6 mice, which also develop a greater degree of neointima formation after coronary artery ligation than nondiabetic mice. The augmented remodeling response is absent in diabetic mice deficient in PAR-4. At the cellular level, glucose-stimulated PAR-4 expression was found to be critically dependent on HuR-mediated mRNA stabilization. These findings, together with a high PAR-4 abundance observed in diabetic versus nondiabetic human atherectomy and a saphenous vein specimen, highlight PAR-4 as an important glucose-regulated thrombin receptor in both mice and humans with a hitherto unsuspected role in the vascular complications of diabetes mellitus.

Materials and Methods

Human Tissue Samples

Human atherectomy samples were obtained from diabetic and nondiabetic patients of both sexes presenting for carotid endarterectomy (ethics approval No. 3944). Patient characteristics are given in Table I (in the online-only Data Supplement). Human saphenous vein segments were obtained from type 2 diabetic and nondiabetic patients presenting for coronary artery bypass grafting (ethics approval No. 3199). Donors gave informed consent. Patient characteristics are provided in Tables I–V in the online-only Data Supplement.

Immunohistochemistry of Human Tissue Sections

Paraffin sections (3 μm) were stained for PAR-4 (ab66103, 1:50; Abcam, Cambridge, United Kingdom) or anti-α smooth muscle actin (αSMA; M0851, 1:200; Dako, Glostrup, Denmark). Nuclear staining used Roti-Mount FluorCare 4′,6-diamidino-2-phenylindole (Roth GmbH, Karlsruhe, Germany). Fluorescence images were captured with an AxioCam HRC camera and AxioVision Software connected to an Axio Imager.M2 microscope (Carl Zeiss).

Western Blot Analysis of PAR-4

Tissue samples (200 mg) were lysed in 500 μL of modified radioimmunoprecipitation assay buffer, followed by centrifugation and homogenization. Lysates (20 μg of protein) were separated by electrophoresis through a 12% SDS-PAGE, transferred onto nitrocellulose membrane, and probed with goat polyclonal PAR-4 (C-20) antibody (1:200, sc-8464; Santa Cruz Biotechnology, Dallas TX) or rabbit polyclonal αSMA (1:1000, ab5694; Abcam). Protein expression was normalized to β-tubulin I (1:10000, T7816; Sigma-Aldrich, St Louis, MO).

Protein expression in cultured human vascular SMCs was detected in whole cell lysates by Western blotting as described, using HuR (3A2, sc-5261) and PAR-4 (sc-8464) and normalized to β-actin. Infrared fluorescent-coupled secondary antibodies were used, allowing fluorescent detection on a LI-COR Odyssey infrared imaging system.

Cell Culture

Human saphenous vein SMCs were cultured in DMEM containing 5.5 mmol/L d-glucose and 15% FCS (GibcoBRL, Rockville, MD), as described. SMCs (passages 5–8) were serum deprived for 48 hours before stimulation.

PAR-4 Immunofluorescence

PAR-4 immunofluorescence (sc-8461, 1:50; Santa Cruz Biotechnology) was assessed in human vascular SMCs after preincubation with the protein kinase A (PKA) inhibitors myr-PK1 and Rp-8-CPT-cAMPS for 30 minutes and stimulation with high glucose or ciprofloxacin for 48 hours. Fluorescence images were captured with an AxioCam HRC camera and AxioVision Software connected to an Axio Imager.M2 microscope (Carl Zeiss).

Mice and STZ-Diabetes Model

Detailed procedures are outlined in the online-only Data Supplement. This study has the approval of the local animal experimentation ethics committee (Landesamt für Natur, Umwelt und Verbraucherschutz NRW, approval 87-51.04.2011.A055). PAR-4–/– mice (C57BL/6 background) were generously provided by Dr Justin Hamilton (Australian Center for Blood Diseases, Monash University, Melbourne, Victoria, Australia) and bred in-house. Type 1 diabetes mellitus was induced with a 180 mg/kg IP bolus of STZ; control mice received an equal volume of citrate buffer solvent. Nonfasted blood glucose levels 3 weeks after injection were 400 to 500 mg/dL and 150 to 250 mg/dL, respectively, for diabetic and nondiabetic mice, regardless of genotype. Pancreatic insulin content was determined by immunohistochemistry as detailed in the online-only Data Supplement. The development of ketoacidosis was monitored using Combur 9 urinary analysis test strips (Roche-Diagnostics, Mannheim, Germany) and by mass spectrometry analysis of urinary β-hydroxybutyrate.

Carotid Artery Ligation

Ligation of the left carotid artery was performed 3 weeks after treatment with STZ or citrate solvent, as described by Kumar and Lindner. Mice were anesthetized with ketamine/xylazine (100 mg/kg per 5 mg/kg IP). Postoperative analgesia was assured with carprofen 5 mg/kg SC. Animals were anesthetized as above 4 weeks after surgery and killed by perfusion with 4% paraformaldehyde. Paraffin-embedded carotid arteries were serially sectioned (5 μm) over 1000 μm proximal to the ligation site, stained with hematoxylin-eosin, and neointimal, medial, and lumen areas were quantified by histomorphometry. Infiltaring macrophages were visualized with Mac-2 primary antibody (CL8942AP, 1:50; Cedarlane Laboratories, Burlington, Ontario, Canada) and goat anti-rat (112-295-167; Dianova, Hamburg, Germany) secondary antibody by immunofluorescence, as detailed above. For analysis of proliferating cells, mice were treated with bromodeoxyuridine (BrdU; 50 mg/kg IP) 24 hours and 1 hour before euthanasia at 7 days. Tissue sections were stained with BrdU antibody (ab6326, 1:50; Abcam) and hematoxylin. Proliferation rates were calculated as the percentage of total cell number.
Statistical Analysis
Data are expressed as mean±SEM. Real-time and Western blot data are normalized to unstimulated controls or nondiabetic patients for human carotid atherectomy specimen and saphenous vein samples. Statistical analysis used the paired Student t test or 1-way ANOVA with Dunnet or Tukey post hoc multiple comparisons procedure, as appropriate. *P<0.05 was accepted as significant.

Results
Increased PAR-4 in Diabetic Human Atherectomy and Saphenous Vein Specimen

We previously reported upregulation of PAR-4 by high glucose in SMCs cultured from the human coronary artery and saphenous vein. Immunohistochemical staining of PAR-4 revealed expression in the tunica media of atherectomy specimens, strongly suggesting expression in SMCs. In addition, PAR-4 staining was evident in the plaque neointima. Consecutive sections stained for αSMA suggested that the expression of PAR-4 in the media occurred mainly in SMCs with low or absent expression of αSMA (Figure IA through ID and Figure IC through IF in the online-only Data Supplement). However, rarely, PAR-4 expression was also associated with clusters of αSMA-positive SMCs (Figure IA and IB in the online-only Data Supplement). In addition in the neointima, PAR-4 expression was found in regions that contained little αSMA-positive cells, as expected in atherosclerotic lesions (Figure IC through IF in the online-only Data Supplement). Importantly, quantification revealed greater PAR-4 immunoreactivity in the media of diabetic patients (Figure IE) than samples from nondiabetic patients (nondiabetic patients, 284.5±13.2 arbitrary fluorescence units versus diabetic patients, 350.3±19.65 arbitrary fluorescence units). In contrast to the media, the expression

Figure 1. Protease-activated receptor 4 (PAR-4) (red) immunofluorescence in human carotid artery atherectomies obtained from (A) nondiabetic (n=13) and (B) diabetic (n=9) patients. α-Smuract muscle actin (SMA; red) immunofluorescence in (C) nondiabetic and (D) diabetic human carotid artery atherectomy samples. Nuclei are stained in blue and autofluorescence of elastin-rich lamellae in green. Arrows point to the inner zone of the tunica media, * indicates the location of αSMA-positive differentiated smooth muscle cells (SMCs); m indicates tunica media; n, neointima. E and F, Quantification of PAR-4 immunofluorescence in the media and intima of nondiabetic and diabetic atherectomy sections. G, PAR-4 mRNA and (H) PAR-4, αSMA, and β-tubulin immunoblotting in samples from human nondiabetic and diabetic atherectomy. Representative immunoblots and quantitative analysis are shown. Data are expressed as fold change of nondiabetic patients after normalization to β-tubulin; PAR-4 and β-tubulin, n=12; αSMA, n=5. Data show mean±SEM, *P<0.05.
in the plaque neointima was not different between the groups (Figure 1F). The area of the media that was included in the analysis was similar between diabetic and nondiabetic samples (Figure IIE in the online-only Data Supplement), proving that upregulation of PAR-4 is not simply a reflection of increased amounts of medial tissue in the diabetic group. Similarly, total PAR-4 protein expression in atherectomies, as determined by Western blotting, was increased (Figure 1H; PAR-4, 2.261±0.500-fold of nondiabetic; n=12). Increased PAR-4 was also observed in human coronary artery samples from diabetic versus nondiabetic patients (n=3 each; Figure IV in the online-only Data Supplement). In addition to immunohistochemistry and immunoblotting, real-time polymerase chain reaction also revealed increased PAR-4 mRNA expression in diabetic atherectomy samples, by ≈9-fold compared with nondiabetic samples (Figure 1G; P<0.05; n=12, n=15).

PAR-4 abundance was also examined in nondiabetic (n=9) versus diabetic (n=9) human saphenous veins (Figure 2A versus Figure 2B). As indicated by morphology and αSMA staining, PAR-4 expression occurred mainly in the media (Figure 2A through 2D). Interestingly, although PAR-4 expression was also localized to areas of αSMA-positive SMCs in the media, the staining revealed that PAR-4 was expressed more strongly in the luminal parts of the media and to a lesser degree in the abluminal part of the media where marked expression of αSMA occurred. Therefore, similar to atherectomies, in veins the expression of PAR-4 might also be attributed to the more dedifferentiated SMC phenotype. Importantly, mean PAR-4 immunofluorescence intensities in the media of nondiabetic and diabetic veins were 223.40±8.13 and 272.00±17.00, respectively, corresponding with an increase of ≈22% in diabetic human saphenous veins (Figure 2E). PAR-4 mRNA expression was increased to ≈19-fold in diabetic versus nondiabetic human saphenous veins (Figure 2F; n=6, n=10; P<0.05). Given the stronger PAR-4 mRNA induction in diabetic veins compared with atherectomies, subsequent in vitro studies examining the mechanisms of glucose-regulated PAR-4 expression were performed in human saphenous vein SMCs.

**PAR-4 Upregulation by High Glucose Requires HuR-Mediated Transcript Stabilization**

To gain further insights into the mechanisms that mediate the increased PAR-4 expression in diabetic vessels, the possible contribution of mRNA stabilization by HuR was investigated. Nucleocytoplasmic shuttling of HuR is a prerequisite for its mRNA-stabilizing activity and was determined by immunofluorescence in high-glucose–treated SMCs. A time-dependent cytosolic HuR accumulation was maximal at 3 hours (Figure 3A; n=5). Similar results were obtained by Western blotting (Figure 3B; n=5; P<0.05). Total HuR protein expression did not change over time (Figure 3B), nor did high glucose influence HuR mRNA expression over 24 hours (data not shown; n=4). HuR small interfering RNA prevented the stimulatory effects of high glucose (6 hours) on PAR-4 mRNA expression (Figure 3C; n=5).

![Figure 2. Protease-activated receptor (PAR)-4 (red) immunofluorescence in human saphenous veins obtained from (A) nondiabetic (n=9) and (B) diabetic (n=9) patients. α-Smooth muscle actin (SMA; red) immunofluorescence in (C) nondiabetic and (D) diabetic saphenous veins. Nuclei are stained in blue and autofluorescence of elastin-rich lamellae in green. Arrows mark the tunica media, * indicates the location of αSMA-positive differentiated smooth muscle cells (SMCs); m indicates tunica media; l, lumen; a, adventitia. E, Quantification of PAR-4 immunofluorescence in the media. F, PAR-4 mRNA expression in human nondiabetic and diabetic saphenous veins (n=10 vs n=6). Data show mean±SEM. *P<0.05.](http://circ.ahajournals.org/doi/abs/10.1161/CIRCULATIONAHA.116.027714)
The specific binding of HuR to PAR-4 mRNA was studied by pull-down polymerase chain reaction. High glucose increased the amount of cytosolic PAR-4 mRNA immunoprecipitated by the HuR antibody >8-fold (Figure 3D; n=5; P<0.05). Only minimal amounts of PAR-4 transcript were immunoprecipitated by the immunoglobulin G control antibody, confirming specificity of the HuR pull-down. High glucose also significantly stabilized PAR-4 mRNA in actinomycin D–pretreated SMCs (Figure 3E; n=4), whereas degradation of PAR-1 mRNA was not influenced (Figure 3F; n=4).

Dependence of PAR-4 regulation on HuR-mediated transcript stabilization was confirmed by use of the well-characterized HuR activator angiotensin II (10 nmol/L), which increased PAR-4 expression in human vascular SMCs (Figure IVA and IVB in the online-only Data Supplement; n=3), an effect abolished by HuR small interfering RNA (Figure V in the online-only Data Supplement; n=5). Similarly, H2O2 (100 μmol/L), which promoted HuR shuttling (Figure VIA in the online-only Data Supplement; n=4; P<0.05), upregulated PAR-4 but not PAR-1 mRNA and total protein expression (both not shown; n=5). Accordingly, high-glucose–stimulated PAR-4 expression could be abolished by nicotinamide adenine dinucleotide phosphate oxidase inhibitors apolipoprotein/diphenylene iodonium (100 μmol/L per 10 μmol/L, prevent H2O2 generation) or cell-permeant polyethylene glycol–catalase (500 U/mL, degrades cellular H2O2; Figure VIB in the online-only Data Supplement; n=6).

**Downregulation of PAR-4 Expression by Agents That Elevate cAMP**

cAMP blocks HuR activation in human vascular SMCs.21 In the present study, basal PAR-4 mRNA expression was suppressed in a time-dependent manner by the adenylate cyclase activator forskolin (10 μmol/L), by the chemically stable prostaglandin I2 analogue cicaprost (10 nmol/L), and by exogenous prostaglandin E1 (1 μmol/L; all n=4; Figure 4A). Cicaprost also accelerated the decay of the PAR-4 transcript in the presence of actinomycin D (Figure 4B; n=4) and counteracted high-glucose–stimulated accumulation of HuR-bound PAR-4 mRNA (3-hour incubation; Figure 4C; all n=3). HuR nucleocytoplasmic shuttling was suppressed by cicaprost with no change in total cellular HuR protein (Figure 4D; n=4). Accordingly, cicaprost completely prevented high-glucose–induced upregulation of PAR-4 mRNA (Figure 4E; n=5) and protein (Figure VII in the online-only Data Supplement; n=5).

**Figure 3.** A, HuR (red) immunofluorescence showing high-glucose (25 mmol/L)–induced nucleocytoplasmic shuttling of HuR in human vascular smooth muscle cells (SMCs). Nuclei are stained in blue. Representative of n=5 individual experiments. B, Western blot analysis of cytosolic HuR accumulation normalized to total cellular HuR in high-glucose–treated SMCs (n=5). C, High-glucose–stimulated protease-activated receptor (PAR)-4 mRNA expression in SMCs transfected with control (siCon) or HuR small interfering RNA (siHuR; all n=5). D, Pull-down polymerase chain reaction (PCR) showing high-glucose–induced binding of HuR to PAR-4 mRNA. Cytosolic fractions were immunoprecipitated (IP) with HuR or immunoglobulin G (IgG) antibody before PCR analysis for PAR-4 mRNA (n=5). Minimal immunoprecipitation by IgG antibody confirms the specificity of the HuR pull-down. E, Influence of high glucose on decay of PAR-4 and F, PAR-1 mRNA in SMCs pretreated with actinomycin D (5 μg/mL; all n=4). Data show mean±SEM, expressed as fold control. *P<0.05.
online-only Data Supplement; n=5). Suppression by cicaprost was similar when cicaprost was added together with high glucose for 48 hours or only for the final 24 hours of incubation. Cicaprost also blunted basal and thrombin (3 U/mL)-evoked tumor necrosis factor-α mRNA expression in high-glucose–treated SMCs (Figure 4F; n=4; *P<0.05).

The contributions of the cAMP effector protein kinase A and the exchange protein directly activated by cAMP (Epac) were subsequently assessed. An Epac activator (8-pCPT-2′-O-Me-cAMP) did not influence PAR-4 expression (data not shown). However, 2 different PKA inhibitors (and Rp-8-CPT-cAMPS) counteracted the effects of cicaprost in high-glucose–stimulated cells. In the absence of PKA inhibition, cicaprost suppressed nucleocytoplasmic shuttling of HuR in response to high glucose; the addition of a PKA inhibitor or Rp-8-CPT-cAMPS restored HuR translocation (Figure 5A and 5B; n=4). Accordingly, high-glucose–stimulated increases in PAR-4 immunofluorescence were abolished by cicaprost and restored in the presence of a PKA inhibitor (Figure 5C and 5D; n=3).

**Systemic Hyperglycemia in STZ-Treated Mice Increases Vascular PAR-4**

The functional significance of PAR-4 upregulation was examined in a mouse model of type 1 diabetes mellitus. Three weeks after treatment of male C57BL/6 mice with STZ, blood glucose levels were increased to >400 mg/dL versus 200 mg/dL (Figure 6A; n=4; *P<0.05), whereas insulin immunoreactivity in pancreatic islets was reduced compared with solvent-treated controls (Figure VIII A in the online-only Data Supplement; n=4). Vascular PAR-4 mRNA expression was significantly increased after 3 weeks in aortas from diabetic mice to 9.0±1.5-fold of nondiabetic controls (Figure 6B; n=4; *P<0.05), as was PAR-4 immunofluorescence in both the aorta (Figure 6C and 6D) and left carotid artery (Figure 6E and 6F; all n=4). PAR-4 expression levels did not differ between the groups at earlier time points (data not shown). Aortic PAR-1 expression was not significantly altered in diabetic versus nondiabetic mice (Figure VIIIIB in the online-only Data Supplement; n=4).

**PAR-4 Deficiency Protects Against Augmented Neointimal Hyperplasia in Diabetic Mice**

Left carotid arteries were ligated 3 weeks after STZ or citrate treatment and collected for analysis 4 weeks after surgery. Wild-type STZ-diabetic mice exhibited dramatically augmented neointimal thickening, which was completely absent in PAR-4−/− diabetic mice. Neointima formation in nondiabetic PAR-4−/− mice was comparable to the wild-type control.
Representative morphologic staining of tissue sections obtained 250-μm below the ligation site are shown in Figure 7A through 7D. Pooled area quantifications >1000 μm for nondiabetic and diabetic mice, respectively, are shown in Figure 7E and 7F, and neointima/media ratios across all sections to 1000 μm proximal to the ligation site are depicted in Figure 7G (all n=6). Nonligated right carotid arteries showed no neointimal thickening (Figure IX in the online-only Data Supplement; n=6). Unlike the neointimal area, neither the medial nor lumen areas differed significantly between the groups (Figure X in the online-only Data Supplement). Furthermore, the intimal cell density was not different between diabetic wild-type and diabetic PAR-4−/− mice, indicating a proportional decrease of cell numbers and matrix (data not shown). β-Hydroxybutyrate was determined by mass spectroscopy in the urine to control for ketoacidosis. This measurement showed a trend to increased concentrations of β-hydroxybutyrate in diabetic mice but no differences between wild-types and PAR-4−/− mice. The average β-hydroxybutyrate concentration in the urine of diabetic mice was in the range of 20 mg/g of creatinine (data not shown), which is well below the threshold of ketoacidosis. Therefore, it is concluded that the observed protection against neointimal hyperplasia after the deletion of PAR-4 is independent of ketoacidosis.

**Increased Proliferation in Ligated Carotid Arteries**

In parallel with quantification of the neointimal area in left carotid arteries, proliferative responses attributed to arterial injury were assessed 7 days after ligation by determining BrdU incorporation. Tissue sections were obtained 50 μm proximal to the ligation site, at which point the degree of neointima formation was comparable across all of the experimental groups. Representative images are depicted in Figure 8A through 8D. Quantification of BrdU-positive cells, normalized
to total neointimal cells, is shown in Figure 8E. The proliferating cell count was significantly higher in diabetic versus nondiabetic wild-type mice (nondiabetic wild-type, 3.0±0.9% BrdU-positive cells per total intimal cells versus diabetic wild-type, 9.8±1.3% BrdU-positive cells per total intimal cells; n=6.5), whereas no such increase was observed in mice lacking PAR-4. A significant decrease of the proliferation index was apparent between wild-type diabetic mice and PAR4-deficient diabetic mice (Figure 8E; n=6 for wild-type controls; n=5 for all other groups). As a measure of inflammatory cell accumulation, Mac-2–positive cell counts were normalized to total cell content within the neointimal area (Figure XI in the online-only Data Supplement; n=6 for wild-type controls; n=5 for all other groups). Wild-type diabetic mice exhibited significantly higher numbers of Mac-2–positive cells than nondiabetic mice, whereas no such increase was observed in PAR-4–deficient animals.

Discussion
The present study identifies PAR-4 as a novel mediator linking hyperglycemia, hypercoagulation, and vascular remodeling in diabetes mellitus. Diabetic patients are particularly prone to thrombotic and proliferative vessel occlusion, in which thrombin plays a key role. Although much attention has focused on inhibiting PAR-1, the classic thrombin receptor, the contribution of PAR-4 has been largely underestimated. We now report increased PAR-4 expression in atherectomy and saphenous vein samples from patients with diabetes mellitus, in keeping with our previous report that high glucose upregulates PAR-4 in human vascular SMCs.

In diabetic human atherectomy samples, increased PAR-4 abundance was observed in the tunica media, as indicated by immunofluorescence, Western blotting, and mRNA expression. In contrast, no difference in expression between nondiabetic and diabetic was detected in the neointima. Medial areas were equivalent, confirming that the increase in PAR-4 expression is not merely because of increased amounts of medial tissue in diabetic atherectomies. PAR-4 immunofluorescence was detected mainly in areas of the media with low expression of αSMA. These data are suggestive of PAR-4 expression in dedifferentiated SMCs and, to a lesser degree, in differentiated SMCs. This hypothesis is in line with the marked upregulation of PAR-4 in response to high glucose in cultured vascular SMCs that also represent the dedifferentiated phenotype. However, it cannot be excluded that other cells also express PAR-4 in human atherectomies. The increased expression of PAR-4 in the tunica media is consistent with a role of PAR-4 in initiating the migration and proliferation of medial SMCs. Human saphenous veins show a greater difference between nondiabetic and diabetic PAR-4 mRNA abundance (≈19-fold difference) than atherectomies. Although diabetes mellitus is not associated with increased rates of venous disease, it is associated with increased rates of vein closure after bypass grafting. Potentially the strong induction in diabetic veins contributes to the enhanced proliferative remodeling and, hence, reduced graft patency reported in patients with diabetes mellitus.
Compared with nondiabetic saphenous veins, diabetic veins express ≈19-fold more PAR-4 mRNA and exhibit a 22% greater PAR-4 immunoreactivity. Human diabetic atherectomies express ≈9-fold more PAR-4 mRNA and exhibit a 23% greater PAR-4 immunoreactivity than nondiabetic samples. The reasons for the differing magnitude of mRNA and protein induction in the specimens are not clear. In isolated SMCs, high glucose increases PAR-4 expression by ≈3-fold at the mRNA level and 2-fold at the cell surface, similarly to our findings with activated factor X-stimulated PAR-2 expression.26 In the present in situ study, the difference in expression is greater, and changes in mRNA levels do not correspond 1:1 with changes in protein. Whether this is the result of differences in translation efficiency, protein degradation, or efficiency of shuttling to the cell surface is not known.

Given the strong PAR-4 upregulation in venous vessels, the cellular pathways underlying PAR-4 regulation were further explored in SMCs cultured from human saphenous vein. Transcript stabilization is a major mechanism of posttranscriptional gene regulation, particularly of inducible genes.19,27 HuR, or ELAV1, the most characterized of a number of mRNA stabilizing proteins,19,27 shows particularly high abundance in human atherosclerosis, intimal hyperplasia, and sclerosed saphenous vein grafts.20 We now show that high glucose promotes HuR translocation in human vascular SMCs and augments cytosolic accumulation of HuR-bound PAR-4 mRNA, whereas HuR small interfering RNA abolishes high-glucose–induced PAR-4 expression. Accordingly, PAR-4 mRNA decay in the presence of the transcription inhibitor actinomycin D is slowed in high-glucose–treated SMCs, and maximal stabilization is seen at
3 hours of stimulation, coinciding with the greatest extent of HuR shuttling. Analysis of the 3′ untranslated region of PAR-4 showed multiple adenylate-uridylate–rich elements that could serve as HuR binding motifs, whereas no such motifs can be found in the PAR-1 sequence. Accordingly, PAR-1 mRNA is not stabilized by high glucose. In our supplementary data, we show that angiotensin II, a classic activator of HuR, transiently upregulates PAR-4 mRNA and protein in an HuR-dependent manner, with no effect on PAR-1. HuR shuttling is also shown to be activated in response to H2O2; accordingly, inhibition of NADPH oxidase with apolipoprotein/diphenylene iodonium to block H2O2 production or catalase, which degrades H2O2, prevents high-glucose–stimulated PAR-4 induction in human vascular cells.

A further candidate regulator of PAR-4 is cAMP, which attenuates the mRNA stabilizing activity of HuR in human vascular SMCs. Several cAMP stimuli, including forskolin, the prostacyclin analogue cicaprost, and exogenous prostaglandin E2, reduce basal PAR-4 mRNA expression in the present study. Cicaprost moreover accelerates PAR-4 mRNA decay in human SMCs. This destabilizing action can be attributed to impaired HuR/PAR-4 mRNA binding and nucleocytosolic shuttling in the presence of the prostacyclin analogue. As a consequence, cicaprost normalizes PAR-4 expression in high-glucose–treated SMCs, both when added concurrently with high glucose for 48 hours or only for the final 24 hours of incubation, and thrombin-stimulated expression of the inflammatory cytokine tumor necrosis factor-α is blunted. Thus, prostacyclin might limit proliferative and inflammatory processes in the vessel wall, both through its direct antitrophic, antithrombotic, and antiadhesive effects and indirectly by suppressing PAR-4. Venous vessels, which are particularly prone to restenosis, exhibit an impaired capacity to generate prostacyclin compared with arteries, and a lower level of endogenous prostacyclin production could explain the stronger PAR-4 immunoreactivity detected in human saphenous veins compared with atherectomy samples.

The precise mechanism by which cAMP suppresses HuR-mediated stabilization of PAR-4 mRNA remains to be defined. cAMP has been reported to downregulate soluble guanylate cyclase by suppressing HuR expression. This effect was, however, evident over 4 to 12 hours, whereas in our study, the destabilizing effects of the cAMP activator cicaprost are observed within 1 to 2 hours, as is inhibition of HuR shuttling, whereas total HuR expression is unaltered. Similarly, the stimulatory effects of high glucose on HuR shuttling and PAR-4 mRNA stability occur within 1 to 3 hours, also with...
no change in total HuR abundance. Thus, HuR downregulation is not likely to be involved in cAMP-regulated PAR-4 expression, nor does the cAMP effector Epac appear to contribute. Phosphorylation is the primary mechanism by which the nucleocytoplasmic translocation of HuR is controlled,16 and although the cAMP effector PKA has not specifically been reported to phosphorylate HuR, its phosphorylation substrates include a number of proteins in the 35- to 52-kDa range,17 which encompasses HuR. We find that inhibition of PKA abolishes the ability of cicaprost to counteract high-glucose–stimulated HuR shuttling in human vascular SMCs; accordingly, high-glucose–stimulated PAR-4 expression is also restored in cicaprost-treated cells on the addition of PKA inhibitors.

PAR-4 contributes to thrombin-stimulated vascular SMC mitogenesis, migration, and inflammatory gene expression.16,17 Moreover, PAR-4 is also activated by cathepsin G, an elastase released by neutrophils, which accumulate in diabetic tissues and contribute to neointimal hyperplasia.18 Thus, glucose-regulated PAR-4 expression might be important for accelerated vascular remodeling in diabetes mellitus.1 In this proof-of-concept study, we show for the first time that PAR-4 deficiency protects against the enhanced neointimal hyperplasia that develops in STZ-diabetic mice after carotid artery ligation. STZ-treated mice exhibited marked hyperglycemia but no evidence of ketoacidosis 3 weeks after the induction of diabetes mellitus; the increase in blood glucose was accompanied by a time-dependent increase in PAR-4 (but not PAR-1) expression in both the aorta and carotid arteries. To test the potential consequences for vascular remodeling, the left carotid artery was ligated according to a conventionally established model of neointimal thickening.24 Diabetic mice are shown to develop greater neointima formation than nondiabetic mice, mirroring an earlier report.14 In that study, oxidative stress, which we here show to posttranscriptionally regulate PAR-4, was found to contribute to the augmented neointima formation. Diabetic mice deficient in PAR-4 showed no such augmented remodeling response; in fact, the neointimal area was smaller even than in nondiabetic wild-type mice. The reasons for this more-than-complete suppression are not clear. Neointima formation in nondiabetic PAR-4−/− mice was comparable to the wild-type controls, suggesting that the involvement of PAR-4 under normoglycemic conditions is negligible.

In an effort to understand why PAR-4 deficiency protects against excessive vessel remodeling in diabetic mice, the impact on macrophage accumulation and on proliferating cell numbers was assessed. Arterial wall injury is associated with the recruitment of inflammatory cells, specifically circulating monocytes, which differentiate into macrophages and actively contribute to neointimal hyperplasia.25 Thrombin and its prototypical receptor PAR-1 have been identified as critical players in this process.26 In this study, carotid arteries from diabetic mice show a greater accumulation of macrophages 7 days after ligation than control mice, whereas no such increase is observed in arteries from PAR-4−/− mice. Similarly, the proportion of BrdU-labeled cells, determined as a measure of proliferation 7 days after ligation, is significantly higher in diabetic wild-type mice compared with nondiabetic controls and diabetic PAR-4−/− mice. Therefore, the augmented neointimal hyperplasia in diabetic animals can mainly be attributed to a greater degree of proliferation resulting from increased expression of PAR-4.

In conclusion, PAR-4 shows increased expression in diabetic human and murine vessels, and PAR-4 deficiency protects against the excessive remodeling induced by carotid artery ligation. At the cellular level, PAR-4 abundance is critically controlled via the mRNA stabilizing actions of HuR, which account for the stimulatory actions of high glucose, angiotensin II, and H2O2 on PAR-4 expression, whereas cAMP agonists, such as the prostacyclin analogue cicaprost, counteract this effect. Such interactions could potentially represent a fine-tuning mechanism to control PAR-4 expression and ultimately also the mitogenic actions of thrombin in the diabetic vessel wall. PAR-4 might potentially serve as a suitable therapeutic target to limit proliferative and inflammatory processes in restenosis-prone diabetic patients, particularly those patients in whom severe bleeding because of selective PAR-1 blockade or complete thrombin inhibition must be avoided or who do not require anticoagulation.

Acknowledgments
We thank Petra Rompel and Kerstin Freidel for excellent technical assistance.

Sources of Funding
This study was supported by funding from the Deutsche Forschungsgemeinschaft (grants RO 3921/2-1 and SFB 612-TP B11) and the Ernst und Berta Grimmke-Stiftung Düsseldorf. Dr Fender was supported by a Rudolf-Marx Stipend from the Gesellschaft für Thrombose und Hämostase-Forschung.

Disclosures
None.

References
11. Andrade-Gordon P, Derian CK, Maryanoff BE, Zhang HC, Addo MF, Cheung WM, Damiano BP, D’Andrea MR, Darrow AL, de Garavilla L,


CLINICAL PERSPECTIVE

The noncoagulant actions of thrombin remain topical given the rising use of the direct inhibitors. Thrombin contributes to thrombotic and proliferative vessel occlusion, which critically limits the success of coronary interventions, particularly in patients with diabetes mellitus. Most thrombin actions are traditionally attributed to the classic receptor protease-activated receptor (PAR)-1, which is, however, an unsuitable target to prevent vessel remodeling, given the associated bleeding complications. PAR-4 is a low-affinity thrombin receptor whose role in vasculopathy, particularly in diabetes mellitus, has not been explored. Here, PAR-4 expression is shown to be increased in human atherectomy and saphenous vein specimen obtained from type 2 diabetic patients compared with nondiabetic patients. At the cellular level, elevated glucose levels drive PAR-4 expression via mechanisms involving increased stabilization of PAR-4 mRNA. The functional consequences of this interaction were examined in a mouse model of vessel remodeling induced by carotid artery ligation. Experimental type 1 diabetes mellitus leads to an increased vascular PAR-4 expression and an augmented vessel hyperplasia, associated with greater numbers of proliferating cells and macrophages. Diabetic mice with genetic deletion of PAR-4 are protected against the excessive neointimal hyperplasia in response to vascular injury, highlighting PAR-4 as an important glucose-regulated thrombin receptor with a hitherto unsuspected role in the vascular complications of diabetes mellitus. Potentially, PAR-4 inhibition might be of benefit in restenosis-prone diabetic patients, particularly those patients in whom severe bleeding resulting from selective PAR-1 blockade or complete thrombin inhibition must be avoided or who do not require anticoagulation.
Thrombin Receptor Protease-Activated Receptor 4 Is a Key Regulator of Exaggerated Intimal Thickening in Diabetes Mellitus

Goran Pavic, Maria Grandoch, Seema Dangwal, Klaus Jobi, Bernhard H. Rauch, Anke Doller, Alexander Oberhuber, Payam Akhyari, Karsten Schröer, Jens W. Fischer and Anke C. Fender

_Circulation_. 2014;130:1700-1711; originally published online September 19, 2014; doi: 10.1161/CIRCULATIONAHA.113.007590

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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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Supplemental Material
Supplemental Methods

Materials

Human thrombin was from American Diagnostica GmbH (Pfungstadt, Germany).

Myristolated proteinase kinase A inhibitor (myr-PKI), actinomycin D and D-glucose were from Calbiochem (San Diego, CA); forskolin, and streptozotocin (STZ) from Sigma (München, Germany). Cicaprost was from Bayer/Schering AG (Berlin, Germany), PGE₂ from Cayman Chemicals (Ann Arbor, MI), Epac activator 8-pCPT-2’-O-Me-cAMP and PKA inhibitor Rp-8-CPT-cAMPS from Biolog (Bremen, Germany). All other materials were from Merck (Darmstadt, Germany) or Roth (Karlsruhe, Germany) unless otherwise stated.

Human tissue samples

Human atherectomy samples were obtained from diabetic and non-diabetic patients of both gender presenting for carotid endarterectomy at the Klinik für Gefäß- und Endovaskularchirurgie, Klinikum der Heinrich-Heine-Universität Düsseldorf, Germany, with approval of the Human Ethics Commission of the Medical Faculty of the Heinrich-Heine Universität Düsseldorf (Approval Nr. #3944) and informed consent of donors. Patient characteristics are given in table 1. All patients were included regardless of specific clinical exclusion/inclusion criteria (“all-comers”). Samples were directly snap-frozen in liquid nitrogen and used either directly for RNA or protein extraction or embedded for paraffin sections after fixation with 4% paraformaldehyde for 24 hours.

Human saphenous vein segments and coronary specimens from type 2 diabetic and non-diabetic patients presenting for coronary artery bypass grafting at the Klinik für
Kardiovaskuläre Chirurgie, Klinikum der Heinrich-Heine-Universität Düsseldorf, Germany, with approval of the Human Ethics Commission of the Medical Faculty of the Heinrich-Heine Universität Düsseldorf (Approval Nr. #3199) and informed consent of donors. Patient characteristics are provided in table 2 of the online data supplement. Samples for RNA extraction were snap-frozen in liquid nitrogen with prior storage in RNAlater® (Qiagen, Hilden, Germany) where necessary. Samples for immunohistochemistry were formaline-fixed and embedded in paraffin.

**Immunohistochemistry of human tissue sections**

Paraffin sections (3 µm) were treated with citrate buffer (pH 6.0, Zytomed Systems, Berlin, Germany) at 98°C for 30 min and blocked with 10% fetal calf serum (FCS)/1% bovine serum albumin (BSA) for 1h at RT. Primary PAR-4 antibody (ab66103, 1:50, Abcam, Cambridge, UK) or anti-alpha smooth muscle actin (αSMA) antibody (M0851, 1:200, Dako, Glostrup, Denmark) was applied overnight at 4°C followed by incubation with secondary antibody (Alexa-Fluor® 647-labelled goat anti rabbit (1:200, Invitrogen, Karlsruhe, Germany) for 1h at RT in the dark. PAR-4 antibody specificity was confirmed by immunoblotting in HEK293 cells transiently transduced with human PAR-4 (data not shown). Collagen was stained with Sirius red in human atherectomy samples. Nuclear staining utilized Roti®-Mount FluorCare DAPI (Roth GmbH). Fluorescence images were captured with an AxioCam HRC camera and AxioVision Software connected to an Axio Imager.M2 microscope (Carl Zeiss, Göttingen, Germany). As negative controls, parallel sections were incubated without primary antibody; these were all negative for PAR-4 and αSMA. Mean PAR-4 fluorescence intensity was quantified using the Zeiss Zen lite 2102 Software. The atherectomy specimens contained large amounts of plaque and variable amounts of the tunica media. To define the tunica media in atherectomies the combination of autofluorescence of elastic lamellae, presence of variable amounts of alpha-SMA positive smooth muscle cells (SMC) and the relative absence of
collagen deposition determined by Sirius red staining was used. Representative images of the atherectomies are shown in supplementary Figure 1. The tunica media of saphenous vein sections was defined by morphology, αSMA staining and autofluorescence (green) of elastin lamellae (supplementary Figure 3).

**Streptozotocin (STZ)-induced diabetes in mice**

Type-1 diabetes was initially induced in male C57BL/6 or PAR-4−/− mice by the low-dose STZ model in which 55 mg/kg STZ is applied intraperitoneally after a 6h fast on 5 consecutive days to induce partial injury of pancreatic β-cells. Control mice received an equal volume of citrate buffer vehicle (pH 4.5). During the study it became apparent that PAR-4−/− mice exhibit a resistance to low-dose STZ, therefore the high-dose model of a single bolus dose of 200 mg/kg STZ was subsequently used. This dose was well-tolerated by both mouse genotypes. Three weeks after injection, non-fasted blood glucose levels (measured in tail vein blood with a standard glucometer) were 400-500 mg/dL and 150-250 mg/dL respectively for diabetic and non-diabetic groups, regardless of genotype.

**Immunohistochemistry of pancreatic insulin**

Pancreatic tissue was collected 3 weeks after treatment of mice with STZ or citrate, fixed in 4% paraformaldehyde and embedded in paraffin. Tissue sections (3 µm) were deparaffinized in xylene, rehydrated in ethanol, and washed with PBS. Antigen retrieval was performed in citrate buffer (pH 6.0, Zytomed Systems, Berlin, Germany) at 98°C for 30 minutes. Sections were blocked with 10% FCS/1% BSA for 1 hour at RT. Insulin antibody (ab63820, 1:500, Abcam, Cambridge, UK) was applied overnight at 4°C and visualised after incubation with horseradish peroxidase-conjugated secondary antibody (sc-2004, 1:200, Santa Cruz Biotechnology, Heidelberg, Germany) and the DAB Substrate Kit (Zytomed Systems). Endogenous peroxidase activity was previously quenched with 3% hydrogen peroxide in PBS.
for 5 minutes. Nuclei were stained with Mayer’s hemalaun solution (Merck). Images were taken on Axio Imager.M2 microscope (Carl Zeiss, Göttingen, Germany) using AxioVision software. As control, parallel sections were incubated without primary antibody.

**Inflammatory cell staining in ligated carotid arteries**

Animals were anaesthetised 7 days after surgery and sacrificed by perfusion with 4% PFA. Paraffin-embedded carotid arteries were serially sectioned (5 µm) over 1000 µm proximal to the ligation site. Infiltrating macrophages were visualized with Mac-2 primary antibody (CL8942AP, 1:50, Cedarlane Laboratories, Burlington, Ontario, Canada) and goat anti-rat (112-295-167, Dianova, Hamburg, Germany) secondary antibody by immunofluorescence.

**Western blot analysis of PAR-4**

Tissue samples (200 mg) were lysed in 500 µl modified RIPA-buffer (137 mmol/l NaCl, 12 mmol/l phosphate, 2.7 mmol/l KCl, 1% NP-40, 0.1% sodium deoxycholate, 0.1 mmol/l PMSF, 0.3 µmol/l aprotinin, 10 µmol/l leupeptin), followed by centrifugation (5 min at 275 x g, 4°C) and homogenization of the supernatant by 20 strokes in a glass douncer. Protein concentration was determined by the butterfly procedure (protein quantitation using bound Coomassie blue stain). Lysates (20 µg protein) lysates were separated by electrophoresis through a 12% SDS-PAGE, transferred onto nitrocellulose membrane and probed with goat polyclonal PAR-4 (C-20) antibody (1:200, sc-8464, Santa Cruz Biotechnology) or rabbit polyclonal αSMA (1:1000, ab5694, Abcam). Protein expression was normalized to beta-tubulin I (1:10000; T7816, Sigma-Aldrich).

Protein expression in cultured human vascular SMC was detected in whole cell lysates by Western blotting as described, utilizing HuR (3A2, #sc-5261) and PAR-4 (#sc-8464) primary and horseradish peroxidase-coupled secondary antibodies (all Santa Cruz Biotechnology).
PAR-4 expression was normalized to β-actin. Infrared fluorescent-coupled secondary antibodies were used allowing fluorescent detection on a LI-COR Odyssey infrared imaging system.

**PAR-4 immunofluorescence in vitro**

PAR-4 immunofluorescence was assessed in human vascular SMC after preincubation with the PKA inhibitors myr-PKI and Rp-8-CPT-cAMPS for 30 minutes and stimulation with high glucose and/or cicaprost for 48h and. Cells were fixed, washed and permeabilized as described above. After blocking with 3% BSA, PAR-4 antibody (sc-8461, 1:50, Santa Cruz Biotechnology) was applied overnight at 4°C, Alexa-Fluor® 568-labelled goat secondary antibody (1:200, Invitrogen) for 1h at RT in the dark. Nuclei were stained with Roti®-Mount FluorCare DAPI (Roth GmbH). Fluorescence images were captured with an AxioCam HRC camera and AxioVision Software connected to an Axio Imager.M2 microscope (Carl Zeiss). Control SMC incubated in parallel without primary antibody were all negative (data not shown).

**RNA extraction and realtime PCR**

Total RNA from cells and tissue was extracted with TriReagent® (Sigma-Adrich, München, Germany); tissue samples were dissociated in Trizol using the gentleMACSTM Dissociator (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). Following treatment with DNaseI (ThermoScientific, St. Leon Rot, Germany) and determination of RNA purity and concentration (Nanodrop ND2000, PeqLab, Erlangen, Germany), reverse transcription and realtime PCR analysis was performed as described.²

**HuR knockdown**
Endogenous HuR mRNA and protein expression was knocked down in human SMC by transfection with control (sc-37007) or HuR siRNA (sc-35619, Santa Cruz Biotechnology) as described.  

**HuR shuttling and pull-down PCR**

Nucleo-cytosolic HuR translocation in SMC stimulated with high glucose was determined by Western blotting and by immunofluorescence. Cells were fixed with 4% paraformaldehyde (PFA, 40 min) washed (PBS) and permeabilized (0.1% Triton X-100, 5 min) then blocked with 3% BSA. HuR antibody (sc-56709, 1:50, Santa Cruz Biotechnology) was applied overnight at 4°C, Alexa-Fluor® 647-labelled goat secondary antibody (1:200, Invitrogen) for 1h at RT in the dark. Nuclei were stained with Roti®-Mount FluorCare DAPI (Roth GmbH). Fluorescence images were captured with an AxioCam HRC camera and AxioVision Software connected to an Axio Imager.M2 microscope (Carl Zeiss). Control SMC incubated in parallel without primary antibody were all negative (data not shown). Binding of HuR to PAR-4 mRNA was determined by immunoprecipitation (IP)-PCR assay as described.  

**Supplemental references**

Supplemental Tables
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Table 2: Patient characteristics of atherectomy specimen used for immunohistochecmistry

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Table 3.: Patient characteristics of atherectomy specimen used for Western Blotting

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**Suppl. Table 1-3.** Human atherectomy samples were obtained from diabetic and non-diabetic patients of both gender presenting for carotid endarterectomy at the Klinik für Gefäß- und Endovaskularchirurgie, Klinikum der Heinrich-Heine-Universität Düsseldorf, Germany, with approval of the Human Ethics Commission of the Medical Faculty of the Heinrich-Heine Universität Düsseldorf (Approval Nr. #3944) and informed consent of donors.
Table 4: Patient characteristics of saphenous vein donors used for immunofluorescence analysis

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Table 5: Patient characteristics of saphenous vein donors used for mRNA analysis

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Suppl. Table 4-5. Saphenous vein segments from type 2 diabetic and non-diabetic patients were obtained from the Klinik für Kardiovaskuläre Chirurgie, Klinikum der Heinrich-Heine-Universität Düsseldorf, Germany, with approval of the Human Ethics Commission of the Medical Faculty of the Heinrich-Heine Universität Düsseldorf (Approval Nr. #3199) and informed consent of donors.
Supplemental Figures
**Supplementary Figure 1:** Representative images of n=9-13 individual specimens stained for PAR-4 (A, C, E and G) and of consecutive sections stained for αSMA (B, D, F and H) immunofluorescence in the tunica media of human non-diabetic and diabetic atherectomies. Nuclei are stained in blue, arrows point to the luminal zone of the tunica media, asterisks indicate the location of αSMA positive differentiated SMC; m, tunica media; n, neointima.
Supplementary Figure 2: A) Representative H&E staining, B) Sirius red staining, C) αSMA (red) immunofluorescence and D) autofluorescence (green) in consecutive sections of human atherectomy samples. Arrows point to the luminal zone of the tunica media; m, tunica media; n, neointima. E) Mean medial area in atherectomy samples obtained from human non-diabetic and diabetic donors. Data shows mean±SEM of n=9-13 patients.
Supplementary Figure 3: A) Representative H&E staining, B) αSMA (red) immunofluorescence and C) autofluorescence (green) in serial sections of human saphenous vein samples. Arrows mark the tunica media; m, tunica media; l, lumen; a, adventitia.
**Supplementary Figure 4:** A) PAR-4 (red) immunofluorescence in the tunica media of human non-diabetic and B) diabetic coronary artery. Nuclei are stained in blue.

Representative images of vessels taken from n=3 individual patients.
Supplementary Figure 5: A) Ang II (10 nmol/L) transiently increases PAR-4 but not PAR-1 mRNA and B) induces PAR-4 protein expression in human vascular SMC (n=3). C) Ang II-stimulated PAR-4 mRNA expression (at 6h) is prevented in SMC transfected with HuR siRNA (n=5). *P<0.05 vs. control.
Supplementary Figure 6: A) H$_2$O$_2$ (100 µmol/L) promotes nucleo-cytosolic shuttling of HuR in human vascular SMC, as determined by western blot analysis of cytosolic and total cellular fractions (n=4, P<0.05). B) Pretreatment (30 min) of human SMC with the NADPH oxidase inhibitors apocynin/diphenyliodonium (apo 100 µmol/L/DPI 10 µmol/L, to prevent H$_2$O$_2$ generation), or cell-permeant pegylated catalase (500 U/mL, to degrade cellular H$_2$O$_2$) prevented the stimulatory effect of high glucose on expression of PAR-4 mRNA (6h incubation, n=4). Data are shown as mean ± SEM, fold of unstimulated control. *P<0.05 vs. control.
**Supplementary Figure 7:** PAR-4 protein expression determined by Western blotting in human vascular SMC stimulated for 48h with high glucose (HG) or cicaprost (10 nmol/L) or the combination, with cicaprost added either concurrently with high glucose for 48h or only for the final 24h of incubation. Data are shown as mean ± SEM, fold of unstimulated control, normalised to β-actin. *P<0.05 vs. control.
Supplementary Figure 8: A) Insulin (brown) expression determined by immunohistochemistry of mouse pancreatic islet sections, 3 weeks after injection of C57Bl/6 mice with STZ or citrate buffer vehicle. Nuclei are stained in blue. Negative control sections were not exposed to primary insulin antibody. Representative of n=4. B) PAR-1 mRNA expression in aortae from non-diabetic and diabetic wildtype mice 3 weeks after treatment with citrate solvent or STZ respectively (n=4). Data shows mean±SEM.
Supplementary Figure 9: A) Non-ligated right carotid arteries from diabetic (STZ-treated) and non-diabetic (citrate-treated) wildtype or PAR-4−/− mice, analysed by H&E-staining as negative controls for ligated left carotid arteries (4 weeks after surgery). Representative of n=6.
Supplementary Figure 10: A) Quantification of neointima area, B) media area and C) lumen area in ligated left carotid arteries 4 weeks after surgery. Shown is the area sum determined at 250 µm intervals up to 1000 µm proximal to the ligation site (all n=6). Shown are mean±SEM. *P<0.05 vs. non-diabetic wildtype, #P<0.001 vs. diabetic wildtype.
**Supplementary Figure 11.** A, B) Mac-2 (red) immunofluorescence in non-diabetic and diabetic ligated carotid arteries from wild-type and C, D) PAR-4−/− mice. Vessel structures are shown by autofluorescence (green) in the FITC channel. Nuclei are stained in blue.

Representative sections taken from n=5-6 individual mice. E) Macrophage count is depicted as % Mac-2 positive cells in relation to all cells counted in neointimal area. Data show mean±SEM. *P<0.05 vs. wild-type non-diabetic.