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High Glucose Regulation of Human Vascular Thrombin Receptors
- Focus on PAR-4 -

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Dedicated to Sri Guru-
'The Knowledge Absolute'

CONTENTS

ABBREVIATIONS

Ang-II	Angiotensin-II
BSA	Bovine serum albumin
cDNA	Complimentary DNA
CVD	Cardiovascular disease
DAB	Diamino benzidine
DAG	Diacylglycerol
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DPI	Diphenyliodinium chloride
DTT	Dithioerithritol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylen glycol tetraacetic acid
ERK	Extracellular regulated kinase
ETS	Electron transport system
FCS	Fetal calf serum
FITC	Fluorescent isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
IgG	Immunoglobulin
IHC	Immunohistochemistry
I-κB	Inhibitory-kappa B
JNK	c-Jun N- terminal kinase
kDa	Kilo Dalton
mAb	Monoclonal antibody
NAD(P)H	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor-kappa B
NP-40	Nonidate P-40 (octyl phenoxylpolyethoxylethanol)
PAGE	Polyacrylamide gel electrophoresis
PAR	Protease-activated receptor

PAR-AP	Protease-activated receptor-activating peptide
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonylfluoride
PKA	Protein kinase A
PKC	Protein kinase C
PVDF	Polyvinyliden fluoride
qRT- PCR	Quantitative realtime-PCR
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
SDS	Sodium dodecylsulphate
SEM	Standard error of mean
SMC	Smooth muscle cell
STAT	Signal transducer and activator of transduction
TBS	Tris buffered saline
TNF-α	Tumor necrosis factor-alpha
Tris	Tris (hydroxymethyl)-aminomethane
Tween-20	Polyoxyethylene (20) sorbitan monolaurate

1. INTRODUCTION

Constrictive vascular remodeling is a common cause of the clinical failure of coronary interventions such as percutaneous transluminal angioplasty or venous bypass grafting, in which vascular smooth muscle cells (SMC) play a pivotal role (Beckman et al. 2002). Neointimal formation after vascular injury resembles an inflammatory tissue-repair response involving vascular SMC proliferation, migration and inflammatory gene expression (Forrester et al. 1991). A central mediator of these processes is the clotting factor thrombin (activated factor II), generated when tissue factor-bearing vascular SMCs or fibroblasts come into contact with blood components. Immediate result of thrombin generation in response to vascular damage is blood clotting. However the majority (more than 95%) of total thrombin released is generated by the mural thrombus after completion of the clotting process, (Brummel et al. 2002) indicating an additional role for thrombin in vessel wall repair and remodeling (fig. 1.1). Subendothelial cells of the vascular wall such as vascular SMCs and fibroblasts are thus likely to be exposed to high levels of thrombin, especially in various pathological conditions associated with disturbed endothelial integrity. This likely plays an important role in the pathogenesis of atherosclerosis and remodeling of the vessel wall (Martorell et al. 2008).

Thrombin stimulates vascular SMC mitogenesis, matrix biosynthesis and expression of inflammatory genes, key processes leading to neointima formation *in-vivo* (Kranzhofer et al. 1996; McNamara et al. 1993). These coagulation independent actions of thrombin are mediated via a unique family of G-protein-coupled receptors, known as protease-activated receptors (PARs) (Coughlin 2000). PARs are involved in hemostasis, thrombosis and a variety of vascular responses to thrombin such as migration, cellular growth, proliferation and inflammatory reactions (Coughlin 2005; Hamilton et al. 2001). PARs are activated through proteolytic cleavage of the extracellular N-terminus, thereby unmasking a new N- terminus which acts as a tethered peptide ligand to initiate

transmembrane signaling by mobilization of intracellular calcium as a consequence of G-protein activation (Coughlin 2000).

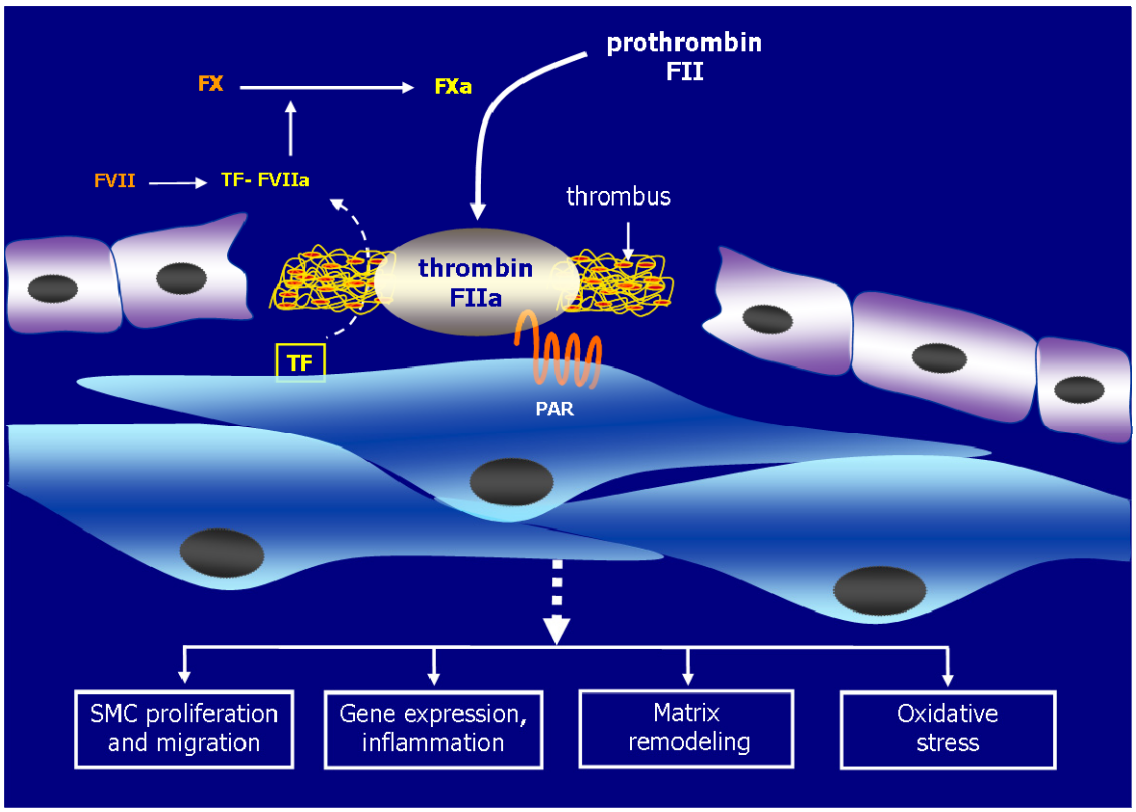

Figure 1.1 Schematic diagram showing haemostatic and cellular effects of thrombin in vasculature

Synthetic peptides corresponding to the tethered ligand domain reproduce most of the biological actions of thrombin independently of receptor cleavage (Hirano 2007). Activated PARs are rapidly uncoupled from signaling and internalized (Coughlin 2000; Hirano 2007), and their reappearance at the cell surface in part requires *de-novo* synthesis. Thus the vascular actions of thrombin are controlled to some extent by transcriptional regulation of PARs. The factors regulating thrombin receptor expression have only recently begun to be defined.

Of the four PARs identified so far, PAR-1, PAR-3 and PAR-4 are activated by thrombin. A further receptor, PAR-2, is activated by other proteases such as trypsin and coagulation factor Xa (Coughlin 2000). PAR-1 is the prototypical receptor to which most thrombin actions in platelets and the vasculature are attributed (Hirano et al. 2003; Wilcox et al. 1994). The role of PAR-1 in vascular remodeling is well described (Chen et al. 2008; Derian et al. 2002; Harker et al.

1995; Stouffer et al. 1996), and inhibition of PAR-1-mediated thrombin effects represents a primary hope for novel anti-restenotic therapeutics (Ahn et al. 2003). PAR-3 acts as a cofactor for PAR-4-induced activation of mouse, but not human platelets (Kahn et al. 1998). Its expression in the vascular SMCs has not been fully elucidated (Borissoff et al. 2009; Bretschneider et al. 2003; Martorell et al. 2008). PAR-4 is a low-affinity receptor with distinct on-off kinetics essential for the sustained platelet response to thrombin of both mouse and human platelets (Kahn et al. 1998; Shapiro et al. 2000), but the role of PAR-4 beyond platelets is poorly understood.

Our laboratory provided the first evidence that functionally active PAR-4 is expressed in human vascular SMCs (Bretschneider et al. 2001). As in platelets, PAR-4 mediates a delayed signaling response to thrombin in human vascular SMC, and is responsible for the second activation of the mitogen activated kinases ERK-1/2. PAR-4 thereby contributes to vascular SMC mitogenesis, and thus the net proliferative effects of thrombin in the vessel wall are likely to involve cooperation of both PAR-1 and PAR-4. Recently, PAR-4 was implicated in myocardial ischemia and reperfusion damage (Strande et al. 2008), cardiomyocyte hypertrophy (Sabri et al. 2003) and pulmonary fibrosis (Ando et al. 2007), and may thus be an appropriate therapeutic target to limit cardiovascular remodeling. Potentially, thrombin generated at the nearby lesion could exert feedback regulation of cellular receptors (Sokolova et al. 2005). PAR-3 and PAR-4 but not PAR-1, are dynamically regulated in response to thrombin in human saphenous vein SMC (Bretschneider et al. 2003). Interestingly, regulation of PAR-4 is more pronounced in human saphenous vein SMCs than in SMCs from mammary artery (K. Schrör, unpublished observations). This might reflect fundamental differences between different vascular beds, which could contribute to the increased failure rates of venous bypass grafts in comparison to arterial grafts (Yang et al. 1998). Thus individual PARs possess distinct properties (Table 1) (Coughlin 2000; Macfarlane et al. 2001; O'Brien et al. 2001; Schrör et al. 2010). Particularly PAR-4 may represent a unique link

between tissue damage and subsequent vascular remodeling. This is currently not systematically studied.

The development of PAR knockout mice has provided the unique opportunity to identify and characterize new members of this novel family of GPCRs, and thereby to evaluate the roles of individual PARs jointly expressed in common cells and tissues and explores their contribution to thrombosis, restenosis, vascular remodeling, angiogenesis and inflammation. Deletion of PAR-1 in mice led to embryonic lethality in some animals and fatal bleeding defects (Major et al. 2003). Curiously, embryonic lethality was not associated with deletion of PAR-4, suggesting perhaps a pathological rather than physiological role in contrast to PAR-1. Studies in murine cells and tissues to date are the only way to define the functional relevance of PARs in the clinical situations, but the differences in species makes extrapolation from mouse to human difficult (Hollenberg et al. 2002).

	PAR-1	**PAR-3**	**PAR-4**
Location	Platelets (human), fibroblasts, VSMC, endothelium , myocardium	Endothelium, megakaryocyte, platelets (mouse)	Platelets (human, mouse), VSMC, megakariocytes, cardiomyocytes
Chromosome	5q13	5q13	19p12
Tethered ligand	TFLLRN/ SFLLRN	TFRGAP	GYPGQV/ AYPGKF
Hirudin-related sequence	YEPFW	FEEFP	None
G-protein coupling	Gi, Gαq, Gα12, Gα13	not known	Gq
Activating enzymes	Thrombin, factor-Xa, granzyme A	Thrombin	Thrombin, trypsin, tryptase, cathepsin G
Affinity	High	High	Low
Thrombin regulation	No	Dynamic	↑
EC_{50} for thrombin	0.05 nmol/L	0.2 nmol/L	5 nmol/L

Table 1. Different properties of thrombin- responsive PARs in the vasculature. (Coughlin 2000; Macfarlane et al. 2001; O'Brien et al. 2001; Schrör et al. 2010).

Potentially thrombin may also serve a connecting link between diabetes and cardiovascular disease. Excessive thrombin generation is strongly associated with diabetes (Undas et al. 2008), one of the major causes of morbidity and mortality in developed countries (Donnelly et al. 2000). A diagnosis of diabetes immediately increases the risk of developing various irreversible clinical complications such as micro- or macro-vascular disorders, which account for 50-70% of all diabetes fatalities, and disabilities (Morrish et al. 2001). In particular type II diabetes predisposes to higher risk to have a heart attack, atherosclerosis (two to four times) and venous graft failure compared to people without diabetes (Ahmed et al. 2000; Haffner et al. 1998). Thrombin plays a central role in hypercoagulation, thrombosis, vascular remodeling and atherosclerosis, all of which are pathological hallmarks of diabetes (Beckman et al. 2002; Undas et al. 2008). Thus increased generation of thrombin in diabetes may underlie the high prevalence of cardiovascular risk in diabetic patients.

Hyperglycemia is an independent risk factor for the development of micro- and macro-vascular complications of diabetes (Ceriello 2005; Laakso 1999; Sheetz et al. 2002). Elevated glucose levels activate protein kinase C (PKC) through *de-novo* synthesis of diacylglycerol (DAG) (Koya et al. 1998; Srivastava 2002). Extensive data support central role of vascular PKC in high glucose regulation of gene expression, cellular proliferation, hypertrophy, inflammation and oxidative stress leading to development of vascular complications associated with diabetes (fig. 1.2) (Busuttil et al. 1996; Ceriello et al. 1995; Dragomir et al. 2008; Inoguchi et al. 2003; Itoh et al. 2001). Human saphenous vein SMCs express PKC-α, -β, -δ, -ε, -μ, -λ and -ζ isozymes (Itoh et al. 2001) of which high glucose is reported to preferentially activate PKC-β or PKC-δ (Koya & King 1998). High glucose stimulated PKC can activate downstream effectors like NAD(P)H oxidase and many transcription factors such as NF-κB, Jnk, cFos and STAT etc. (Hashim et al. 2004; Hattori et al. 2000; Srivastava 2002). These effectors are critical factors for the development of vascular diseases, atherothrombosis and inflammation in diabetes (Hattori et al. 2000; Inoguchi et al. 2003; Schubl et al. 2009). High glucose may also alter membrane integrity of

cells via its osmolar effects, leading to deleterious cellular effects of high glucose.

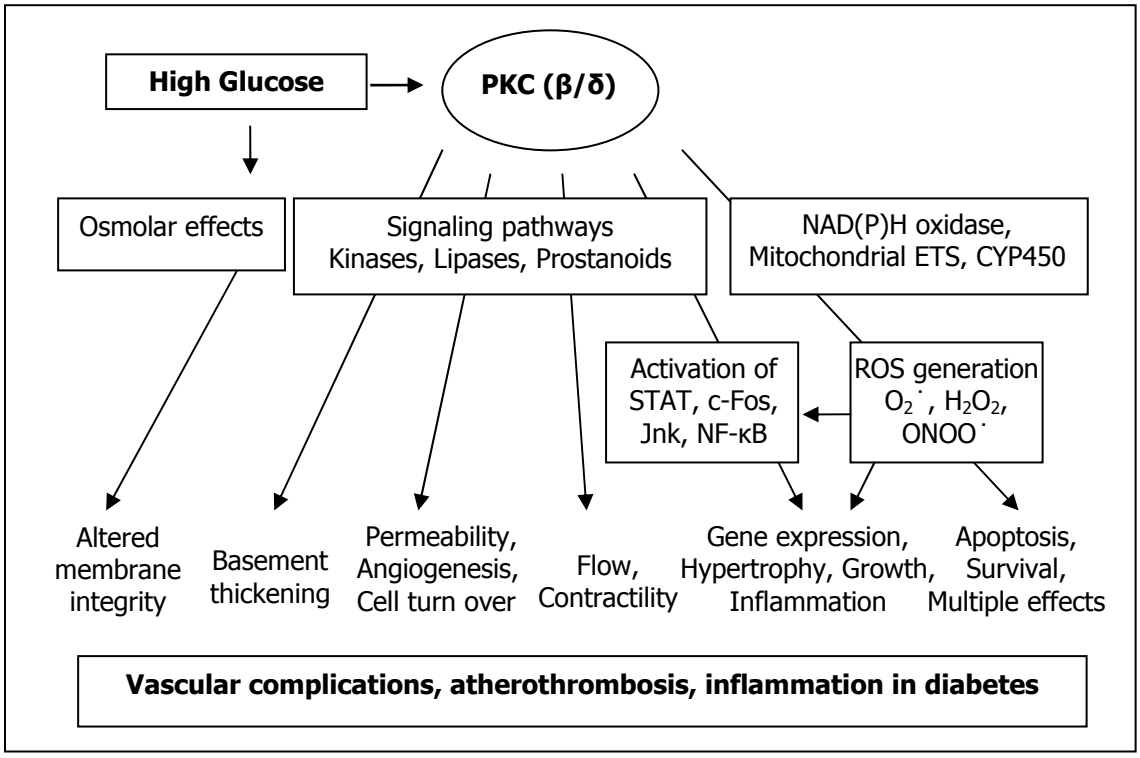

Figure 1.2 Simplified illustration of various deleterious effects of high glucose and its effectors in vasculature (Hattori et al. 2000; Inoguchi et al. 2003; Koya & King 1998; Schubl et al. 2009; Srivastava 2002).

One central target downstream of PKC is nuclear factor-kappa B (NF-κB), which plays an important role in high glucose regulation of gene transcription (Dragomir et al. 2008; Hattori et al. 2000). Activated NF-κB has been detected in SMCs of carotid artery after balloon injury (Landry et al. 1997) and in the intima and media of atherosclerotic vessel sections (Wilson et al. 2002), suggesting its role in development of atherosclerosis. NF-κB is an inducible dimeric transcription factor composed of members of the Rel family of DNA-binding proteins that recognize a common sequence motif. The Rel proteins differ in their abilities to activate transcription, such that only p65/RelA and c-Rel were found to contain potent transcriptional-activation domains among the mammalian family members. The p65:p50 heterodimers were the first form of NF-κB to be identified and are the most abundant in most cell types.

Consequently, the term NF-κB most often is used to describe the p50:p65 complex (Karin et al. 2000).

Currently, it appears that all NF-κB complexes are regulated in the same manner—primarily through interactions with inhibitory-kappa B (I-κB). All of these proteins share a highly conserved 300-amino-acid Rel homology region (RHR), which is responsible for dimerization, DNA binding, and interaction with I-κB proteins (Chen et al. 1998). These also contain a nuclear localization sequence (NLS). Different NF-κB dimers exhibit different binding affinities for κB sites bearing the consensus sequence GGGRNNYYCC, where R is purine, Y is pyrimidine, and N is any base (Miyamoto et al. 1995). NFκB is normally sequestered in the cytoplasm of unstimulated cells as an inactive trimeric complex (p50:p65:I-κB). In classical NF-κB signaling pathway (fig. 1.3) cellular activation by agonists such as IL-1, TNF-α or LPS activates an I-κB kinase complex to phosphorylate I-κB proteins and subsequent polyubiquitination and proteosomal degradation of I-κB leads to the translocation of free NF-κB into the nucleus, where it binds to NF-κB recognition site of DNA to regulate gene transcription (Gilmore 2006; Karin & Ben-Neriah 2000).

Beside activation of deleterious signaling cascade, diabetes represents a state of compromised oxidative defense in human cells (Mooradian 2006). Activation of NAD(P)H oxidase through PKC is implicated in oxidative stress associated with hyperglycemia (Gao et al. 2009; Rask-Madsen et al. 2005). NAD(P)H oxidase catalyses the transfer of electron from NADPH to molecular oxygen via their catalytic subunits to generate superoxide and hydrogen peroxide (H_2O_2) (Thomas et al. 2008). Enhanced oxidative stress can activate various signaling targets such as NF-κB and MAPKs, which in turn promote mitogenic, survival and apoptotic responses in vascular cells (Irani 2000). Reactive oxygen species (ROS) play a key role in various pathological settings including diabetes and inflammation (Inoguchi et al. 2003; Madamanchi et al. 2005). Moreover

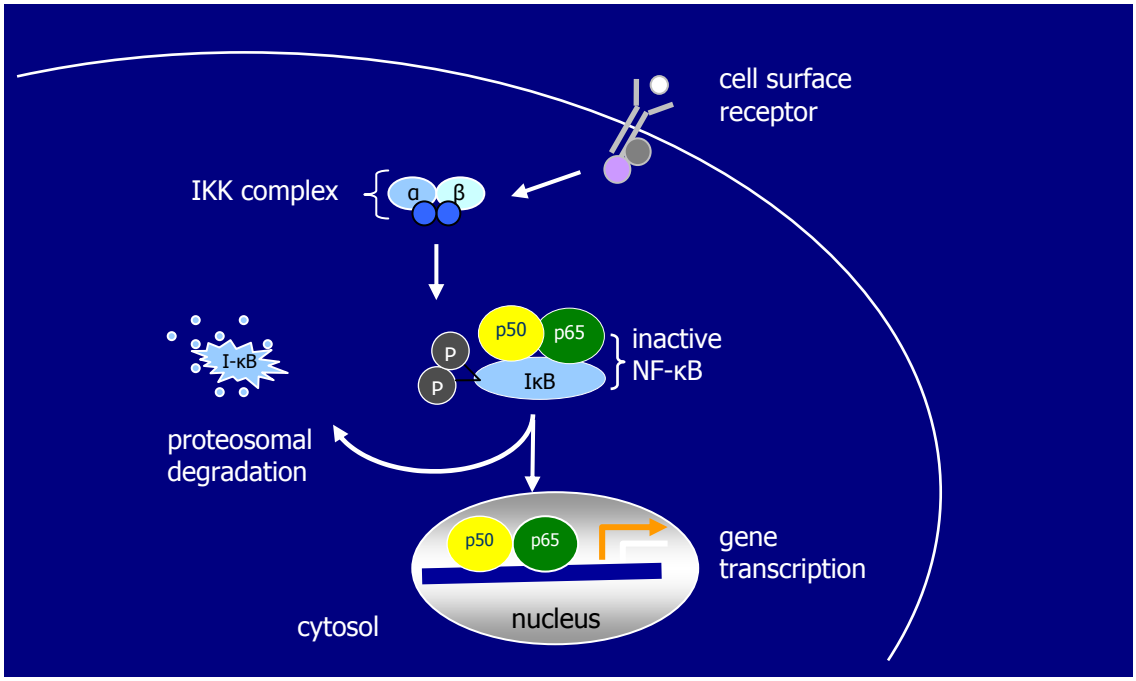

Figure 1.3 A Classical NF-κB signal transduction pathway. NF-κB homo- or hetero- dimers such as p50/p65 are maintained in the cytoplasm by interaction with an independent inhibitor κB (I-κB) molecule (often I-κBα) to form inactive trimers. In many cases, the binding of a ligand to a cell surface receptor e.g. tumor necrosis factor-receptor (TNF-R) recruits an inhibitory κB kinase (IKK) complex, containing two molecules of the regulatory scaffold and α and β catalytic subunits. IKK phosphorylates I-κB at two serine residues, which then undergoes ubiquitination and subsequent proteosomal degradation in cytosol, setting free the NF-κB-dimer. This active NF-κB-dimer then enters the nucleus to turn on target genes (Gilmore 2006).

hyperglycemia induced oxidative stress, via NAD(P)H oxidase, enhances thrombin formation in diabetes (Ceriello et al. 1995) and PAR-1 may be regulated in redox dependent manner, but it is not known if other thrombin receptors are also be regulated in this manner (Capers et al. 1997; Nguyen et al. 2001).

In summary, diabetes is associated with enhanced thrombin generation, modest chronic inflammation, increased rates of vascular proliferation and oxidative stress leading to vascular remodeling, restenosis and bypass graft failure (Ceriello et al. 1995; Heidland et al. 2001; Undas et al. 2008). Thrombin plays a central role in all of these vascular pathologies. Moreover, both thrombin and high glucose activate PKC and redox dependent signaling pathways to induce vascular SMC migration, proliferation and matrix biosynthesis (Galis et al. 1997; Maruyama et al. 1997). Thus high glucose and thrombin may interact functionally to modulate the migratory or proliferative behaviour of vascular

SMCs. Such cooperation may possibly start at the level of thrombin receptor regulation, which may serve as deciding control point of diabetes, particularly if regulated by high glucose and could underlie the high rates of atherosclerosis and vein graft failure in diabetes. Improved understanding of the role and regulation of thrombin receptors could identify these as appropriate targets for novel anti-restenotic therapies, especially in diabetic patients.

Broad aims and questions to address:

1. Does high glucose modulate expression of thrombin receptors (PARs) in human vascular SMCs?
2. Does high glucose alter the signaling and functional responses to PAR-activation?
3. By which mechanism does high glucose produce these effects and which cell signaling intermediates are involved?
4. Are these processes relevant to the clinical setting?

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Drugs/ stimuli

Angiotensin-II (100 nmol/L)	Growth factor	Sigma- Aldrich, Deisenhofen, Germany
Apocynin (100 µmol/L)	NAD(P)H oxidase inhibitor	Sigma-Aldrich, Schnelldorf, Germany
Bovine α-thrombin (3 NIH Units/mL)	Serine protease	late Dr. J. Stürzebecher, Zentrum für Vaskuläre Biologie und Medizin, Jena, Germany
Calphostin C (200 nmol/L)	Nonspecific PKC inhibitor	Enzo Lifescience GmbH, Lörrach, Germany
Diphenyliodinium chloride (10 µmol/L)	NAD(P)H oxidase inhibitor	Sigma-Aldrich, Schnelldorf, Germany
D-glucose (25 mmol/L)	Hyperglycemic stimulus	Calbiochem, San Diego, CA
H_2O_2 (100 µmol/L)	Oxidant	Merck, Darmstadt, Germany
Mannitol (19.5 mmol/L+ 5.5 mmol/L glucose)	Osmolar control	Calbiochem, San Diego, CA
NF-κB activation inhibitor (100 nmol/L)	NF-κB inhibitor	Calbiochem, San Diego, CA
PAR-1AP (TFLLRN) (20- 100 µmol/L)	Synthetic hexapeptide PAR-1 agonist	Biosyntan, Berlin, Germany
PAR-4AP (AYPGKF) (200- 400 µmol/L)	Synthetic hexapeptide PAR-4 agonist	Biosyntan, Berlin, Germany
PD-98059 (20 µmol/L)	ERK inhibitor	Calbiochem, San Diego, CA

PKC-β inhibitor (50 nmol/L)	PKC-β isozyme inhibitor	Calbiochem, San Diego, CA
Rottlerin (1 µmol/L)	PKC-δ isozyme inhibitor	Biotrend Chemicals AG, Zürich, Switzerland
Staurosporine (1 µmol/L)	General PKC inhibitor	Calbiochem, San Diego, CA
U-73122 (10 µmol/L)	PLC inhibitor	Calbiochem, San Diego, CA
Y-27632 (10 µmol/L)	ROCK inhibitor	Calbiochem, San Diego, CA

2.1.2. Antibodies

Human PAR-4 (sc-6420) (goat pAb)	Santa Cruz Biotechnology (SCBT) Santacruz, CA, USA	1:200
Human PAR-4 (M1-clone) (mouse mAb)	Abnova, Heidelberg	1:200
Human PAR-4 (M1-clone) (mouse mAb)	Sigma Aldrich, Munich Germany	1:200
FITC conjugated PAR-4 extracellular N-terminal (rabbit pAb for FACS)	Alomone labs, Israel	1 µg (1:50)
FITC conjugated rabbit IgG (Isotype control for FACS)	SCBT, Santacruz, CA, USA	1 µg (1:50)
β-actin (mouse mAb)	Sigma, Schnelldorf, Germany	1:50,000
NF-κB p65 (goat pAb)	SCBT, Santacruz, CA, USA	1:200
NF-κB p65 (rabbit mAb)	Cell signaling	1:200
Phospho I-κBα (mouse mAb)	SCBT, Santacruz, CA, USA	1:200
Total I-κBα (mouse mAb)	SCBT, Santacruz, CA, USA	1:200
PKC-δ (rabbit pAb)	Cell Signaling, Danvers, MA	1:200
SM-actin (mouse mAb for IHC, ICC)	DAKO, Hamburg, Germany	1:10
Donkey anti-goat IgG, HRP conjugated	SCBT, Santacruz, CA, USA	1:3000
Goat anti-mouse IgG, HRP conjugated	SCBT, Santacruz, CA, USA	1:3000
Goat anti-rabbit IgG, HRP conjugated	SCBT, Santacruz, CA, USA	1:3000
Goat anti-mouse IgG, alkaline phosphatase conjugated	Abcam, UK	1:40

2.1.3. Buffers and Solutions

All reagents used were of high grade quality from either Merck (Darmstadt, Germany) or Sigma (Deisenhofen, Germany) unless otherwise stated.

Blotting buffer (1X)	190 mmol/L glycine, 25 mmol/L Tris, 20% (v/v) methanol
Buffer A (hypotonic homogenisation buffer) 100 mL Store at 4°C	10 mmol/L HEPES (pH 7.5) 10 mmol/L KCl 0.1 mmol/L EDTA 0.1 mmol/L EGTA + 1 tablet protease inhibitor Makeup volume with dH_2O Immediately prior to use + 1 mmol/L DTT + 1% PMSF (add IMMEDIATELY prior to use)
Buffer C (nuclear extract buffer) 100 mL, store at 4°C	20 mmol/L HEPES (pH 7.5) 25% glycerol 0.4 mol/L NaCl 1 mmol/L EDTA 1 mmol/L EGTA Makeup volume with dH_2O + 1 tablet protease inhibitor 1 mmol/L DTT+ 1% PMSF (add IMMEDIATELY prior to use)
Cell lysis buffer: Laemmli Buffer (2X)	125 mmol/L $Na_{2\,h}PO_4/NaH_2PO_4$ (pH 7.0), 100 mmol/L DTT, 20% (v/v) Glycerol, 4% (w/v) SDS, 0.002% bromophenol blue
Citric saline (10X), Autoclave and store at 4°C	1.35 mol/L potassium chloride, 0.15 mol/L sodium citrate

Complete DMEM	DMEM 500 mL (glucose 5.5 mmol/L), 15 % (v/v) FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1.9 mmol/L L-glutamine, 9.6 mmol/L sodium pyruvate
HEPES Buffer	HEPES 10 mmol/L (pH 7.4), NaCl 145 mmol/L, $Na_{2\,h}PO_4$ 0.5 mmol/L, glucose 5.5 mmol/L, $MgSO_4$ 1 mmol/L and $CaCl_2$ 1.5 mmol/L
PBS/EDTA	0.1 mmol/L EDTA in PBS
Resolving Gel (10%) (2 gels)	1.5 mol/L Tris HCL pH 8.8 2.5 mL 10% SDS 100 µL dH_2O 4.0 ml 30%Acrylamide/Bis-acrylamide (37.5:1) 3.3 ml TEMED 4 µL APS 10% (0.1 g/ml) 100 µL
Running buffer (1X)	190 mmol/L Glycine, 25 mmol/L Tris, 0.1% (m/v) SDS
Stacking Gel (5%) (2 gels)	1 mol/L Tris HCL pH 6.8 0.75 mL 10% SDS 60 µL dH_2O 4.1 ml 30% Acrylamide/Bis-acrylamide (37.5:1) 1.0 ml TEMED 6 µL APS 10% (0.1 g/ml) 60 µL

TBS (10X)	1.5 mol/L NaCl, 100 mmol/L Tris/HCl pH 7.4
TBS-T (0.1%)	0.1% (v/v) Tween-20 in 1X TBS
TBS-TB (5%)	5% (w/v) BSA in TBS-T

2.1.4. Kits and reagents

BAC DNA isolation kit	Princeton Separations, Philadelphia, PA
cDNA reverse transcription kit	Applied Biosystems, Darmstadt, Germany
DAB substrate kit	Zytomed Systems, Berlin, Germany
DMEM	Gibco BRL, Rockville, MD, USA
Fast red kit	Thermo Scientific, Germany
Hoechst-33342	Invitrogen, Karlsruhe, Germany
Immobilon kit	Millipore, Schwalbach, Germany
Lipofectamine2000®	Invitrogen, Karlsruhe, Germany
Luciferase assay system, leuciferin	Promega, Mannheim, Germany
Mayer's hemalaun solution	Merck, Darmstadt, Germany
Midi-prep kits	Qiagen, Hilden, Germany
QuantiTect primer assays	Qiagen, Hilden, Germany
Sensi-mix SYBR® green reagent	Quantace, London, UK

2.1.5. Apparatus

7300 realtime PCR system	Applied Biosystems, Germany
BioRad mini gel electrophoresis set	BioRad, München, Germany
BioRad GelDoc 8	BioRad, München, Germany
IX-50, IX-70, BX-50 (4X, 10X, 20X objectives)	Olympus, Germany
Nanodrop spectrophotometer	Peqlab Biotechnologie GmbH, Germany
Back-illuminated EMCCD camera	iXon^{EM}+897, Andor, Connecticut, USA
Argon/Krypton ion laser (488nM)	Stabilite 2017, Newport Spectra Physics GmbH, Darmstadt, Germany
Thermo-cycler	Eppendorf, Hamburg, Germany
Tabletop centrifuge	Eppendorf, Hamburg, Germany
EPIC- XL cytometer	Backman Coulter, Germany

2.1.6. Softwares

7300 system sequence detection software (SDS)	Applied Biosystems, Germany
Quantity One	Bio-Rad Laboratories, Inc.
imageJ software, NIH, USA	http://rsbweb.nih.gov/ij
Genomatix software	http://www.genomatix.de/
Transfac, PATCH	http://www.gene-regulation.com/
SECentral clone manager 5	Scientific and Educational Software, NC
Endnote X	Thomson Reuters Inc., http://www.endnote.com/
System II (30) software	Backman Coulter, Germany

2.2. Methods

2.2.1. Cell culture and incubations

Human saphenous vein specimens were obtained through the Department of Cardiac Surgery at the University Hospital Düsseldorf with approval of the Human Ethics Commission of the Medical Faculty of the Heinrich-Heine Universität Düsseldorf and informed consent of donors. Vascular SMCs were isolated by the explant technique after Faillier-Becker (Fallier-Becker et al. 1990). In brief, vessels were opened longitudinally and kept immersed in DMEM. After mechanical removal of the endothelium with gentle scraping, the medial layer was carefully removed from the adventitia and cut into about 1 mm segments. These were placed in 6-well culture plates and incubated in complete DMEM containing 5.5 mmol/L glucose (GibcoBRL, Rockville, MD, USA) at 37 °C and 5% CO_2. Medium was changed every 48 h. Within 1-2 weeks SMCs grew out from the medial explants and proliferated. Vascular SMC phenotype was confirmed in primary cultures by typical hill and valley appearance and staining for SM-actin. Upon reaching confluence, cells were subcultured by detachment with TE (trypsin-EDTA) only upto passage 4 or citric saline (5 min) and adding complete DMEM. Cells were collected by centrifugation (900 rpm, 5 min). SMCs were resuspended in fresh complete DMEM prior to seeding into tissue culture plates. Vascular SMCs at passage 4-10 were used for experiments and were synchronized by serum-deprivation (48 h) prior to stimulation with various stimuli in presence or absence of high glucose.

2.2.2. Quantitative real-time PCR

Gene expression was analyzed by quantitative real-time PCR (qRT-PCR) as described (Rosenkranz et al. 2009). Total RNA was extracted from vascular SMCs using Tri® Reagent (Sigma-Aldrich, München, Germany) as instructed by the manufacturer. RNA concentration and purity were determined measuring

absorption at 260 nm and 280 nm using nanodrop® spectrophotometer (Peqlab Biotechnologie GmbH, Darmstadt, Germany).

For realtime-PCR, 0.5-1 µg RNA was reverse-transcribed with the cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) according to manufacturers' instructions. Target gene mRNA expression levels were determined by real-time PCR using Sensi-mix SYBR® Green Reagent (Quantace, London, UK) and QuantiTect Primer Assays (Qiagen, Hilden, Germany) in the 7300 Real Time PCR System (Applied Biosystems) according to manufacturers' instructions. Target gene expression levels were normalized to GAPDH using the ΔΔCt method (Winer et al. 1999), and effects of treatment expressed as fold change vs control. GAPDH mRNA expression was not changed over the entire high glucose time course and thus served as a suitable house keeping gene in human vascular SMC.

2.2.3. Immunoblotting

Protein expression levels were determined by Western blotting (Rauch et al. 2002). Cells were lysed in Laemmli buffer, denatured at 95 °C for 5 min and sonicated for 3 sec and then cleared by centrifugation at 13,200 rpm for 1 min. Proteins were loaded onto 10% SDS-ploy acrylamide gels and separated in electrophoresis chamber (BioRad, München, Germany) using 1X running buffer at 160 V for 60- 90 min. A prestained protein ladder (~10- 130 kDa, Fermentas, St. Leon-Rot, Germany) was used as molecular weight marker. Separated proteins were transferred to PVDF membrane (Millipore, Bedford, MA, USA) in a semi-dry blotting chamber (Bio-Rad, München, Germany) saturated with blotting buffer. Non-specific binding was blocked for 2 h at room temperature to 12 h at 4 °C with 5% bovine serum albumin (BSA) in TBS-T, followed by overnight incubation at 4 °C with specific primary antibodies. This was followed by incubation with respective horseradish peroxidase-coupled secondary antibodies (1:3000, 1 h at room temperature (RT); Santa Cruz

Biotechnology, CA, USA). After washing 3x10 min in TBS-T, bands were visualized by enhanced chemiluminescence (Immobilon kit, Millipore, Schwalbach, Germany) as instructed by the manufacturer and quantified by densitometry (BioRad GelDoc8, QuantityOne software). Expression was normalized to β-actin (mouse anti-human, Sigma, Schnelldorf, Germany) after stripping (0.2N NaOH, 5 min) and reprobing of membranes.

2.2.4. Immunocytochemistry

Subconfluent human saphenous vein SMCs seeded on 10 mm glass coverslips were stimulated as indicated and fixed in paraformaldehyde (4% in PBS) for 20 min at RT, then permeabilized in 0.1% Triton X-100 for 5 min at RT. Cells were incubated with primary goat anti-PAR-4 antibody (1:100, Santa Cruz Biotechnology, Heidelberg, Germany) overnight at 4 °C followed by washing with PBS (3x5 min) and then incubation with FITC conjugated secondary anti-goat antibody (1:400, Santa Cruz Biotechnology) for 1 h at room temperature in the dark. Cells were again washed 3x10 min PBS (3x5 min) followed by nuclear staining with Hoechst-33342 (Invitrogen, Karlsruhe, Germany) for 20-30 sec and again washing with PBS (3x5 min). Fluorescent images were immediately captured by Colorview II camera and Soft Imaging System connected to an Olympus BX50 microscope (Hamburg, Germany).

2.2.5. Fluorescence cytometry

For analysis of surface expression of PAR-4, SMCs were seeded in 6-well plates and stimulated as indicated. After non-enzymatic detachment with citric saline buffer for 5-10 minutes at 37 °C, cell suspensions were centrifuged and resulted pellets were resuspended in PBS. Cell suspensions (50 µL) were incubated with 1 µL FITC-conjugated anti-human PAR-4 extracellular N-terminal antibody

(Almone Labs, Israel) for 45 min- 1 h at 4 °C in the dark. Isotype-matched FITC-conjugated antibody was used to assess nonspecific binding. Samples were diluted with 500 µL isotone and immediately analyzed on an EPIC-XL cytometer (Beckman Coulter). SMC populations were identified according to forward and side scatter distributions. Detectors were set to logarithmic amplification and fluorescence was measured in 5000 cells using the System II (3.0) software. For quantification, the ratios of the mean fluorescence signals of PAR-4 and nonspecific IgG-stained cells were normalized to the unstimulated control.

2.2.6. Luciferase reporter assay

PAR-1, PAR-3 and PAR-4 luciferase reporter vectors were utilized to access thrombin receptor promoter activities. Human PAR-promoter vectors were constructed as described (Rosenkranz et al. 2009). The human PAR-3 and PAR-4 genomic clones RZPDB737C121013D and pBeloBAC11 (RZPD, Heidelberg, Germany) were obtained to construct PAR-3 and PAR-4 promoter vectors respectively. DNA was isolated using the big BAC DNA Isolation Kit (Princeton Separations, Philadelphia, PA, USA). An 8271bp fragment containing the PAR-3 promoter or 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. A final 3467 bp *KpnI/Esp3I* fragment of PAR-3 promoter or 2617 bp *SmaI* fragment of PAR-4 promoter was ligated into the pGL3basic luciferase reporter vector (Promega, Mannheim, Germany).

These constructs as well as empty pGL3 basic vector were transfected into saphenous vein SMCs using Lipofectamine2000® (Invitrogen, Karlsruhe, Germany) as per manufacturer's instructions. Stimuli were added 24 h post-transfection for indicated times and cell lysates were collected to measure luciferase reporter activity using the Luciferase Assay System (Promega,

Mannheim, Germany). The mean of at least three replicates per treatment group was taken for each experiment.

2.2.7. siRNA-mediated gene silencing

Subconfluent human saphenous vein SMCs were transfected with 40 nmol/L of Signalsilence™ NF-κB p65 siRNA (Cell Signaling Technology, Frankfurt, Germany), PAR-4, PKC-δ siRNA or control siRNA (Santa Cruz Biotechnology) using Lipofactamine2000® (Invitrogen). Transfection was validated by western blotting for NF-κB p65, PKC-δ proteins or by qRT-PCR for PAR-4 mRNA. Cells were stimulated 48 h post-transfection with various stimuli as indicated in different procedures.

2.2.8. Cell fractionation and NF-κB translocation study

Accumulation of nuclear NF-κB p65 and cytosolic phospho- I-κBα was determined by western blotting as a measure of NF-κB activation. Saphenous vein SMCs were stimulated with high glucose for indicated times prior to extraction of cellular fractions as described (Rauch et al. 2000). For collection of cytosolic and nuclear fractions, SMCs were washed in PBS and detached with EDTA (0.1 mmol/L in PBS). Cells were then scraped, centrifuged (2 min, 6000 rpm) and resuspended in 200 µL of ice-cold hypotonic homogenization buffer 'Buffer A'. After being allowed to swell on ice for 5-20 min, cells were sheared with a small-gauge needle upon addition of 1% NP-40 and centrifuged for 10 min, 4 °C at 14,000 rpm. Resulting supernatants were taken as cytosolic fractions and the pellets were resuspended in 50 µL 'Buffer C'. Nuclear NF-κB p65 or cytosolic phospho- and total I-κBα were determined by western blotting using mouse monoclonal anti-human phospho- I-κBα, total I-κBα or NF-κB p65 antibodies.

2.2.9. Chromatin Immunoprecipitation (ChIP) assay

Specific binding of NF-κB to the PAR-4 promoter was investigated in saphenous vein SMCs stimulated with high glucose (3 h) in the presence or absence of staurosporine (1 μmol/L) by a modified ChIP assay essentially as described (Rosenkranz et al. 2009). SMCs (2×10^6 cells), stimulated with high glucose for 3 h in presence or absence of staurosporine (1 μmol/L, added 30 min prior to high glucose), were fixed with 1.5% formaldehyde at RT for 20 min. Cross-linking was stopped by adding 0.125 mol/L glycine. Cells were scraped, collected and centrifuged. Resulting pellets were hypotonically lysed and nuclei were collected by centrifugation. After sonication, chromatin was precleared with Protein G PLUS-Agarose (Santa Cruz Biotechnology, CA, USA) and immunoprecipitated with NF-κB p65 antibody (4 °C, overnight). Protein–antibody complexes were collected by addition of Protein G PLUS-Agarose for 6 h, and the beads were extensively washed. Protein–DNA cross-links were eluted, reversed and treated with proteinase K. DNA was purified by phenol/chloroform/isoamylalcohol extraction, and finally precipitated with ethanol.

PCR with primers (5'-GAGAACAGTGGCTGCAGATG-3' (forward) and 5'-GGAGACTGGAGTGTGGGT-3' (reverse) covering the NF-κB binding site amplified a 212 bp region of human PAR-4 promoter. Negative control primers, binding to the 3′ UTR region of GAPDH were:

5′-ATGGTTGCCACTGGGGATCT-3′ (forward),

5′-TGCCAAAGCCTAGGGGAAGA-3′ (reverse) (Invitrogen, Germany)

These primers amplified a 174 bp region of genomic DNA between the GAPDH gene and the CNAP1 gene. Cycler conditions were 5 cycles of 94 °C/ 30sec, 72 °C/ 60sec; 5 cycles of 94 °C/ 30sec, 70 °C/ 30sec, 72 °C/ 60sec then 32 cycles 94 °C/ 30sec, 58 °C/ 30sec, 72 °C/ 60sec; and then 72 °C/ 15min. PCR products

were resolved on a 1.8% agarose/ ethidiumbromide gel and were visualized under UV light.

2.2.10. Intracellular calcium measurements

Calcium release from intracellular stores $[Ca^{2+}]_i$ was measured as described (Bretschneider et al. 2001) with some modifications. Subconfluent saphenous vein SMCs seeded on 10 mm glass coverslips were serum-deprived for 24 h and then pretreated ± high glucose for 48 h. After washing twice with HEPES buffer cells were loaded with the calcium-sensitive fluorescent dye Fluo-4 acetoxymethyl ester (10 µmol/L, Invitrogen) in HEPES buffer for 30 min at room temperature (RT). Cells were again washed and kept immersed in fresh HEPES buffer. Transient $[Ca^{2+}]_i$ release induced by thrombin or receptor agonist peptide was observed by recording the fluorescence changes of Fluo-4 upon Ca^{2+} binding at 1 Hz with back-illuminated EMCCD camera (iXon^{EM}+897, Andor, Connecticut, USA) attached to an inverted fluorescence microscope (Olympus IX-70, 20X objective). Fluo-4 was excited with a 488 nm laser beam from an Argon/Krypton ion laser (Stabilite 2017, Newport Spectra Physics GmbH, Darmstadt, Germany) and the emitted light was passed through a dichroic mirror and a long pass filter (505DLRPXR and 500ALP, Omega Filters, Brattleboro, VT) before detection. Image sequences were analyzed using imageJ software.

2.2.11. Migration assay

Migration was studied by wound-scratch assay as described (Weber et al. 2000). In brief synchronized SMCs were stimulated with high (25 mmol/L) glucose or the osmolar control mannitol (19.5 mmol/L mannitol added to DMEM containing 5.5 mmol/L glucose) for 48 h. A 1 mm cleft was scratched into cell

monolayer with a sterile pipette tip and medium was replaced with serum-free DMEM containing hydroxyurea (5 mmol/L) to prevent proliferation. Cells were stimulated with thrombin (3 U/mL) or activating peptides for PAR-1 (100 µmol/L) or PAR-4 (400 µmol/L). Migration into the cleft was monitored daily upto 72 h. Cells were then fixed with ice-cold methanol for 5 min at RT and washed thrice with PBS. Images were taken with a Colorview-II camera and Soft Imaging System connected to an Olympus IX50 microscope at multiple sites along the cleft. Data were analyzed using ImageJ software by counting the number of cells migrated to the cleft.

2.2.12. Immunohistochemistry

PAR-4 immunostaining was performed on paraffin embedded sections of healthy human artery and human diabetic plaque specimens 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 thick) were deparaffinized in xylene, rehydrated in ethanol and washed with PBS. Endogenous peroxidase activity was quenched with H_2O_2 (3% in methanol) for 20 minutes at RT. Antigen retrieval was performed in 0.1 mmol/L sodium citrate with 0.1 mol/L citric acid (pH 6) at 96 °C for 20 minutes. Sections were blocked at RT with 10% FCS/ 1% BSA for 60 min. Goat polyclonal PAR-4 antibody (8 µg/mL ie 1:50 in 1% BSA/ PBS, SantaCruz Biotechnology, USA) was applied overnight at 4 °C and visualized with HRP-conjugated secondary antibodies (1:400 in PBS) and the DAB Substrate Kit (Zytomed Systems, Berlin, Germany). Actin staining utilized a primary mouse monoclonal m-actin antibody (1:10, Dako, Hamburg, Germany) and alkaline phosphatase-conjugated secondary antibody (1:40, Abcam, UK) with final detection by Fast Red (Thermo Scientific, Germany). Nuclei were stained with Mayer's haemalaun solution (Merck, Darmstadt, Germany). Nonspecific isotype-matched IgG were used to control non-specific staining.

Images were taken with a Colorview-II camera and Soft Imaging System connected to an Olympus BX50 microscope.

2.3. STATISTICAL ANALYSIS

Data are expressed as mean± S.E.M of at least three different experiments and normalized to untreated controls. Statistical analysis utilized one-way analysis of variance (ANOVA) applied with Bonferroni's post-hoc multiple comparison procedure. p value ≤0.05 was accepted as significant.

3. RESULTS

3.1. Regulation of thrombin receptors by high glucose in human vascular SMC

3.1.1. Thrombin receptor mRNA Expression

High glucose selectively enhances PAR-4 mRNA in human saphenous vein SMC

Expression of all thrombin receptors has been previously reported in human vascular SMC (Bretschneider et al. 2001; Chaikof et al. 1995), but the potential regulation of these receptors by high glucose is not known. In human saphenous vein SMCs constitutive PAR-1 or PAR-3 expression was not significantly altered by high glucose either over short or long-term incubation (fig 3.1). By contrast PAR-4 mRNA was rapidly upregulated, significantly to 3.2±07 fold by 1.5h, and an approximately 2-fold induction was sustained to 96 h (fig. 3.1; n=7, $p<0.05$).

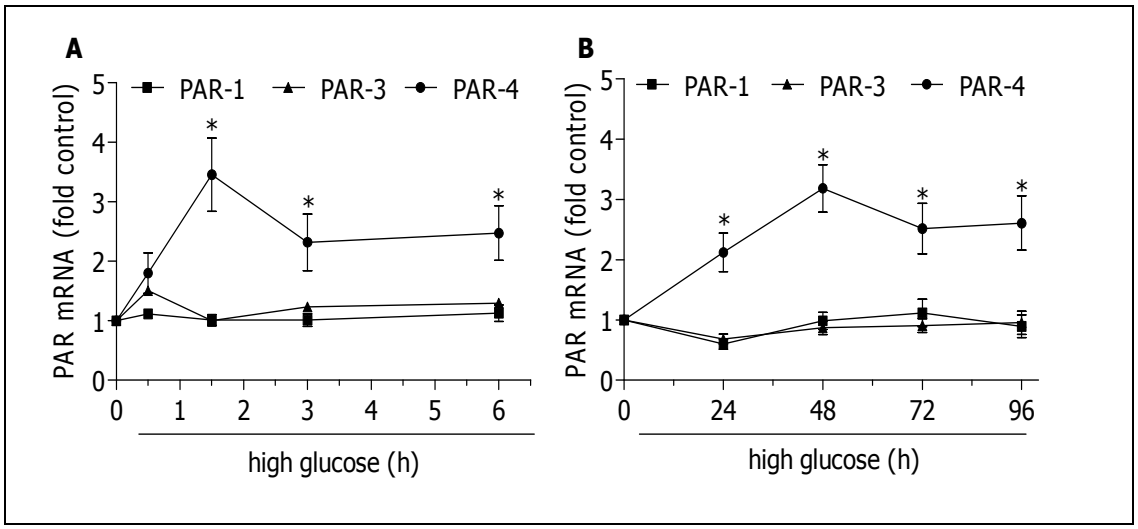

Figure 3.1 Influence of high glucose (25 mmol/L) on PAR-1, PAR-3 and PAR-4 mRNA after **(A)** short or **(B)** long term treatment in human saphenous vein SMC measured by qRT-PCR and normalized to GAPDH (n=7, *$p<0.05$ vs unstimulated normal glucose control).

The osmolar control mannitol did not influence PAR-4 mRNA expression after 6 to 96 h treatment (n=3, fig 3.2), indicating the regulatory effects of high glucose are not due to changes in osmolarity.

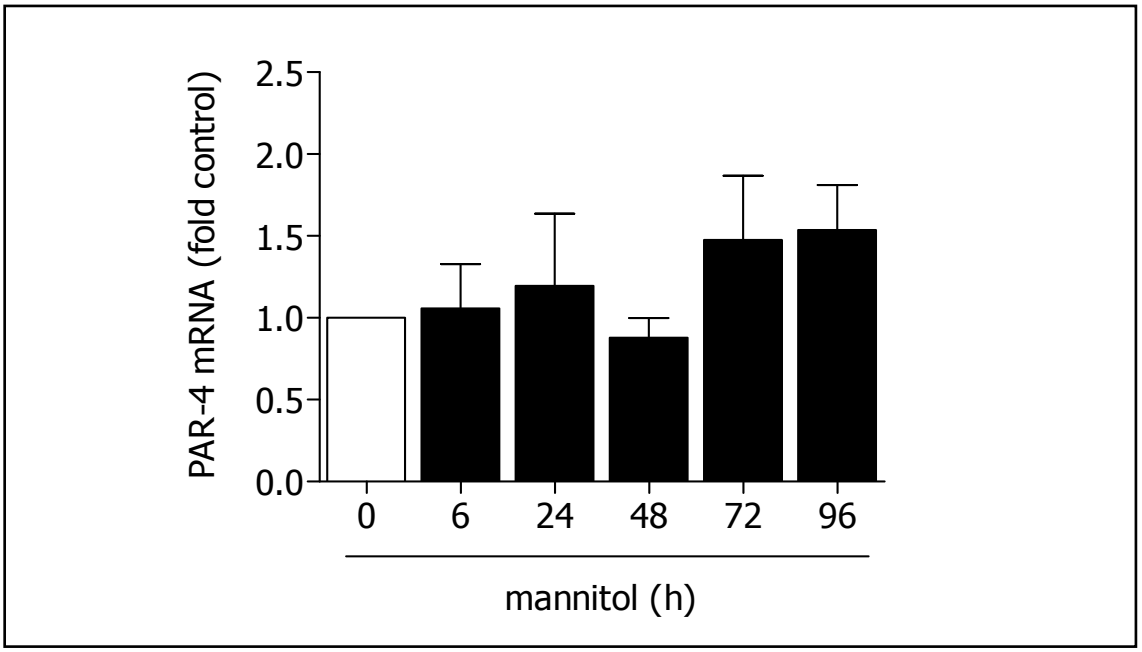

Figure 3.2 Time course of iso-osmolar mannitol (19.5 mmol/L + 5.5 mmol/L glucose) on PAR-4 mRNA in human saphenous vein SMC measured by qRT-PCR and normalized to GAPDH (n=3).

Effect of high glucose concentrations on PAR-4 mRNA expression in human saphenous vein SMC

The effect of high glucose on PAR-4 mRNA expression was found to be concentration dependent. Treatment of human saphenous vein SMCs with 5.5 to 30 mmol/L D-glucose concentrations for 48 h showed the most reproducible effect with 25 mmol/L glucose (fig. 3.3; n=4, $p<0.05$). All subsequent studies therefore utilized this concentration.

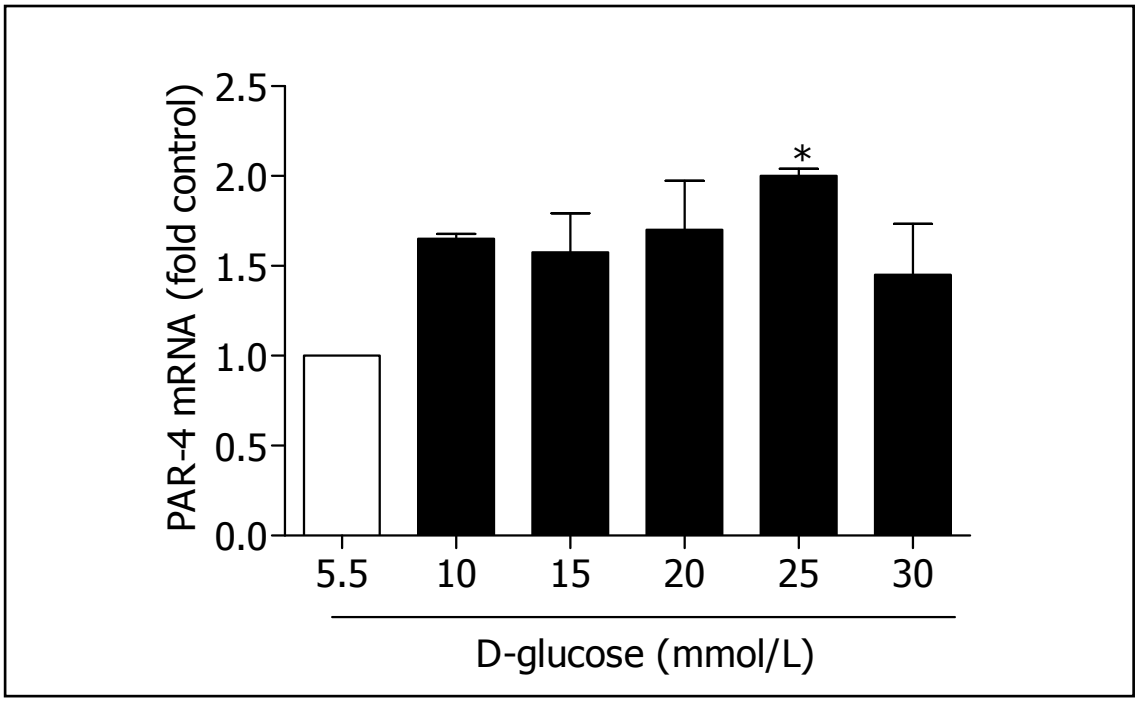

Figure 3.3 Effect of high glucose (48 h) on PAR-4 mRNA expression in human saphenous vein SMC, determined by qRT-PCR and normalized to GAPDH (n=4, *p<0.05 vs normal (5.5 mmol/L) glucose control).

High glucose regulation of thrombin receptors in human coronary artery SMC

Comparable regulatory effects of high glucose on thrombin receptor were observed in human coronary artery SMCs. As in saphenous vein SMCs, PAR-4 upregulation was more rapid and pronounced (more than 7 fold at 3 h and sustained to 2-fold till 48 h, fig. 3.4; n=5, p<0.05) in comparison to PAR-3 (2.5 fold at 6-24 h), while PAR-1 was negligibly influenced.

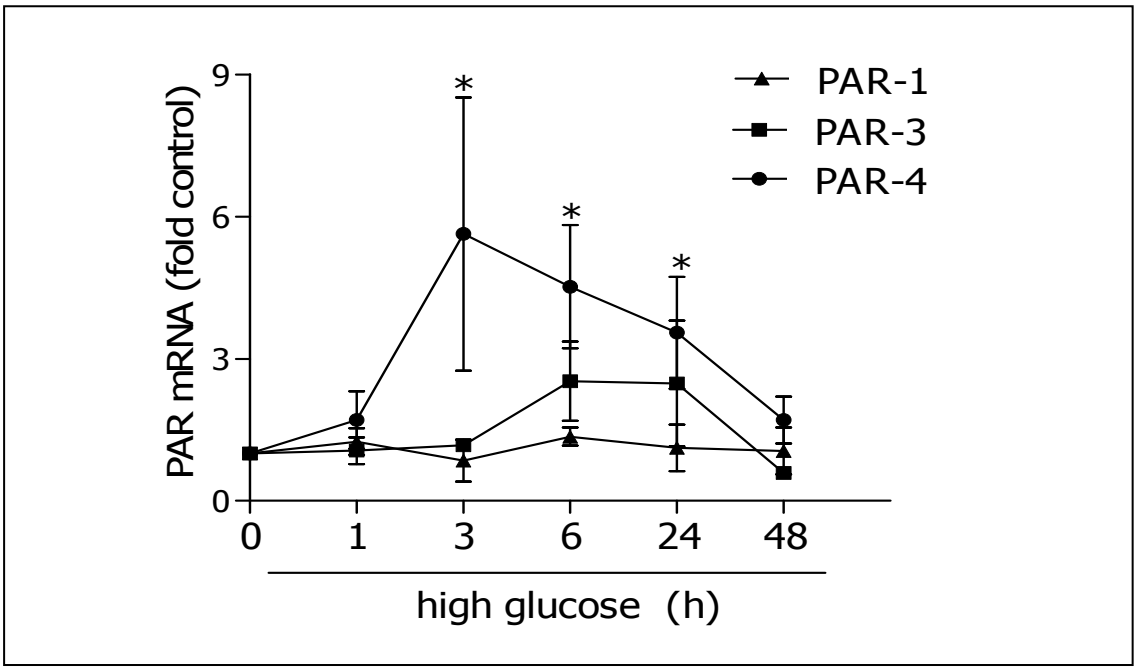

Figure 3.4 Influence of high glucose (25 mmol/L) time course on PAR-1, PAR-3 and PAR-4 mRNA expression in human coronary artery SMC measured by qRT-PCR. (n=4, *p<0.05 vs unstimulated normal glucose control).

3.1.2. Thrombin receptor protein expression

The regulatory action of PAR-4 was validated at the protein levels using western blot and immunofluorescence techniques.

High glucose selectively increases PAR-4 total protein in human saphenous vein SMC

Western blotting experiments showed time dependent changes in PAR-4 protein by high glucose. As seen with mRNA, PAR-1 and PAR-3 protein did not change while PAR-4 protein levels were increased approximately 2-fold over 48-96 h of high glucose stimulation (representative blots and pooled data, fig 3.5 A, B; n=5, $p<0.05$).

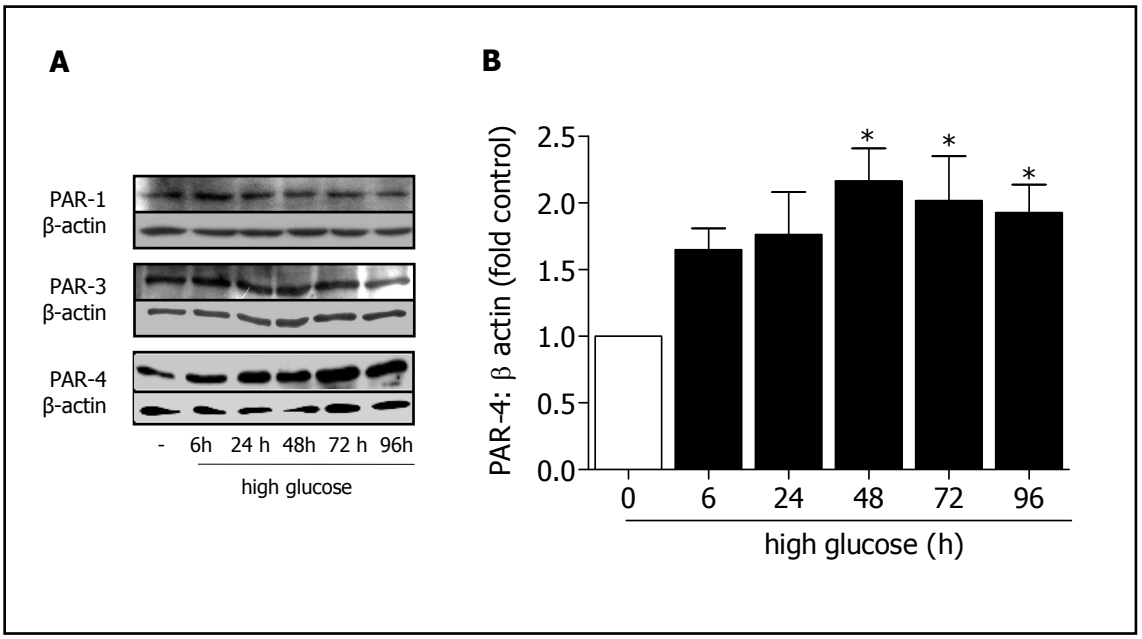

Figure 3.5 Effect of high glucose (25 mmol/L) on thrombin receptor total protein levels in human saphenous vein SMC. **(A)** Representative western blots showing bands for PAR-1 (66 kDa), PAR-3 (43 kDa), PAR-4 (38 kDa) and corresponding β-actin band (42 kDa). **(B)** Pooled data for PAR-4 total protein levels normalized to β-actin (n=5, *p<0.05 vs unstimulated normal glucose control).

High glucose enhances PAR-4 immunofluorescence in human saphenous vein SMC

Similar to total protein, PAR-4 immunofluorescence was increased by high glucose at 48- 72 h, while no effect was seen with the iso-osmolar control mannitol (19.5 mmol/L mannitol in DMEM containing 5.5 mmol/L glucose) either at 6 h or at 48 h treatment (fig. 3.6; n=3). This again indicates that regulation of PAR-4 by high glucose in human saphenous vein SMC is independent of changes in osmolarity.

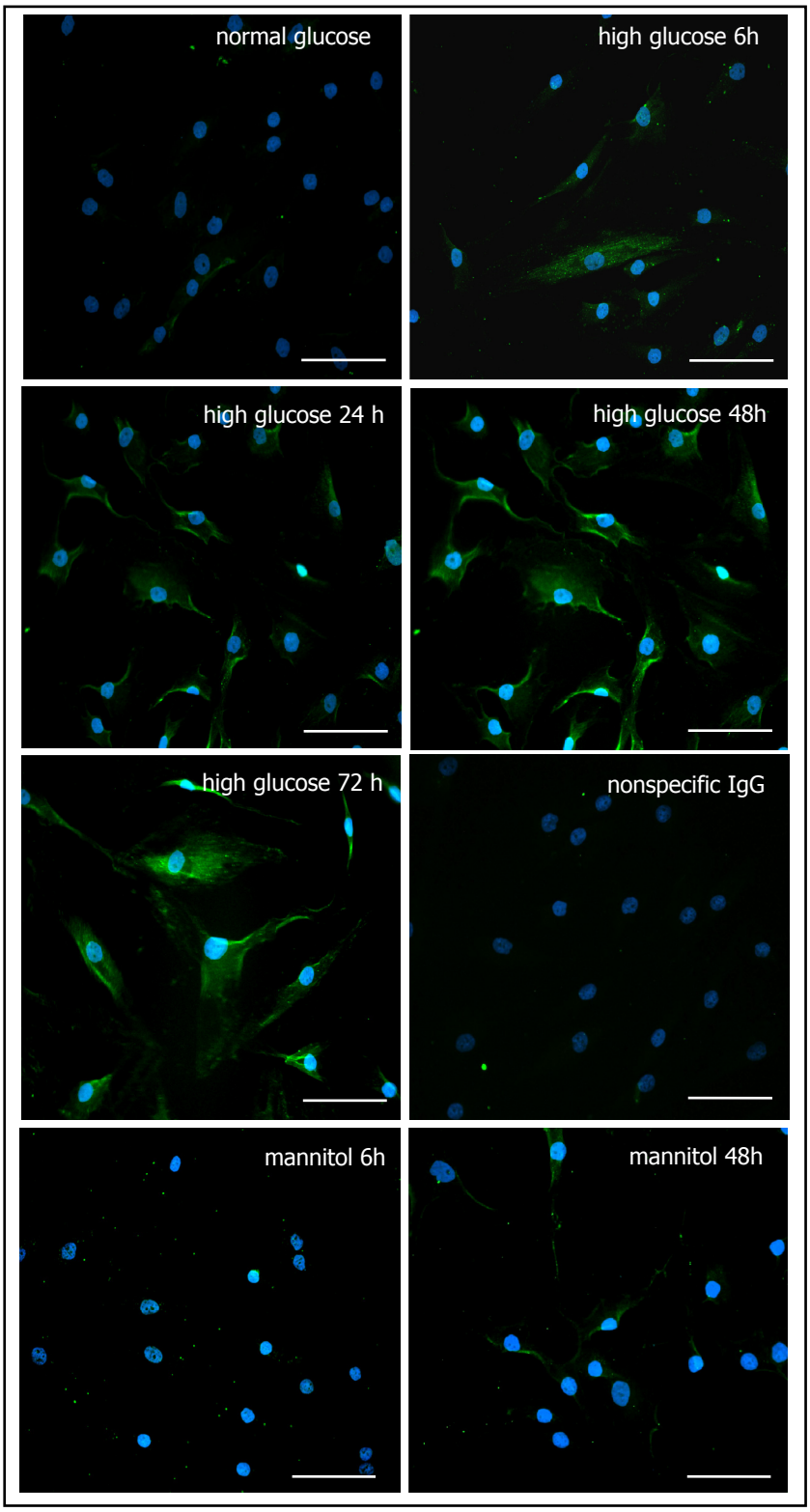

Figure 3.6 Influence of high glucose (6-72 h) or iso-osmolar mannitol (6 and 48 h) on PAR-4 immunofluorescence vs normal glucose control and nonspecific IgG control in human saphenous vein SMC. Representative images are showing green fluorescent signal of FITC for PAR-4 and blue signal for nuclear stain hoechst as captured by fluorescence microscope (representative of 3 individual experiments).

3.1.3. PAR-4 cell surface expression

PAR-4 cell surface expression was determined by fluorescence cytometry. In unstimulated cells approximately 75% of total cells exhibited low fluorescence and approximately 25% showed relatively high fluorescence, indicating two different cell populations with different levels of PAR-4 expression (fig 3.7 A). Upon stimulation with high glucose (48-72 h) the proportion of cells expressing high levels of PAR-4 was increased by more than 2 fold (pooled data, fig 3.7 B; n=4, $p<0.05$). The maximum increase in PAR-4 cell surface expression was seen at 48 h, similar to the time course observed in western blot studies. Therefore this time point was chosen as a high glucose pretreatment interval in subsequent functional studies prior to addition of thrombin or other thrombin receptor agonists.

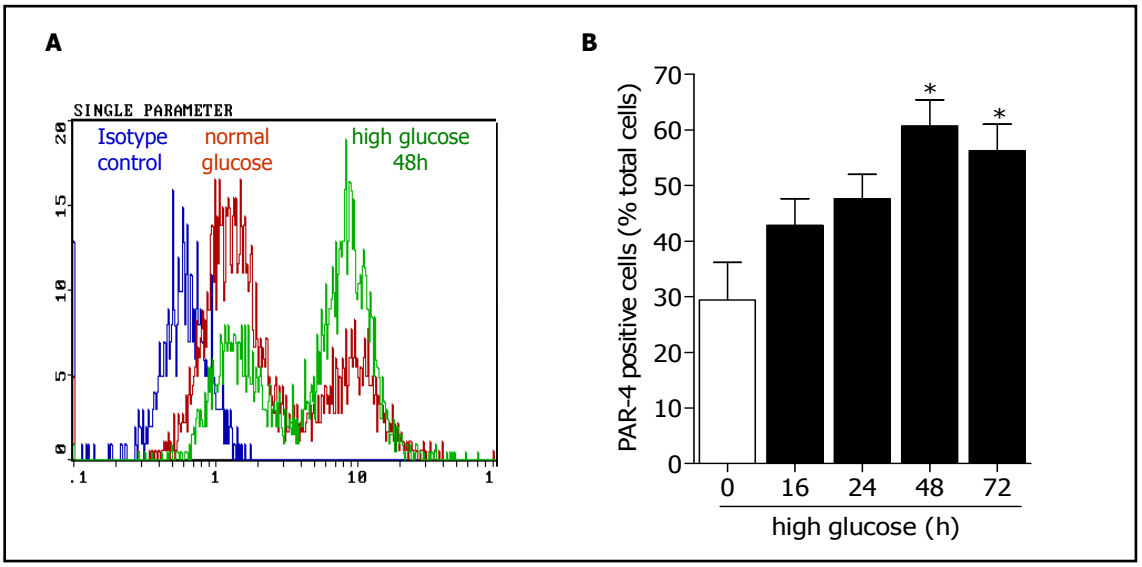

Figure 3.7 Influence of high glucose on PAR-4 cell surface expression **(A)** representative fluorescence trace in human saphenous vein SMC maintained in normal (red) or high glucose for 48 h (green). **(B)** Pooled data showing time course of high glucose stimulated PAR-4 surface expression in human saphenous vein SMC (n=4, *p<0.05 vs unstimulated control).

3.2. Functional outcomes of high glucose mediated PAR-4 upregulation in human vascular SMC

High glucose selectively and rapidly enhanced PAR-4 mRNA expression in human vascular SMC, while PAR-1 and PAR-3 were not influenced. Significant increases in PAR-4 protein and cell surface expression were seen after 48 h of high glucose treatment. The subsequent functional studies were designed to examine the impact of enhanced PAR-4 expression on classical cellular actions of thrombin such as calcium signaling leading to vascular SMC migration and inflammatory gene expression.

3.2.1. Thrombin receptor mediated calcium transients

Calcium signals evoked by thrombin and PAR receptor activation

Thrombin elicits intracellular calcium ($[Ca^{+2}]_i$) mobilization in human vascular SMCs with contribution of all three subtypes of thrombin receptors expressed in these cells. In this study, the relative contributions of PAR-1, PAR-3 and PAR-4 to the overall thrombin response were accessed. Thrombin alone (3 U/mL) elicited a rapid and marked increase in intracellular calcium as shown by a transient fluorescent signal observed in human saphenous vein SMCs loaded with calcium sensitive fluorescent dye Fluo-4 AM. However, as previously shown no calcium signal was seen with repeated application of thrombin confirming complete disappearance of all thrombin receptors (Bretschneider et al. 2001) (fig 3.8 A). Selective activating peptides for PAR-4 (PAR-4AP, AYPGKF, 200 µmol/L) and PAR-1 (PAR-1AP, TFLLRN, 20 µmol/L) applied in sequence, elicited comparable calcium signals. Subsequent application of thrombin– now to cells, in which PAR-1 and PAR-4 were desensitized– showed

a residual response reflecting relatively low contribution of PAR-3 in this effect (fig. 3.8 B).

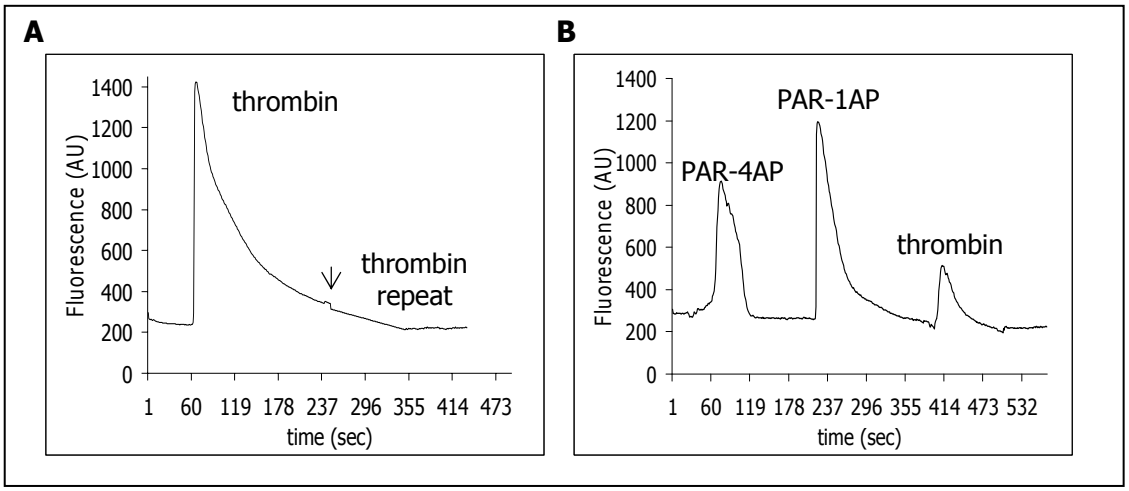

Figure 3.8 Representative fluorescence (arbitrary units) vs time curves showing intracellular calcium changes evoked **(A)** by thrombin alone and **(B)** by sequential application of PAR-APs followed by thrombin in human saphenous vein SMC using calcium sensitive fluorescent dye Fluo-4AM (representative of n=5).

High glucose enhances $[Ca^{+2}]_i$ transient evoked by thrombin

Subsequent studies examined the impact of high glucose on thrombin receptor mediated intracellular calcium mobilization. Human saphenous vein SMCs were maintained in normal or high glucose for 48 h prior to study and fluorescence changes in response to increasing concentrations of thrombin was investigated. To obtain the maximum calcium signal, cells were stimulated with an ionophore ionomycin (10 µmol/L in 6 mmol/L $CaCl_2$/HEPES buffer) at the end of each experiment. Ionomycin induced calcium signals were taken as $[Ca^{+2}]_{i\ max}$ and the signal produced by thrombin was calculated as % $[Ca^{+2}]_{i\ max}$. Concentration response curve showed EC_{50} value of 1.2 or 0.5 U/mL in normal or high glucose pretreated saphenous vein SMCs respectively (fig 3.9). This shows that cells become more sensitive to thrombin upon high glucose treatment, and more importantly, the differences in intracellular calcium release can be seen at 1 U/mL (approx. 10 nmol/L) or higher thrombin concentrations. Potentially these differences may be due to receptors other than PAR-1, which has higher affinity towards thrombin and thus can be activated at very low

thrombin concentration (picomolar range). PAR-4, by contrast is a low affinity receptor, activated by thrombin at higher concentrations only (Covic et al. 2000; Steinberg 2005).

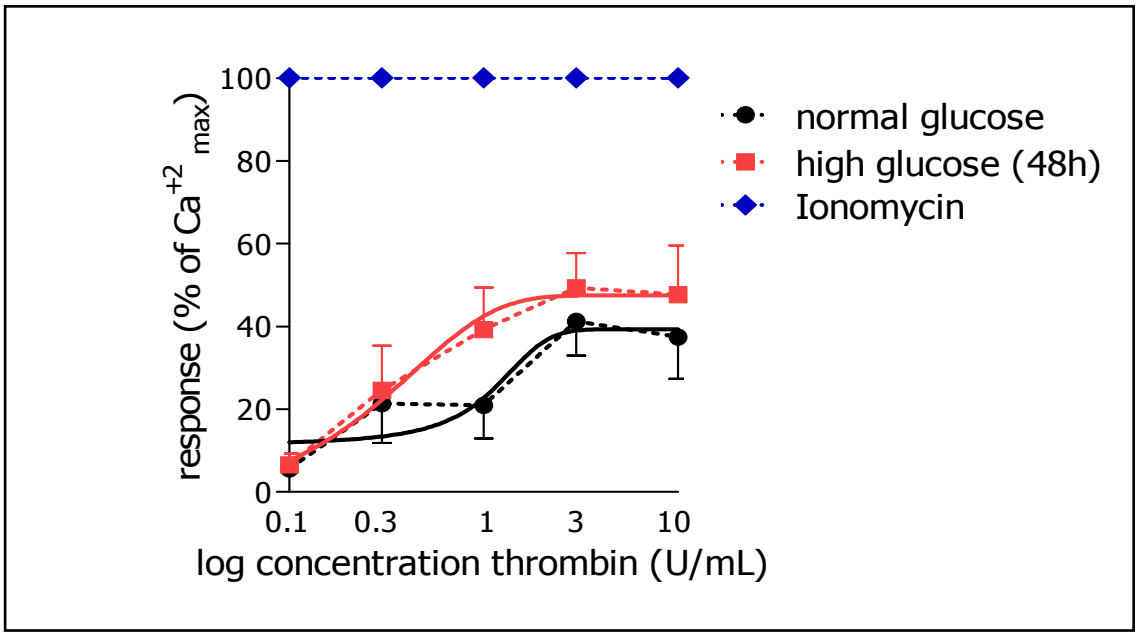

Figure 3.9 Log concentration-response curve of thrombin in human saphenous vein SMC pretreated with normal and high glucose for 48 h. Points connected with dotted lines indicate the real observations and smooth lines represent log transform fitting curve. Response to ionomycin (10 µmol/L) in 6 mmol/L $CaCl_2$/ HEPES was taken as 100%, (n=3).

Further, the $[Ca^{+2}]_i$ transients evoked by PAR-1AP or PAR-4AP were observed to investigate their relative contribution to thrombin induced $[Ca^{+2}]_i$ signal. Calcium is a ubiquitous second messenger activating various calcium dependent kinases such as PKC or MAPK, and transcription factors like NFAT, CREB or NF-κB to control a broad range of cellular functions such as gene transcription, growth, proliferation and migration (Berridge et al. 2003; Crabtree et al. 2009; Lipskaia et al. 2004; Lipskaia et al. 2003). Cells treated with high glucose (48 h) showed significantly higher $[Ca^{2+}]_i$ peak in response to PAR-4AP or thrombin, while response to PAR-1AP did not alter (fig. 3.10 A, B; n=5, $p<0.05$). This is consistent with expression studies that high glucose enhanced PAR-4 but not PAR-1 in human saphenous vein SMCs after 48 h treatment.

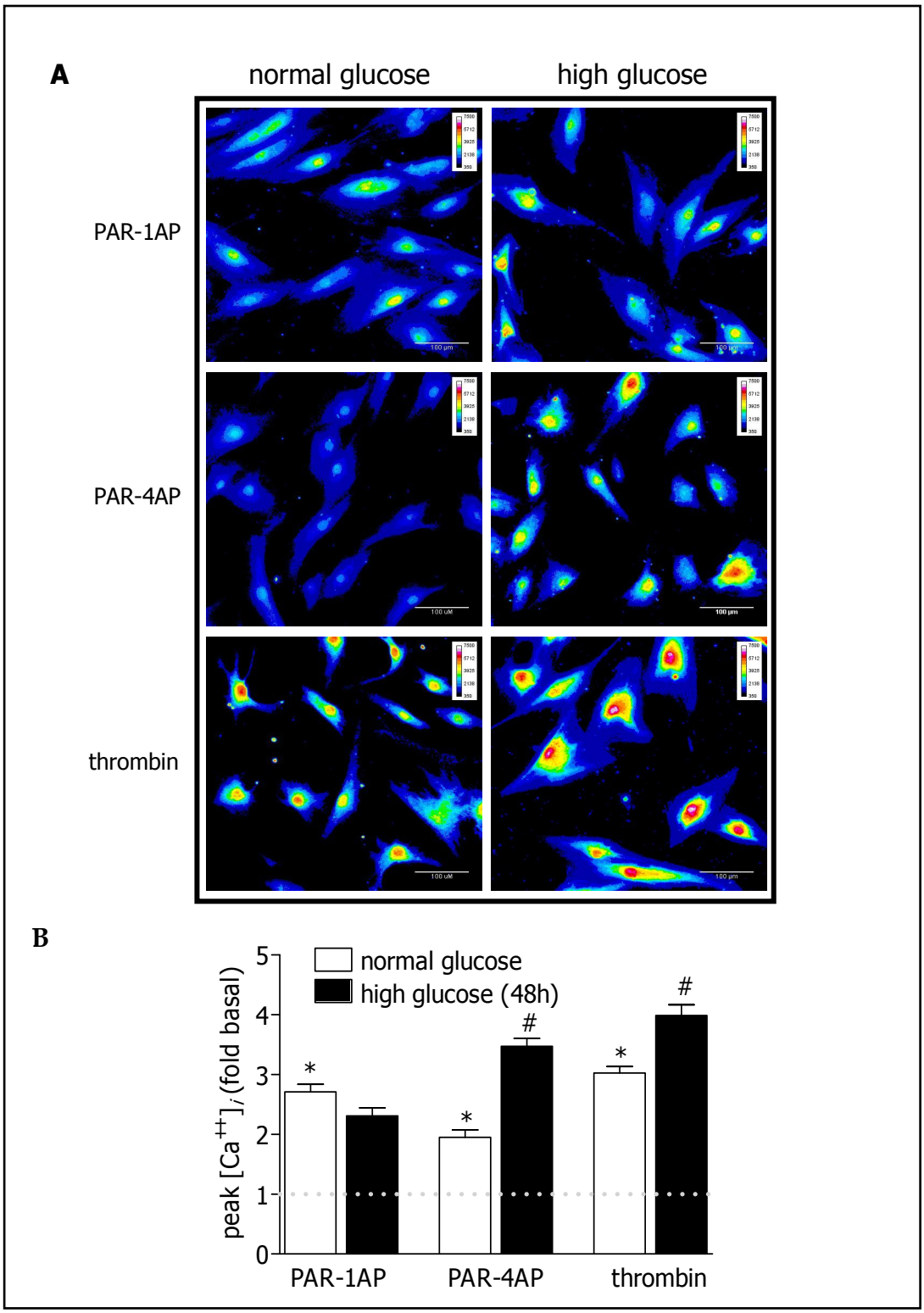

Figure 3.10 (A) Images of PAR-1AP (20 µmol/L), PAR-4 AP (200 µmol/L) or thrombin (3 U/mL) induced peak $[Ca^{+2}]_i$ in human vascular SMC pretreated with normal or high glucose (48 h). **(B)** Pooled data showing quantification of peak $[Ca^{+2}]_i$. (n=5; *p<0.05 vs basal (dotted line), #p<0.05 vs normal glucose counterpart.

3.2.2. Thrombin receptor mediated human vascular SMC migration

High glucose enhances PAR-4-mediated SMC migration

The functional consequence of the selective PAR-4 induction by high glucose was further examined in a wound-scratch migration assay, a model of wound healing. An artificial wound area was scratched into the human saphenous vein SMCs monolayer maintained in normal or high glcose (48 h). Cell monolayers were then washed with PBS and the total migration of cells was observed in the presence of a proliferation inhibitor 5-hydroxy urea (5 mmol/L in serum free DMEM) for next 72 h. SMCs maintained in normal glucose showed modest degree of migration in response to PAR-4AP (400 µmol/L). This was markedly enhanced when cells were previously exposed to high glucose for 48 h (fig. 3.11).

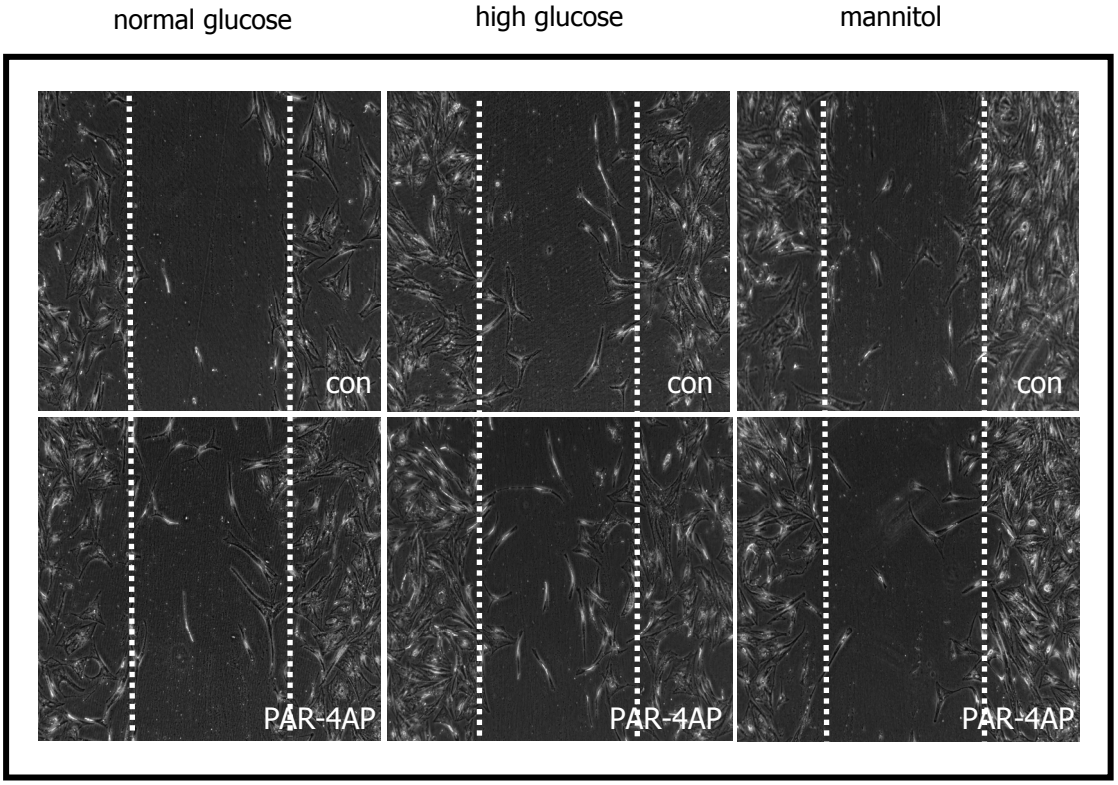

Figure 3.11 Effect of high glucose or mannitol pretreatment (48 h) on PAR-4AP (400 µmol/L) induced migration of human saphenous vein SMCs in a wound-scratch assay. Upper and lower panels show SMC migration in absence and presence of PAR-4AP respectively. Images are representative of 3 individual experiments.

The isoosmolar control mannitol did not influence the migratory responses to PAR-4 activation, which reflects the stimulatory effect of high glucose but not mannitol on functional PAR-4 expression (fig. 3.11).

Comparison of thrombin and PAR-AP induced SMC migration

The migratory responses to PAR-1AP, PAR-4AP and thrombin were compared to confirm selective enhancement of functional PAR-4 expression. While high glucose (48 h) enhanced migratory response to both PAR-4AP and thrombin, migration elicited by PAR-1AP was not increased (fig. 3.12 A representative images and 3.12 B pooled data of n=4, $p<0.05$). Again this is consistent with selective induction of PAR-4 but not PAR-1 expression by high glucose.

Effect of PAR-4 silencing on thrombin induced SMC migration

As in last experiments high glucose enhanced thrombin mediated human saphenous vein SMC migration, in order to further validate if these responses were attributed to increased PAR-4 expression in high glucose cultures, the effects of PAR-4 knock down in these SMCs were examined. In human saphenous vein SMCs transfected with specific siRNA against PAR-4, the migratory ability of thrombin in cells previously exposed to normal or high glucose, was significantly reduced. This confirms the significant contribution of PAR-4 receptors in thrombin mediated migration of SMCs under high glucose conditions (fig. 3.13 A, B; $p<0.05$, n=3).

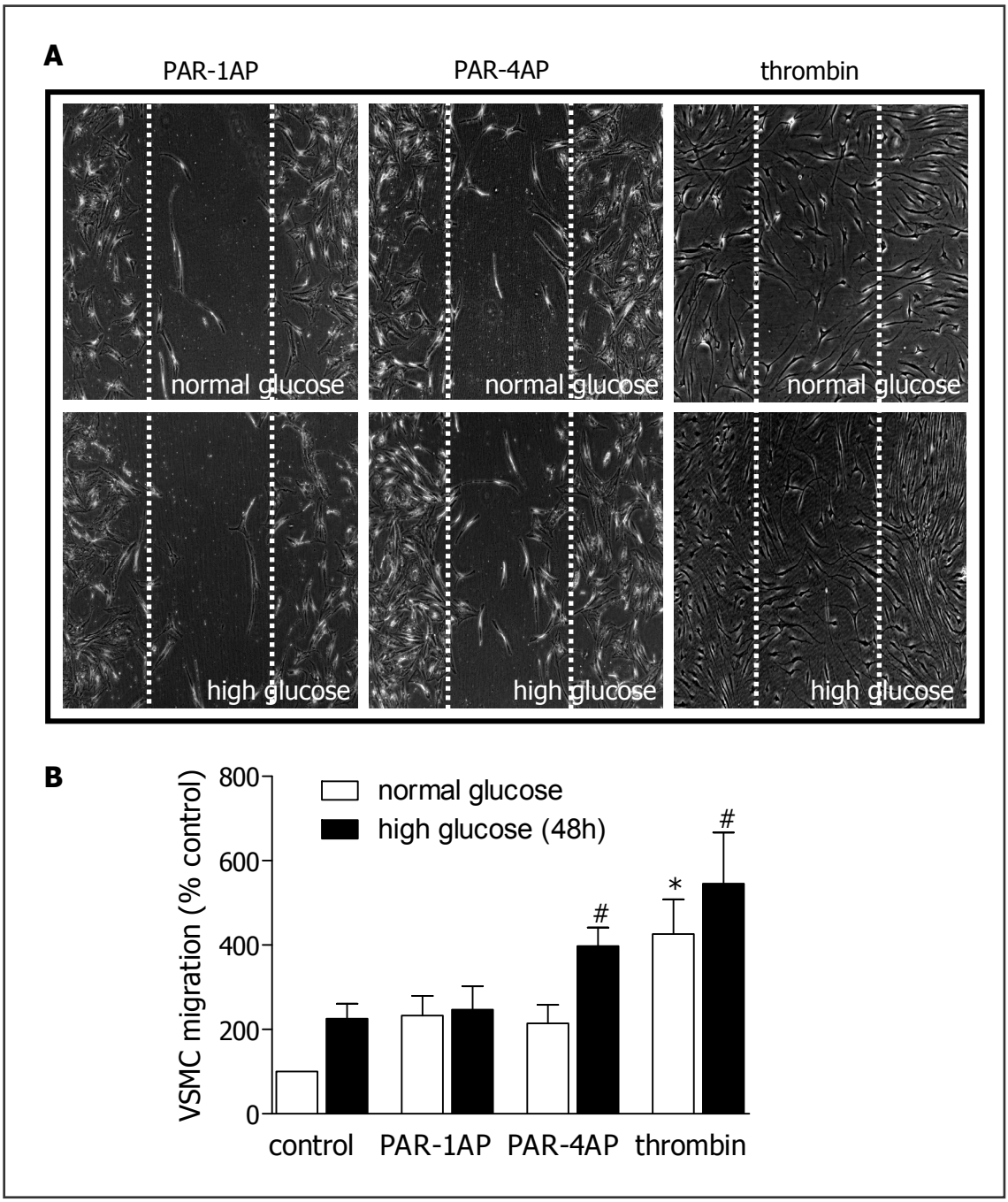

Figure 3.12 Effect of high glucose pretreatment (48 h) on PAR-1AP (100 µmol/L), PAR-4AP (400 µmol/L) or thrombin (3 U/mL) induced migration of human saphenous vein SMC in a wound-scratch assay. **(A)** Representative images of normal glucose or high glucose pretreatment (48 h) and **(B)** pooled data. (n=4, *$p<0.05$ vs unstimulated normal glucose control, #$p<0.05$ vs corresponding normal glucose counterpart).

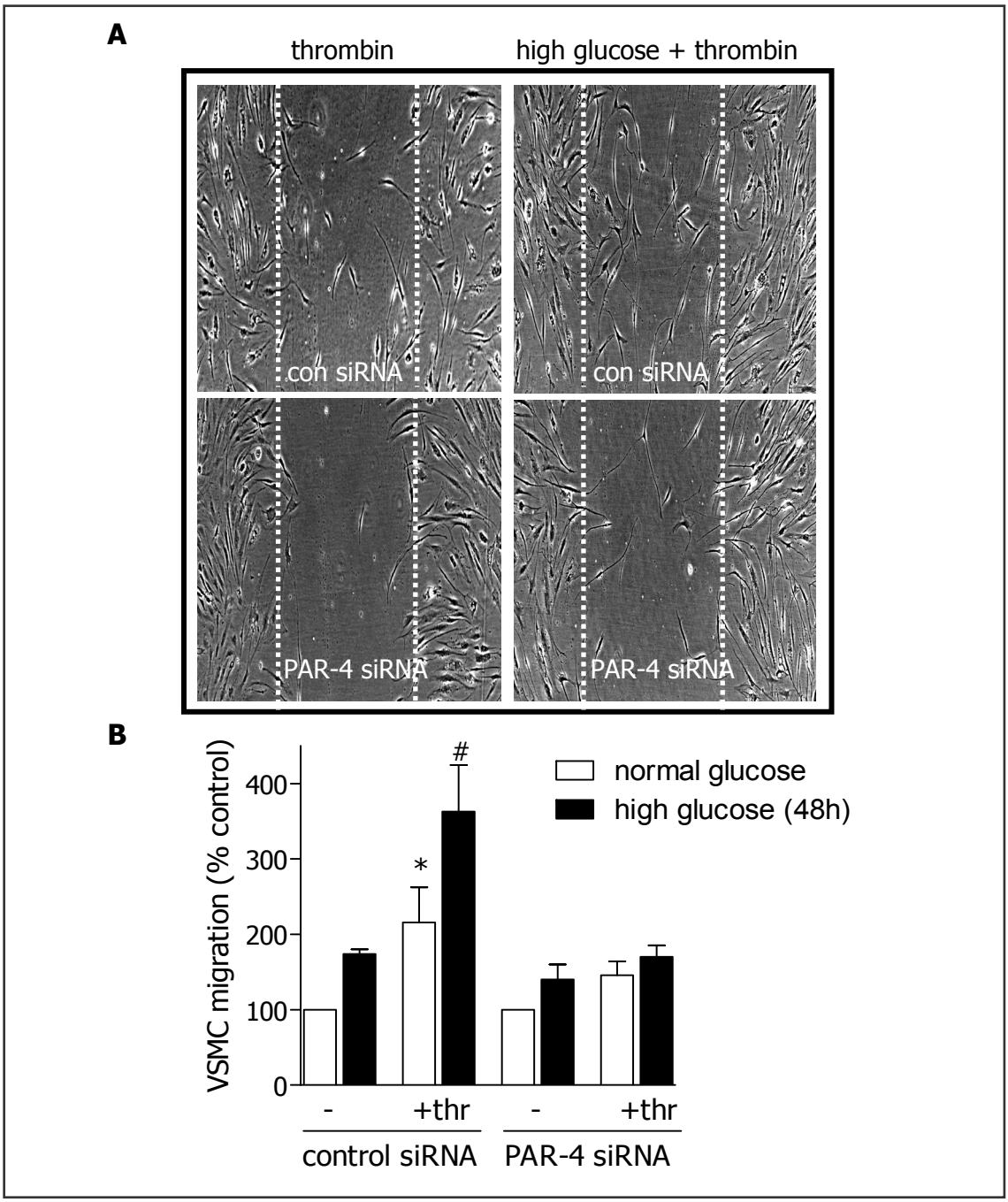

Figure 3.13 Effect of PAR-4 silencing on thrombin (3 U/mL) induced migration in normal and high glucose pretreated (48 h) human saphenous vein SMC in a wound-scratch assay **(A)** representative images and **(B)** pooled data of 3 individual experiments. (*$p<0.05$ vs unstimulated normal glucose control and #$p<0.05$ vs normal glucose counterpart).

3.2.3. PAR-4 induced inflammatory gene expression

Inflammation is a key player of tissue repair and remodeling (Ridker 2009), thus TNF-α was chosen as a further functional parameter, in the cells exposed to high glucose. TNF-α is an inflammatory cytokine crucial for the development of atherothrombosis and diabetes (Pickup et al. 2000; Ridker 2009), and may be stimulated by thrombin in certain cells such as cultured glial cells (Kim KY 2002).

In human saphenous vein SMCs maintained in normal glucose, acute stimulation with PAR-4AP (200 µmol/L, 3 h) induced TNF-α mRNA expression to approximately 2-fold. High glucose pretreatment (48 h) further enhanced this acute PAR-4-mediated TNF-α expression to 3.5±0.8 fold (fig. 3.14 A; n=4, $p<0.05$). Interestingly, similar changes were seen at the level of PAR-4 mRNA expression (fig. 3.14 B; n=4, $p<0.05$) indicating a possible autoregulation of PAR-4.

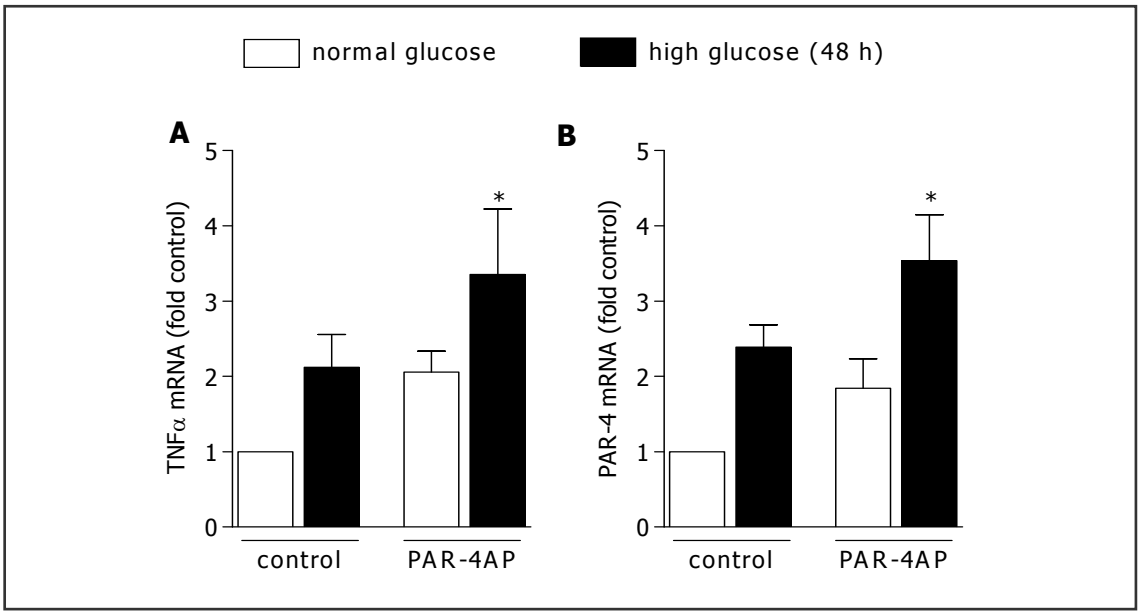

Figure 3.14 Effect of high glucose pretreatment (48 h) on PAR-4AP (200 µmol/L) induced **(A)** TNF-α and **(B)** PAR-4 mRNA expression in human saphenous vein SMCs measured by qRT-PCR (n=4, *$p<0.05$ vs unstimulated normal glucose control)

To further confirm that enhanced TNF-α expression is attributable to selective increase in PAR-4 expression, the impact of PAR-4 gene silencing was examined. PAR-4 siRNA led to approximate 80% knockdown of PAR-4 gene in saphenous vein SMCs. High glucose enhanced thrombin stimulated TNF-α expression was completely abolished by PAR-4 knock down in these cells, indicating proinflammatory role of thrombin mediated via PAR-4 (fig. 3.15 A, B).

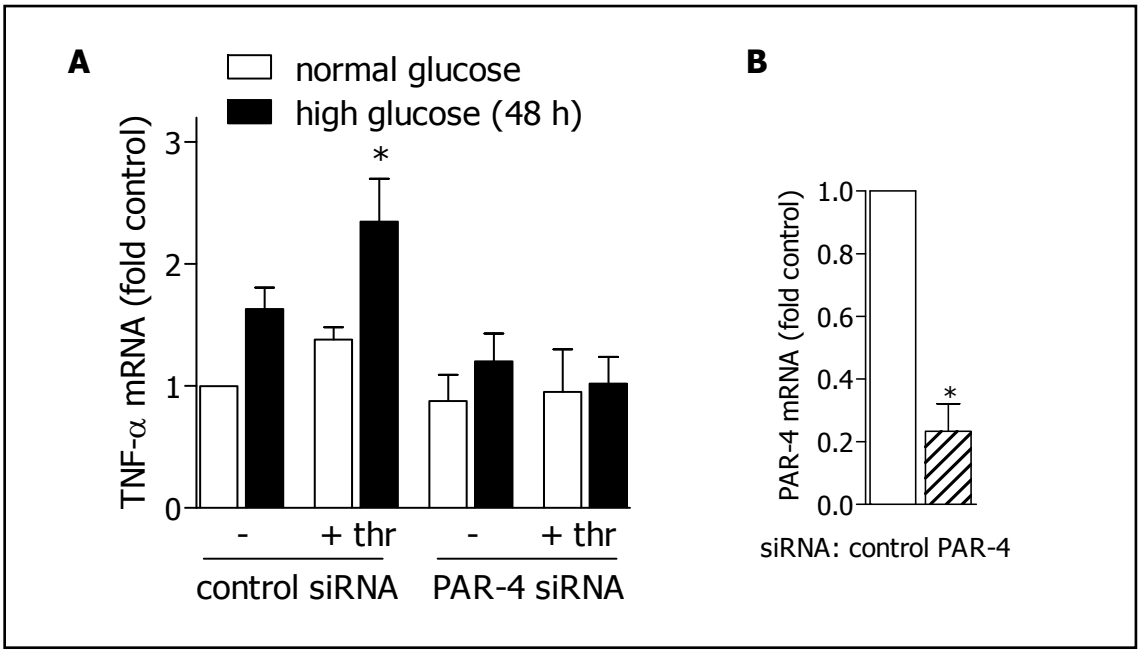

Figure 3.15 (A) High glucose enhances thrombin (3 U/mL) mediated TNF-α mRNA expression in human saphenous vein SMC, this effect is abolished in presence of PAR-4 siRNA (40 nmol/L). **(B)** Validation of siRNA mediated PAR-4 knockdown in parallel experiment measured by qRT-PCR (n≥4, *p<0.05 vs unstimulated normal glucose control).

3.3. Mechanisms of high glucose induced PAR-4 upregulation

3.3.1. Transcriptional regulation of PAR-4 by high glucose

Thrombin receptors are rapidly internalized after activation and reappearance of active receptors at the cell surface may in part involve transcriptional upregulation. Luciferase-reporter assays were utilized to investigate the possible transcriptional regulation of thrombin receptors by high glucose. SMCs from human saphenous vein were transfected with luciferase reporter vectors under control of different human thrombin receptor promoters. High glucose (24 h) selectively enhanced PAR-4 but not PAR-1 or PAR-3 promoter activity (fig. 3.16 A, n=4, $p<0.05$). This is consistent with the changes seen at the level of PAR-4 mRNA expression. A time course of high glucose stimulated PAR-4 promoter activity reflected the time course of changes in mRNA levels. Significant enhancement of PAR-4 promoter activity was observed at 6 h (to 642±80%) and 48 h (to 501±125%, fig. 3.16 B; n=4, both $p<0.01$). Luciferase reporter assay sometimes showed much variability in PAR-4 promoter activity, especially in experiments with signaling inhibitors, therefore the results were not included in present study.

To identify the possible mediators to control the transcriptional regulation of thrombin receptors and of PAR-4 in particular, sequence analysis of thrombin receptor promoters was performed using transfac® (www.gene-regulation.com/) and genomatix® databases (www.genomatix.de/). This revealed distinct transcription factor binding motifs within the individual PAR promoters. Several sites for transcription factors binding motifs known to be activated by high glucose (in bold letters) and its main effector PKC were identified in PAR-4 promoter, but were absent in the promoters of either PAR-1 or PAR-3 (table 2). Among these candidate regulators of PAR-4, NF-κB was of particular interest given its central role in cardiovascular disorders and diabetes (Landry et al. 1997; Wilson et al. 2002).

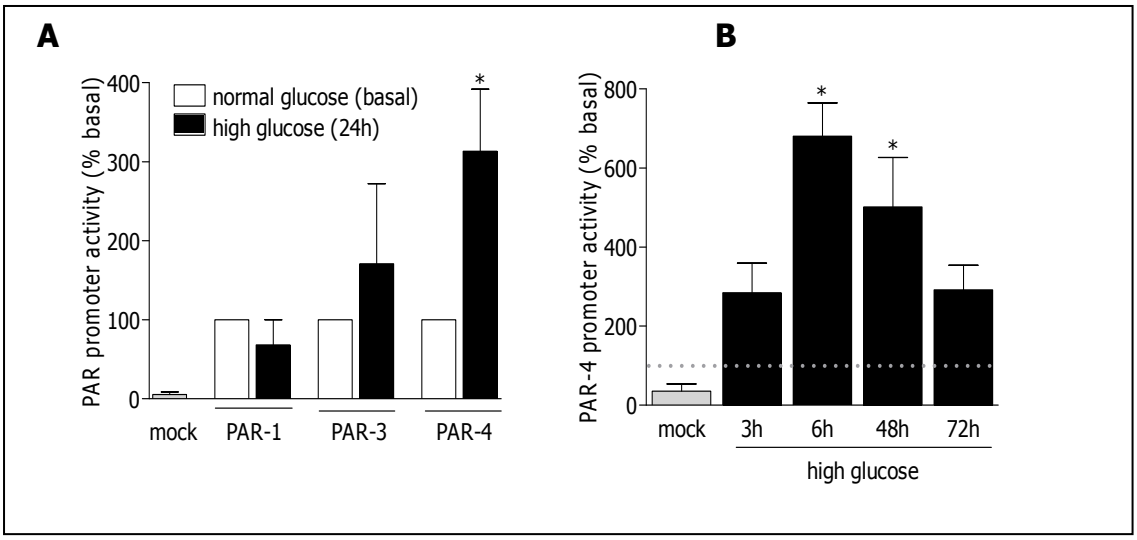

Figure 3.16 (A) Promoter activities of all thrombin receptors in human saphenous vein SMCs after 24 h normal or high glucose treatment. **(B)** Time dependent increase in high glucose induced PAR-4 promoter activity in human saphenous vein SMCs measured by luciferase reporter assay (n=4, *p<0.01 vs unstimulated control).

Transcription factors binding motifs in PAR-4 promoter only			
ACAAT	EGR3	***MAFA***	PEA3
AIRE	ELF2	MEF3	***PPARγ***
AML1	ER	MIT	PRE
AP4	FAST1	MTF1	RBPJK
CDX1	FIXRE	***MYOD***	RREB1
CHOP	HEN1	MYOGENIN	TAL1_E2A
CHREBP_MLX	***HSF2***	***NEUROD1***	TAL1αE47
CREL	IK-2	***NFκB***	TAXCREB
DEC2	***KAISO***	NMYC	***THR***
δEF1	LYF1	PAX6	***ZNF76_143***

Table 2. Putative transcription factors binding motifs present only in PAR-4 promoter but not in PAR-1 or PAR-3 promoters. Transcription factors in bold are known to be regulated by high glucose.

3.3.2. Central role of PKC

PKC is a key mediator of the detrimental effects of diabetes (Koya & King 1998). The activation of PKC initiated by hyperglycemia regulates contractility, extracellular matrix formation, cell proliferation, angiogenesis, cytokine actions and leukocyte adhesions, all of which are abnormal in diabetes (Nishizuka 1992; Nishizuka 1995). Therefore the role of PKC isozymes in the glucose stimulated expression of PAR-4 was examined using various pharmacological tools and siRNA applications.

Inhibitors of PKC prevent high glucose regulated PAR-4 expression

The ability of high glucose to induce PAR-4 mRNA expression was prevented by PKC inhibition in human saphenous vein SMCs using various selective and non-selective inhibitors to PKC isozymes. Inhibitors were added to cells 30 minutes prior to high glucose stimulation and were present till the end of study period. Cells were collected after 6 h and 48 h of high glucose stimulation for mRNA and protein expression studies respectively, as changes in PAR-4 mRNA expression were significant from 3 h onwards, while PAR-4 protein levels were significant only after 48 h of high glucose stimulation.

Both calphostin-C and staurosporine (the nonselective PKC inhibitors) prevented high glucose stimulated PAR-4 expression by inhibiting total PKC protein (fig 3.17, $n=4$, $p<0.05$).

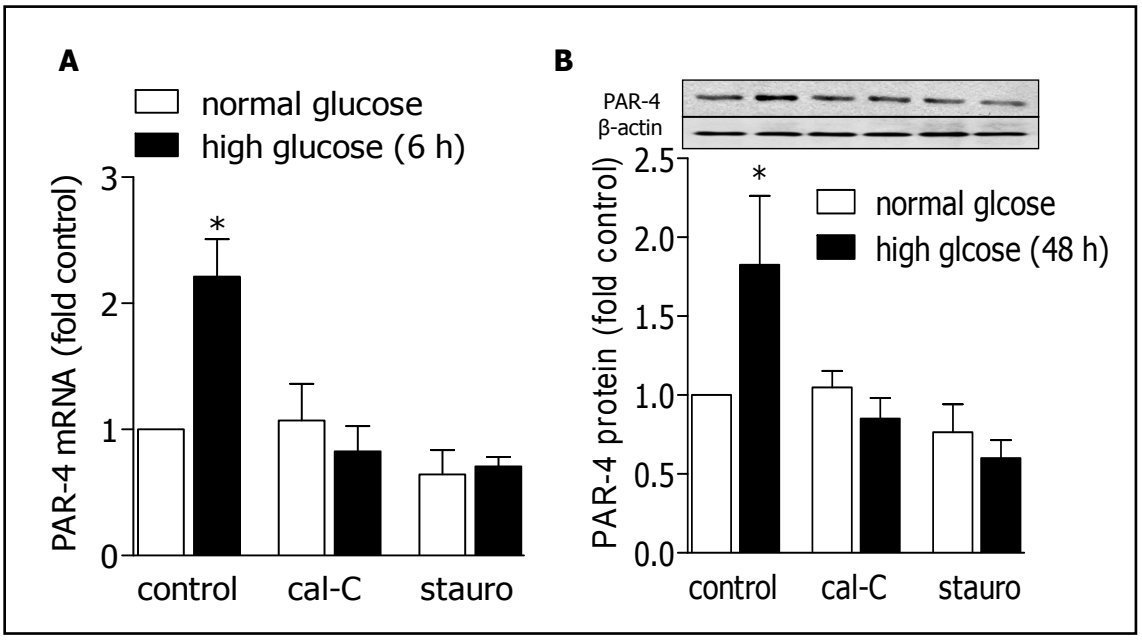

Figure 3.17 PAR-4 expression in human saphenous vein SMCs stimulated with high glucose ±nonselective PKC inhibitors: calphostin-C (200 nmol/L), staurosporine (1 μmol/L). Cells were collected at **(A)** 6 h for mRNA expression by qRT-PCR or **(B)** 48 h for total protein determination by western blotting (n=4; *P<0.05 vs unstimulated normal glucose control).

To further confirm which specific PKC isozyme is involved in this regulation, selective inhibitors to PKC-β or δ were used as most of the deleterious effects of high glucose are attributed to these isozymes (Koya & King 1998). A selective PKC-β inhibitor (PKC-βI) or PKC-δ inhibitor (rottlerin) could also inhibit the effect of high glucose on PAR-4 mRNA expression or protein expression in human saphenous vein SMCs (fig 3.18 A, B; n=4, p<0.05).

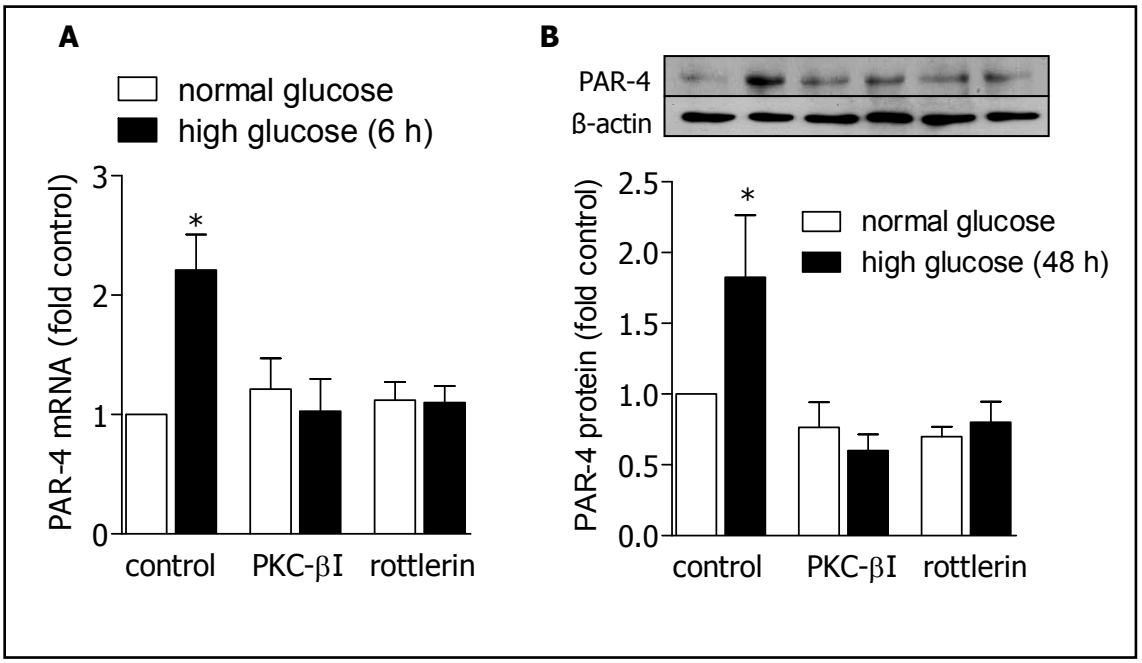

Figure 3.18 PAR-4 expressions in human saphenous vein SMCs stimulated with high glucose ±isozyme selective PKC inhibitors: specific PKC-β inhibitor (50 nmol/L), PKC-δ inhibitor rottlerin (1 μmol/L). Cells were collected at **(A)** 6 h for mRNA expression by qRT-PCR or **(B)** 48 h for total protein determination by western blotting (n=4; *P<0.05 vs unstimulated normal glucose control).

siRNA mediated PKCδ knockdown in saphenous vein SMC

Role of PKCδ was further confirmed by siRNA mediated knockdown in human SMCs from saphenous vein, as some recent reports question on specificity of rottlerin to inhibit PKC-δ due to its ability to inhibit other kinases such as calmodulin kinase (Soltoff 2007). SMCs transfected with siRNA targeted to PKC-δ showed prevention against stimulatory effect of high glucose on PAR-4 mRNA expression at 6 h and protein levels at 48 h (fig. 3.19 A, B; n=5, $p<0.05$). The validation of siRNA mediated knockdown was determined by western blotting after harvesting SMCs 48 h post transfection from duplicate wells in same plate. About 45% knock down of PKC-δ protein levels were found in SMCs prior to high glucose stimulation (fig. 3.19 C).

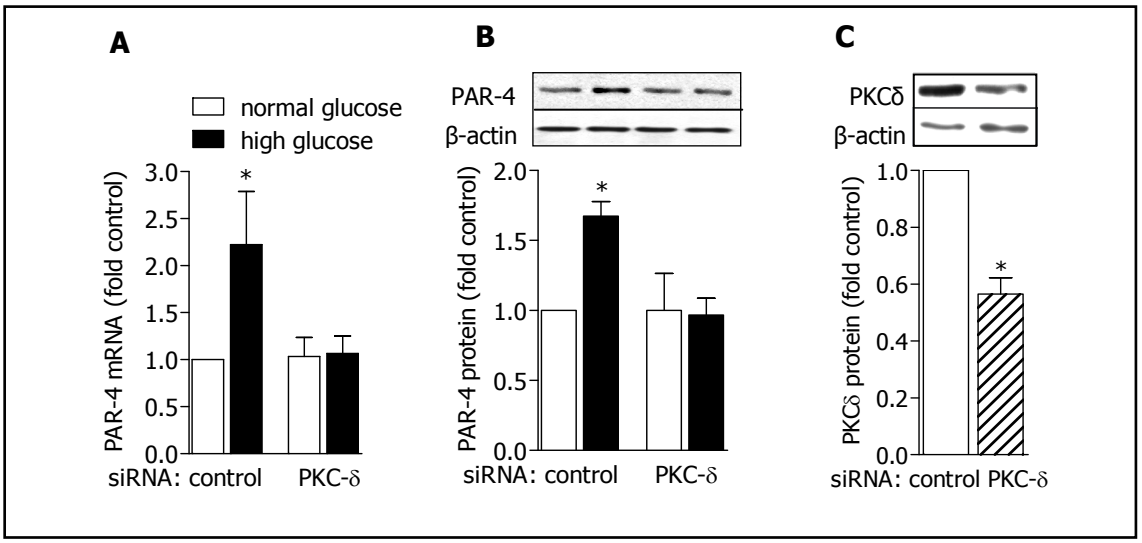

Figure 3.19 PKC-δ silencing prevents **(A)** PAR-4 mRNA expression after 6 h high glucose treatment as measured by qRT-PCR (normalized to GAPDH) and **(B)** PAR-4 protein (38 kDa) after 48 h high glucose stimulation of human saphenous vein SMCs (normalized to β-actin, 43 kDa). **(C)** Validation of siRNA mediated knockdown of PKC-δ protein (57kD, normalized to β-actin) at 48 h post-transfection in same experiments by western blotting (n=4, *p<0.05 vs unstimulated normal glucose control).

3.3.3. Role of NF-κB

NF-κB, a potential down stream effector to PKC, is an inducible transcription factor, highly abundant in atherosclerosis and inflammation (Wilson et al. 2002). Interestingly NF-κB is found to be present in the PAR-4 promoter, but not in PAR-1 or PAR-3 promoters. Therefore the role of this important effector in the glucose stimulated expression of PAR-4 was examined using inhibitors and siRNA mediated silencing.

High glucose enhances nuclear translocation of NF-κB

Further the potential ability of high glucose to activate NF-κB in human saphenous vein SMCs was examined by a translocation assay. Human saphenous vein SMCs were stimulated with high glucose and subcellular fractions were collected for western blot analysis of NF-κB p65 subunit and phosphorylated or total I-κBα proteins. High glucose induces a rapid shuttling

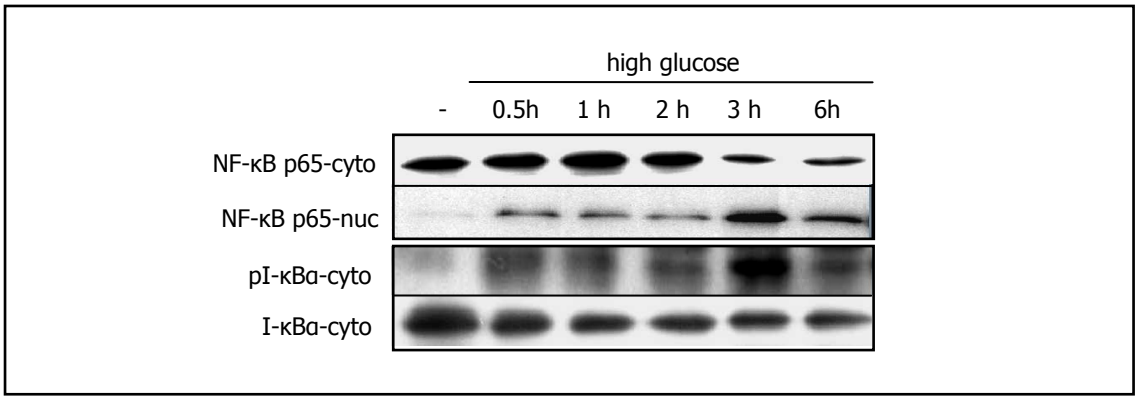

Figure 3.20 High glucose induced shuttling of NF-κB p65 (65 kDa) subunit from the cytosol to the nucleus, accumulation of phospho I-κBα and degradation of total I-κBα (37 kDa) in the cytosol of human saphenous vein SMC as determined by western blot (representative of n=3).

of NF-κB p65 subunit from the cytosol to the nucleus, which was maximal at 3 h. Simultaneous accumulation of phosphorylated I-κBα and subsequent degradation of total I-κBα in the cytosol in same time frame showed consistency with NF-κB activation (fig. 3.20; representative of n=3).

NF-κB inhibitor prevents stimulatory effect of high glucose on PAR-4 in SMC

To investigate the possible role of NF-κB in high glucose regulation of PAR-4, a synthetic inhibitor to NF-κB activation was utilized. Addition of this inhibitor (100 nmol/L, 30 minutes prior stimulation to high glucose) completely suppressed high glucose enhanced PAR-4 mRNA expression and protein levels in human saphenous vein SMC (fig. 3.21 A,B, n=3; $p<0.05$).

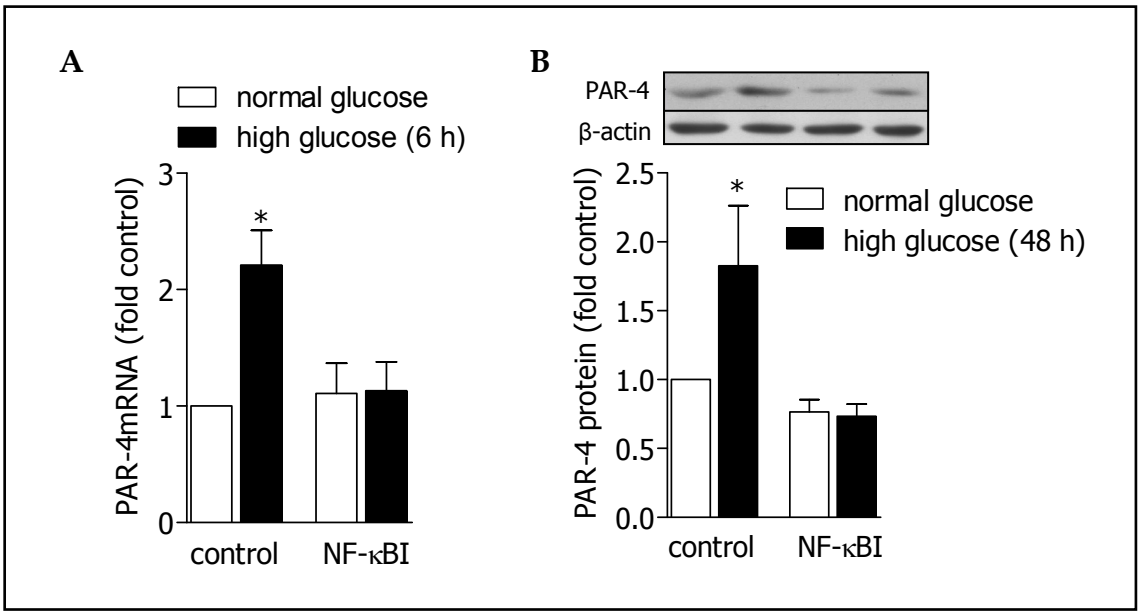

Figure 3.21 PAR-4 expressions in human saphenous vein SMCs stimulated with high glucose ± NF-κB activation inhibitor (100 nmol/L). **(A)** PAR-4 mRNA expression measured by qRT-PCR. **(B)** PAR-4 total protein levels determined by western blotting. Cells were collected at 6 or 48 h for mRNA or protein expression studies respectively (n=4; *P<0.05 vs unstimulated normal glucose control).

siRNA mediated NF-κB gene silencing

In order to confirm the role of NF-κB, impact of siRNA mediated gene silencing in human saphenous vein SMCs was examined. SMCs were transfected with NF-κB siRNA-1 and 2 (1:1) and to validate siRNA mediated knockdown, cells were harvested at 48 h post transfection prior to high glucose stimulation. A knockdown of approximately 60% of NF-κB p65 protein levels was observed (fig. 3.22 C; n=3, $p<0.05$). In these cells, the ability of high glucose to stimulate PAR-4 mRNA expression and protein levels was abolished. (fig. 3.22 A, B; n=5, $p<0.05$).

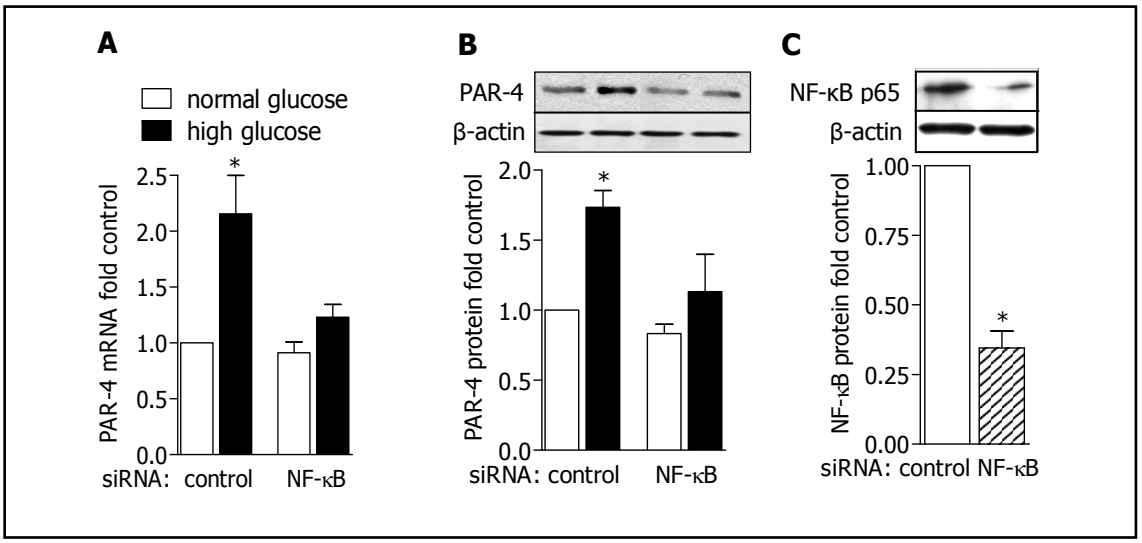

Figure 3.22 NF-κB silencing prevents high glucose induced **(A)** PAR-4 mRNA expression (6 h study; normalized to GAPDH) and **(B)** PAR-4 protein (38 kDa band; 48 h study; normalized to β-actin) in human saphenous vein SMC. **(C)** Validation of NF-κB p65 siRNA mediated knockdown (65kD NF-κB-p65 protein; normalized to β-actin) at 48 h post-transfection in same experiments by western blotting (n≥3, *p<0.05 vs unstimulated normal glucose control).

High glucose enhances binding of active NF-κB to PAR-4 promoter

A modified ChIP assay was performed to examine if high glucose activated NF-κB indeed binds to the human PAR-4 promoter. Since shuttling of active NF-κB to the nucleus was maximal at 3 h, human saphenous vein SMCs were stimulated with high glucose for this interval. Chromatin: NF-κB complexs were immunoprecipitated for NF-κB p65 subunit. After purification the resultant products were amplified by PCR using specific human PAR-4 primers. GAPDH primers were used as a control to observe purity of ChIP samples. A part of starting genomic material for ChIP, taken prior to immunoprecipitation, served as positive or input control. The ChIP assay revealed that high glucose enhanced NF-κB binding to the human PAR-4 promoter at 3 h. Furthermore, this binding was prevented by PKC inhibitor staurosporine, indicating the PKC dependency of NF-κB/PAR-4 promoter binding. Absence of any product signal of GAPDH PCR of ChIP samples indicates absence of contamination with genomic DNA in these samples (fig. 3.23; representative of n=3).

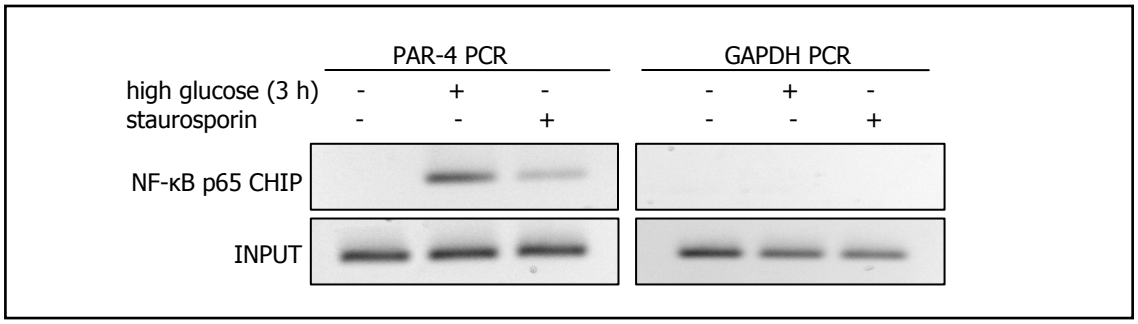

Figure 3.23 High glucose (3 h) stimulated NF-κB/PAR-4 promoter binding in human saphenous vein SMCs by ChIP assay. Inhibition of total PKC with addition of staurosporine (1 µmol/L, 30 minutes prior high glucose) reduced this binding. Input samples indicate starting whole genomic chromatin sample and serve as positive control, while ChIP samples indicate chromatin after NF-κB immunoprecipitation. The absence of bands in GAPDH PCR confirms that these samples were free of DNA contamination. Images are representative of 4 individual experiments.

3.3.4. Other mediators

Beside PKC high glucose is known to activate a number of signaling pathways in human vascular SMCs such as activation of mitogen activated kinases (MAPK), Rho kinases (ROCK) etc (Hashim et al. 2004; Kawamura et al. 2004; Lafuente et al. 2008). To investigate the effect of these signaling intermediates, specific inhibitor of phospholipase C (U-73122), ERK1/2 (PD-98059) or ROCK (Y-27632) was used. Both PLC and ERK inhibitors were found to prevent high glucose induced PAR-4 upregulation. Phospholipase C (PLC) is upstream of PKC, while ERK is involved in multiple pathways such as mitogenic signaling. This suggests that ERK contributes to PAR-4 mRNA expression in human saphenous vein SMCs exposed to high glucose (fig 3.24; $n=3$, $p<0.05$).

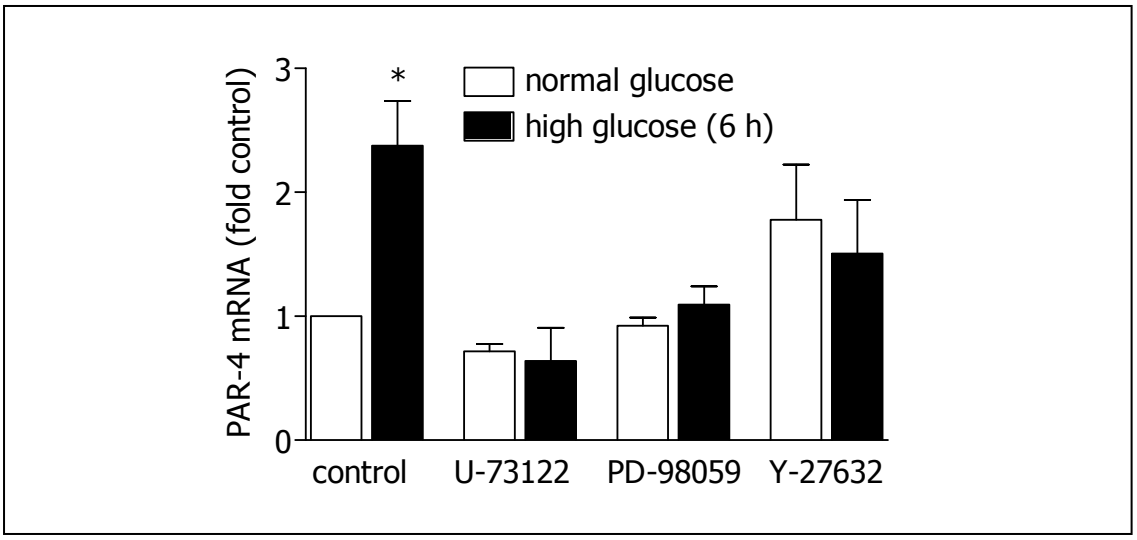

Figure 3.24 PAR-4 mRNA (6 h) in human saphenous vein SMCs stimulated with high glucose ±inhibitors: PLC inhibitor: U-73122 (10 µmol/L), ERK1/2 inhibitor: PD-98059 (20 µmol/L) or ROCK inhibitor: Y-27632 (10 µmol/L) (n=4; *P<0.05 vs unstimulated normal glucose control).

3.3.5. Role of oxidative stress

ROS mediate various signaling pathways that underlie vascular inflammation in atherogenesis and the complications of diabetes (Baynes 1991; Madamanchi et al. 2005). Recent evidences indicate that membrane-bound NAD(P)H oxidases are the major sources of $O^{\cdot}_2$ free radical generation in the vasculature and this is a critical event in gene regulation (Harrison et al. 2003a). The potential contribution of ROS dependent mechanism in high glucose regulated PAR-4 expression is to date not reported.

Inhibition of NAD(P)H Oxidase prevents high glucose induced PAR-4 regulation in human vascular SMC

To investigate the involvement of NAD(P)H oxidase to trigger PAR-4 regulation by high glucose inhibitors of NAD(P)H oxidase were utilized. Diphenyliodinium (DPI, an inhibitor of flavon containing oxidase) and apocynin are conventionally used to inhibit assembly and activation of the multimeric NAD(P)H oxidase complex (Mohan et al. 2007). In this study the

combination of DPI and apocynin completely prevented the stimulatory effect of high glucose on PAR-4 mRNA and total protein expression, indicating involvement of oxidative stress in this effect (fig 3.25 A, B; n=4, $p<0.05$).

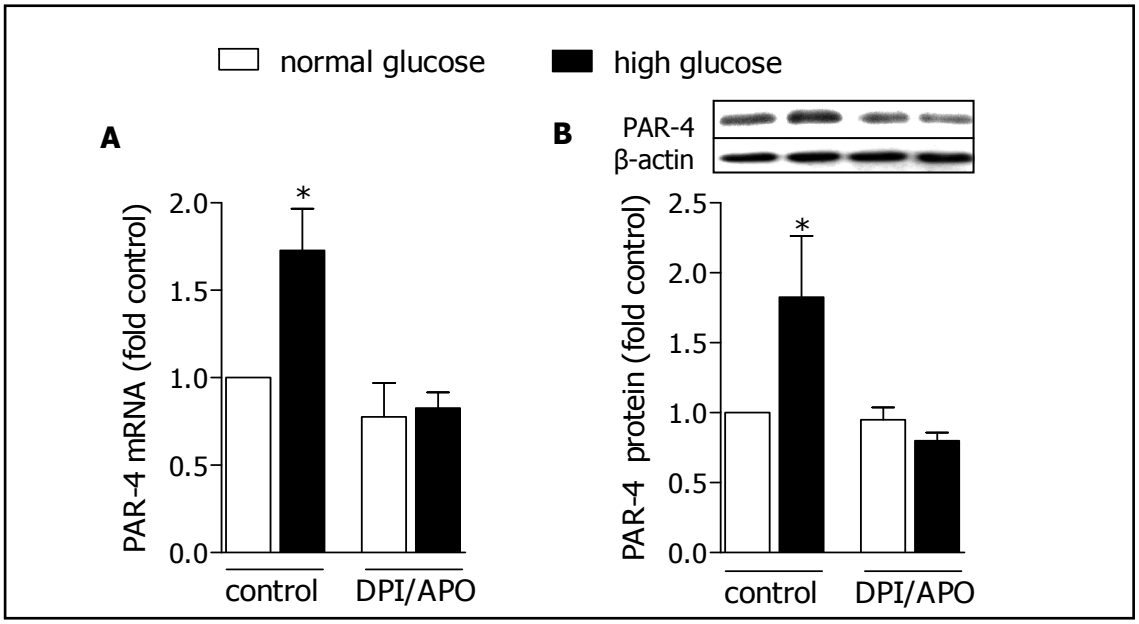

Figure 3.25 NAD(P)H oxidase inhibitors, DPI (10 µmol/L) and apocynin (100 µmol/L), prevent stimulatory effects of high glucose on **(A)** PAR-4 mRNA (collected after 6 h) and **(B)** PAR-4 total protein expression (collected after 48 h) in human saphenous vein SMC (n=4, *$p<0.05$).

This aspect of PAR-4 regulation was further examined using the classical stimulus for vascular NAD(P)H oxidase, the vasoactive peptide angiotensin-II (Ang II) (Browatzki et al. 2005; Harrison et al. 2003b). Enhanced Ang-II activity has been implicated in development of atherosclerosis, cardiac hypertrophy and vascular complications of diabetes and generation of ROS such as superoxide anion and H_2O_2 has been implicated in these pathologies (Browatzki et al. 2005; Marrero et al. 2005; Natarajan et al. 1999; Zafari et al. 1998). In this study, Ang-II enhanced PAR-4 mRNA expression within 1-3 h and also increased PAR-4 protein in human saphenous vein SMCs to a similar extent as high glucose (fig. 3.26 A, B; $n\geq3$, $p<0.05$).

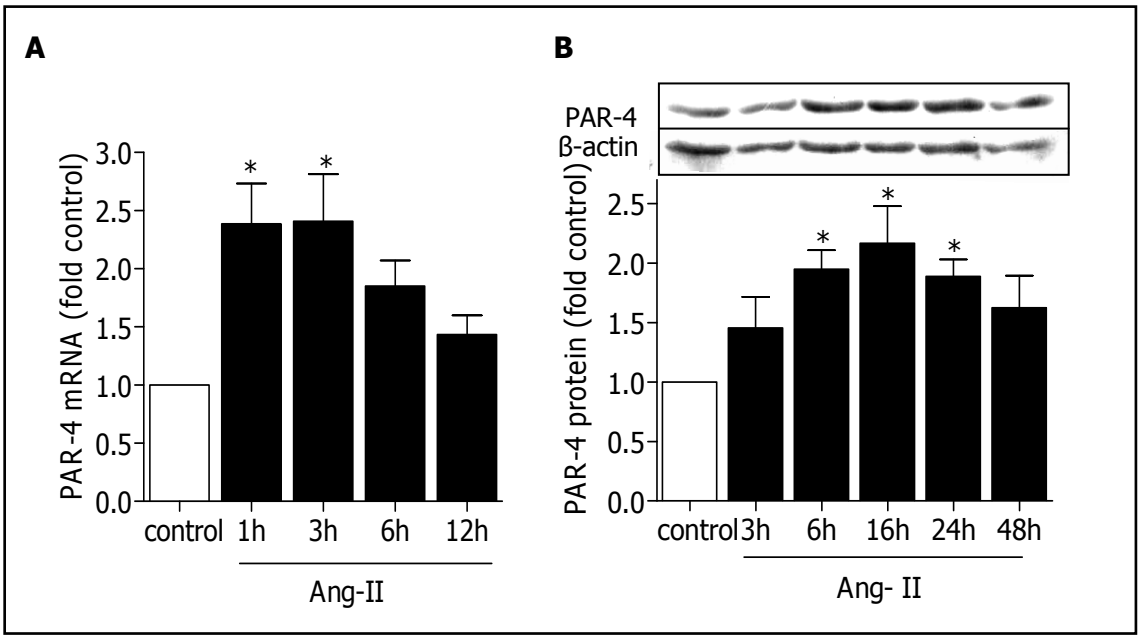

Figure 3.26 Ang-II enhances **(A)** PAR-4 mRNA expression relative to GAPDH as measured by qRT-PCR and **(B)** PAR-4 protein levels by western blotting in human saphenous vein SMCs (n=3-5, *p<0.05 vs unstimulated control).

The impact of the cellular oxidant H_2O_2 was also examined. H_2O_2 is generated enzymatically from superoxide anions in cells through the action of superoxide dismutase (Madamanchi et al. 2005). H_2O_2 performs important functions such as reacting with transition metals to produce highly reactive hydroxyl radicals ($OH^{\cdot}$) to destroy biomolecules through oxidation (Madamanchi et al. 2005). H_2O_2 also stimulates gene regulation, vascular SMC proliferation and triggers calcium signal leading to shape change of vascular SMC (Gonzalez-Pacheco et al. 2002; Lin et al. 2009). At higher concentrations however, H_2O_2 acts as a direct cellular oxidant involved in cell damage, apoptosis and progression of atherosclerosis (Li et al. 1997; Ross 1999). Here, H_2O_2 was found to rapidly induce PAR-4 mRNA expression within 1 h of treatment while changes in PAR-4 protein levels were seen from 3 h. Increased protein levels in contrast to mRNA levels, were sustained to 24 h (fig. 3.27 A, B; $n \geq 3$, $p<0.05$). Potentially additional post-translational mechanisms are likely to contribute to the PAR-4 regulation by H_2O_2.

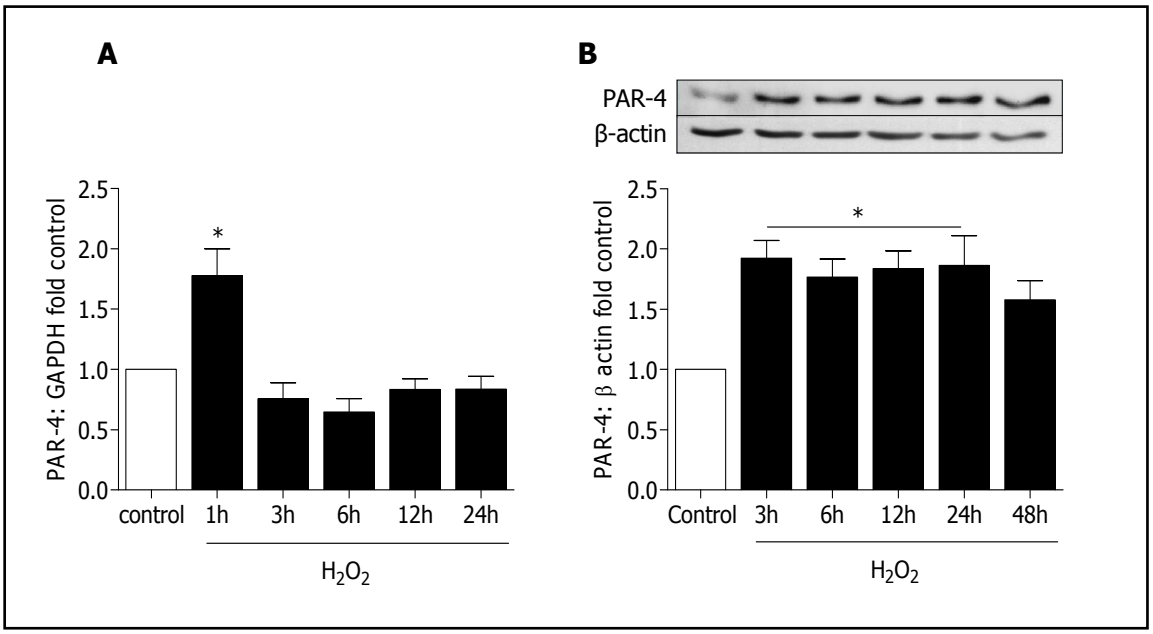

Figure 3.27 The oxidant species hydrogen peroxide (100 µmol/L) enhances **(A)** PAR-4 mRNA and **(B)** PAR-4 protein levels in human saphenous vein SMCs (n=3-5, *p<0.05 vs unstimulated control).

Collectively these findings implicate oxidative stress, specifically the NAD(P)H oxidase, in the high glucose regulated PAR-4 expression in human saphenous vein SMCs.

3.4. Immunohistochemical detection of PAR-4 in human diabetic atherosclerotic plaques

Thrombin and its receptor PAR-1 are intimately involved in the development of vascular disease such as vein graft failure or atherosclerosis, and are likely to also contribute to the cardiovascular complications of diabetes. Clearly high glucose has a direct and specific impact on the abundance of thrombin receptors, particularly PAR-4, in human vascular SMCs. To date there is no evidence to extrapolate these observations to the clinical setting. Therefore the distribution of PAR-4 in human diabetic carotid plaque was investigated by immunohistochemistry (fig.3.28 A-D). Actin staining to localize SMCs was performed in parallel tissue sections. In comparison to control staining, positive

PAR-4 immunoreactivity was seen in both vessel media and in the vicinity of the intimal plaque region (fig. 3.28 A, B: low power and D: high power). Actin staining for localization of vascular SMCs was seen in near-intimal (leuminal side) and medial regions (abluminal) of the plaque which corresponded to the regions showing positive PAR-4 immunoreactivity (fig. 3.28 C). No PAR-4 staining could be seen in vessel sections from nondiabetic patients (pictures not shown). This is the first evidence of presence of PAR-4 in human diabetic plaques.

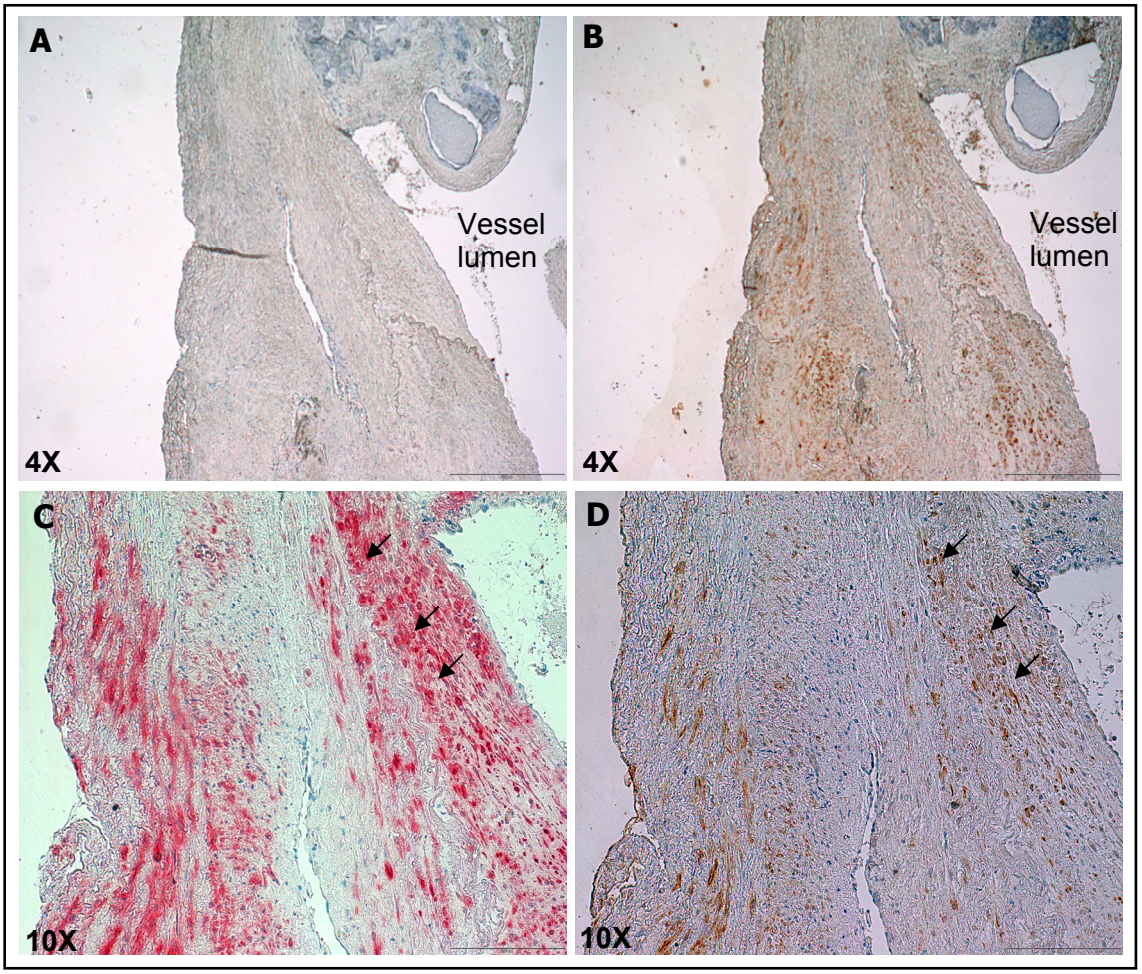

Figure 3.28 Immunohistochemical detection of PAR-4 in carotid atherosclerotic plaque from diabetic patient **(A)** control **(B)** PAR-4 (DAB stain in brown, using 4X objective, indicated with arrows) **(C)** SMC-actin (Fast red in pink) and **(D)** PAR-4 using 10X objective indicating high PAR-4 expression in near-lumeninal regions corresponding to smooth muscle cells rich regions as indicated by black arrows.

4. DISCUSSION

This study provides the first evidence of a direct regulatory action of high glucose on expression and function of vascular thrombin receptors. High glucose is shown to selectively induce PAR-4 mRNA, protein and cell surface expression in human saphenous vein SMCs, with no change in the constitutive expression levels of other thrombin receptors, PAR-1 and PAR-3. This high glucose regulation of PAR-4 is independent of changes in osmolarity, and involves PKC-δ activation leading to subsequent release of NF-κB from NF-κB:I-κB complex following phosphorylation and proteosomal degradation of I-κB in cytosol. Nuclear shuttling of free NF-κB to cell nucleus and binding to PAR-4 promoter then initiates PAR-4 transcription. Beside PKC and NF-κB activation, high glucose mediated-ROS generation via NAD(P)H oxidase is also found to contribute to high glucose regulation of PAR-4 in human vascular SMCs. Accordingly other pro-oxidant factors such as vasoactive peptide Ang-II or exogenous H_2O_2 also regulate PAR-4 in a similar manner.

The increased expression of PAR-4 is associated with enhanced calcium signaling, migration and TNF-α expression in response to PAR-4 activating peptide and thrombin in human saphenous vein SMCs. Responses to PAR-1 activating peptide are not influenced, regarding the unaltered expression levels of this receptor in high glucose treated cells. As thrombin exerts its responses via all thrombin receptor subtypes, this suggests that increased thrombin response in high glucose condition is due to enhanced PAR-4 component of thrombin response. Together with the high abundance of PAR-4 in human diabetic plaques, these findings implicate the importance of PAR-4 thrombin receptor in the vasculature, and strongly suggest that enhanced PAR-4 expression and function by high glucose may influence vascular responses of thrombin, especially in diabetic settings. Fig. 4.1 depicts the schematic overview of the present study.

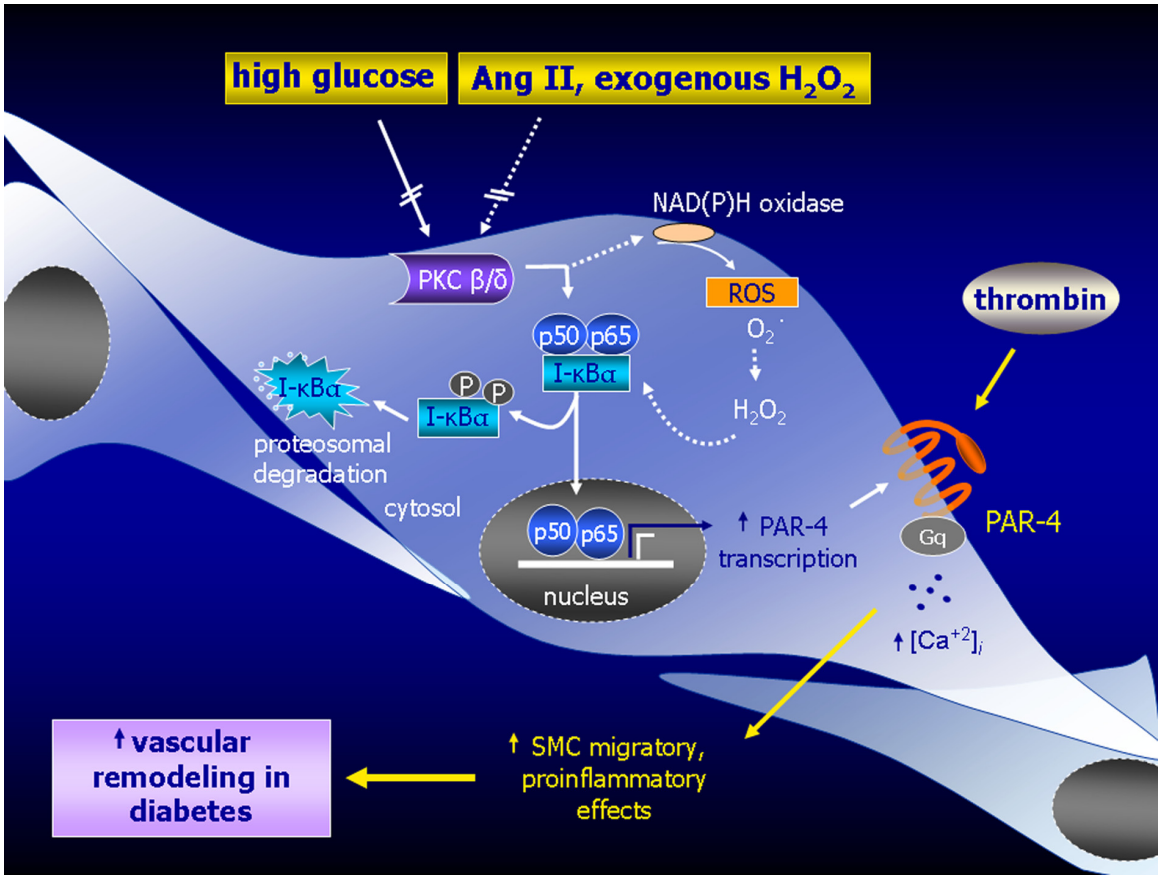

Figure 4.1 Schematic overview of the present study: High glucose activates PKC isoforms β or δ followed by phoshphorylation led degradation of I- κBα and consequent translocation of NF-κB p65 to nucleus to selectively increase PAR-4 transcription and surface expression in human vascular SMCs. High glucose also increases NAD(P)H oxidase dependent ROS generation ($O_2^{\cdot}$ and H_2O_2). This is likely to activate NF-κB and thereby to regulate PAR-4 in vascular SMCs via a similar mechanism. Ang-II and exogenous H_2O_2 also elicit PAR-4 upregulation. Enhanced PAR-4 expression, in association with its enhanced calcium signaling, contributes to enhance the migratory and proinflammatory responses of thrombin under high glucose setting. Solid lines indicate mechanism elucidated in the present study, dotted lines show signaling events previously reported by other groups.

4.1. Human vascular thrombin receptor regulation by high glucose

Diabetes is strongly associated with vascular complications and increased thrombin generation leading to atherothrombosis (Beckman et al. 2002; Undas et al. 2008). Thus thrombin may play a key role in diabetese associated vascular pathologies. The relative contribution of individual PARs in these vascular complications is not known. PAR-1, the prototypical thrombin receptor, is conventionally considered as the key player in vascular thrombin response, while relatively little is known about the contribution of other thrombin

receptor subtypes. Here, an *in-vitro* model to mimic *in-vivo* hyperglycemia was utilized. Concentration- response studies show high glucose enhances PAR-4 mRNA expression in a concentration- dependent manner. The most reproducible responses were observed at 25 mmol/L glucose, which corresponds to 450 mg/dL and may be observed in poorly controlled hyperglycemic patients and in diabetic emergency situations. This relatively high concentration of glucose is conventionally used to mimic chronic deleterious effects of hyperglycemia *in-vitro* (Little et al. 2007; Totary-Jain et al. 2005), as in the cell culture system, exogenous glucose is the sole nutrient available for cells.

Exposure of vascular SMCs to 25 mmol/L glucose led to a significant and sustained increase in PAR-4 mRNA and total protein and cell surface expression. PAR-1 and PAR-3 by contrast were not regulated. Similarly, PAR-4 promoter activity, as determined by luciferase reporter assay was stimulated by high glucose, while activities of the PAR-1 or PAR-3 promoters were not influenced. Such a differential regulation reflects previous reports by our laboratory that thrombin elicits a differential regulation of PARs. Both PAR-3 and PAR-4, but not PAR-1, are dynamically regulated thrombin receptors and PAR-4 regulation is more pronounced in human saphenous vein SMCs than in arterial SMCs exposed to thrombin (K. Schrör, unpublished data). This raises the interesting possibility that different PARs, particularly PAR-4, play distinct roles in platelet aggregation, myocardial infarction and cardiovascular remodeling (Coughlin 2005).

In the present study, high glucose was found to also regulate PAR-4 in vascular SMCs from human coronary artery, however with different kinetics. The induction of PAR-4 mRNA occurred relatively late (at 3 h) and was of shorter duration (to 48 h) in comparison to early onset (1.5 h) and sustained effect (to 96 h) in human saphenous vein SMCs. Thus regulation of thrombin receptors may be tissue specific and such an intrinsic difference may underlie the lower patency, higher risk of restenosis and failure rates of bypass vein grafts than arterial grafts (Cho et al. 2006).

The cellular response of any agonist does not only depend on agonist concentration or affinity of receptors to agonist, but may also be affected by the availability of receptors. However, the affinity of PAR-1 to thrombin (0.05 nmol/L) is about 100 fold lower than that of PAR-4 (5 nmol/L) (Steinberg 2005), in pathological settings such as in atherothrombosis, or after vascular injury, the overwhelming generation of thrombin in the vicinity of vascular SMCs would suffice to activate PAR-4 despite of its lower affinity to thrombin (Schrör et al. 2010). In this situation, the availability of regulated receptors would become an important variable rather than the intrinsic activity of different receptors to their agonist. The findings of the present study clearly demonstrate that PAR-4, in setting of high glucose unlike PAR-1 or PAR-3, is a dynamically regulated thrombin receptor. Therefore PAR-4 may play a unique role as an intermediate between atherothrombosis and vascular complications of diabetes.

4.2. Functional significance of PAR-4 regulation by high glucose

The selective regulation of PAR-4 by high glucose is likely to be associated with enhanced net responses to thrombin in human vascular SMC. The immediate response to thrombin receptor activation in vascular SMC is a transient increase in intracellular calcium, an important signal for a diverse range of cellular functions such as cellular growth and migration as well as gene transcription (Berridge et al. 2003; Crabtree & Schreiber 2009; House et al. 2008; Lipskaia & Lompre 2004). Thrombin, PAR-4AP and PAR-1AP induced a similar pattern of transient $[Ca^{2+}]_i$ increases in vascular SMC. In a sequence application of PAR-4AP followed by PAR-1AP and then thrombin, all stimuli were able to elicit increases in $[Ca^{2+}]_i$, but when applied in reverse sequence (thrombin followed by PAR-4AP or PAR-1AP), or with repeated application of same agonist (thrombin followed by thrombin or PAR-4AP followed by PAR-4AP), no such $[Ca^{2+}]_i$ increase in response to second thrombin application or PAR-AP was

detected. These observations are consistent with rapid desensitization of thrombin receptors (Coughlin 2000).

Under resting conditions, all thrombin receptors PAR-1, PAR-3 and PAR-4, are expressed at the cell surface of vascular SMCs and are responsive to thrombin. Upon activation receptors are rapidly uncoupled from the cellular signaling or are desensitized by other mechanisms and would no longer be available for repeated application of the same stimuli (Vouret-Craviari et al. 1995). The ability of thrombin to induce a residual $[Ca^{2+}]_i$ elevation when applied after PAR-4AP and PAR-1AP can possibly be explained by the activation of a further thrombin receptor, PAR-3 (Bretschneider et al. 2003). This residual calcium signal was relatively modest indicating that PAR-1 and PAR-4 are the predominant receptor subtypes mediating thrombin induced calcium signaling in these SMCs. Pretreatment of human saphenous vein SMCs with high glucose (48 h) selectively enhanced the transient $[Ca^{2+}]_i$ elevation elicited by a first application of either thrombin or by PAR-4AP, but not of PAR-1. This reflects the specific induction of PAR-4 but not other thrombin receptors by high glucose in vascular SMCs.

Vascular SMC migration is a central event in the tissue-repair process subsequent to injury, but excessive migration facilitates neointima formation and lumen narrowing (Rudijanto 2007). Thrombin has been shown to increase migration *in-vitro* and contribute to tissue repair *in-vivo*. In a wound-scratch migration assay, a modest migratory effect with PAR-4AP or PAR-1AP was seen under normal glucose conditions. Similar to calcium signals, pretreatment of human saphenous vein SMCs with high glucose (48 h) strongly enhanced the migratory responses to both thrombin and PAR-4AP but not to PAR-1AP. This indicates a significant contribution of PAR-4 to the enhanced net migratory response of thrombin under hyperglycemic conditions. Enhanced migration of vascular SMCs is essential for neointima formation, restenosis and tissue repair processes *in-vitro* as well as *in-vivo* (Pinkaew et al. 2009; Rudijanto 2007). Potentiating effect of high glucose on thrombin or PAR-4 mediated migration would be consistent with the higher rates of in-stent restenosis in diabetic

patients (Scheen et al. 2004; Stone et al. 2007). Intuitively this might seem to contradict the impaired healing of surface wounds such as foot ulcer in diabetic patients (Jeffcoate et al. 2004), however, this discrepancy may likely to be due to differences in tissue properties and the relative contribution of thrombin and its receptors in response to injury in extravascular regions.

Beside vascular SMC migration and proliferation, inflammation is a key factor in atherosclerosis and remodeling (Fan et al. 2003; Ross 1993). Diabetes is associated with a chronic inflammation, as shown by increased plasma levels of inflammatory cytokines such as TNF-α, IL-6 and others (Pickup et al. 2000), which are involved in the development of atherosclerosis (Ridker 2009). Thrombin, acting via PARs, is also known to promote inflammatory state by inducing early TNF-α expression (Kim KY 2002). In the current study the potential impact of high glucose on inflammatory signaling of PAR-4 and thrombin was examined. High glucose pretreatment enhanced TNF-α gene expression both at the basal level and upon stimulation with PAR-4AP or thrombin. Augmented local inflammatory actions of thrombin and specifically PAR-4, could therefore contribute to the net detrimental action of high glucose in the vessel wall.

To clearly identify if increased PAR-4 expression underlies the enhanced net cellular response of thrombin by high glucose, PAR-4 expression was knocked down using specific siRNA. In cells transfected with siRNA against PAR-4, the high glucose induced enhancement of thrombin actions both migration and inflammatory gene expression was lost. These observations highlight PAR-4 as a critical mediator of the vascular effect of thrombin, which to date has received little attention.

4.3. Mechanisms of vascular PAR-4 regulation by high glucose

Thrombin receptors being single-use receptors are likely to be controlled in part by transcriptional mechanism. In order to investigate the effect of high glucose on promoter activities of thrombin receptors in human vascular SMC, luciferase reporter assay revealed the selective transcriptional regulation of PAR-4, but not of PAR-1 or PAR-3. Also the effect of osmolar control mannitol did not regulate PAR-4 mRNA. Thus the PAR-4 regulatory actions of high glucose are unlikely to involve changes in osmolarity but rather a specific signaling leading to transcriptional changes in PAR-4. To investigate the intermediate signaling molecules responsible for the regulatory effects of high glucose on PAR-4, pharmacological inhibitors of PKC were used. PKC, an ubiquitous enzyme, is a critical intracellular mediator of high glucose signaling in human vascular SMCs (Koya & King 1998; Sheetz & King 2002), and extensive data support a central role of PKC in high glucose- stimulated gene expression, vascular SMC proliferation, and atherothrombosis (Devaraj et al. 2009; Itoh et al. 2001). Investigations on selective or isoform-specific PKC inhibitors have attracted great attention during last decades as PKC-mediated cellular processes are mostly tissue- and isoform- specific. PKC-δ in particular is reported to promote vascular SMC proliferation in diabetes (Rask-Madsen & King 2005; Yamaguchi et al. 2004). Recent studies also demonstrated that LY333531, a PKC-β specific inhibitor, prevented hyperglycemia-induced impairment of endothelial-dependent vasodilation in healthy subjects (Shen 2003). Thus both isoforms are likely to be involved in diabetic vascular pathologies.

Human saphenous vein SMCs express conventional (α, β), novel (δ, ε and μ) and atypical (λ, ζ) PKC isozymes (Itoh et al. 2001). Of these, the PKC- β and -δ isozymes appear to be preferentially activated by high glucose (Koya & King 1998). Here, inhibition of PKC with nonsubtype specific inhibitors-staurosporine or calphostin-C, or of PKC-δ with rottlerin or specific siRNA against PKC-δ, identified this isozyme as a critical mediator of high glucose stimulated PAR-4 expression in human vascular SMCs. An inhibitor of PKC-β

could also prevent high glucose induced PAR-4 mRNA and protein upregulation, indicating involvement of other PKC subtypes in this effect.

In present study, cellular responses such as vascular SMC migration or calcium signaling in response to PAR-1 activation were not altered in high glucose pretreated SMCs, whereas selective PAR-1 activation was able to induce vascular SMC migration or calcium signal in normal glucose cultures. Since high glucose did not influence PAR-1 expression, possible explanations may be the altered coupling to signaling molecules or even the desensitization of PAR-1 receptor. Actions of PAR-1 and PAR-4 are mostly attributed to their coupling to Gi, $G_{12/13}$ or Gq proteins respectively (Coughlin 2005; Offermanns et al. 1994). In diabetic settings, Gi expression and coupling to receptors is suppressed, whereas Gq expression is enhanced (Hashim et al. 2004; Wichelhaus et al. 1994). Furthermore, PKC-β activation by phorbolester desensitizes PAR-1 receptors and reduces the calcium signal in response to PAR-1 activation (Yan et al. 1998). In present study, high glucose stimulated PAR-4 regulation involved PKC-β, which could additionally desensitize PAR-1 at the same time. This suggests that either or both proposed explanations may fit to the high glucose setting. Thus under high glucose conditions, enhanced expression and resultant cellular responses of PAR-4 would be more influential to net cellular responses of thrombin in human vascular SMCs.

A major target downstream of PKC is the transcription factor NF-κB (Hattori et al. 2000). Activated NF-κB has been detected in vascular SMCs of carotid artery after balloon injury (Landry et al. 1997) and in the intima and media of atherosclerotic vessel sections (Wilson et al. 2002), suggesting an important role in development of atherosclerosis. There is some evidence of NF-κB activation leading to thrombin receptor regulation in vasculature. One study carried out in endothelial cells shows that PAR-4 but not PAR-1 mRNA is upregulated by inflammatory cytokine TNF-α or IL-1, most probably via NF-κB activation (Hamilton et al. 2001). The role of NF-κB to regulate functional thrombin receptors in vascular SMCs still needs to be defined.

A sequence analysis of the human PAR-1, PAR-3 and PAR-4 promoters was performed using two different databases- www.gene-regulation.com/ and www.genomatix.de/. This revealed the presence of an NF-κB binding motif (GGGACCCCCC) at position 543 upstream of the ATG. No such site could be identified in the human PAR-1 or PAR-3 promoters, which could explain the selective regulation of PARs by high glucose. Pharmacological inhibition or siRNA-induced knockdown of NF-κB prevented high glucose-stimulated PAR-4 mRNA and protein in human saphenous vein SMCs.

NF-κB is normally sequestered in the cytoplasm of non-stimulated cells as an inactive trimeric complex of I-κB/p65/p50, but rapidly translocates to nucleus upon cellular stimulation for example with TNF-α or IL-6 etc (Ghanim et al. 2004). Activation of NF-κB involves phosphorylation of I-κB (isoforms α or β) by an I-κB kinase complex, with subsequent polyubiquitination and proteosomal degradation. This allows the translocation of the active NF-κB dimer (p65/p50) to the nucleus, where it binds to cognate DNA sequence to regulate gene transcription (Karin & Ben-Neriah 2000).

NF-κB signaling is involved in dysregulation of vascular SMCs *in-vitro* or in human atherosclerosis (Bourcier et al. 1997). Further NF-κB gene polymorphism can presents a risk for pathogenesis of diabetes mellitus in human (Romzova et al. 2006). In the human saphenous vein SMCs used in the present study, high glucose induced a rapid (within 3 hours) phosphorylation of cytosolic I-κBα and as a consequence a stimulated translocation of the free NF-κB p65 to the nucleus. The specific binding of free NF-κB p65 subunit to the NF-κB binding site of the human PAR-4 promoter was validated by ChIP analysis. The interaction between NF-κB and PAR-4 was suppressed by the PKC inhibitor staurosporine further highlighting the central role of PKC as a critical regulator of PAR-4 upstream of NF-κB.

A further player in diabetes associated vascular complications is oxidative stress, characterized by overburden of ROS production and suppressed oxidative defense mechanism in the cells, which then leads to hypertrophy, deleterious redox signaling and vascular tissue damage (Gao & Mann 2009).

Redox enzyme- vascular NAD(P)H oxidase serves as a major source of vascular ROS generation (Lassegue et al. 2003), which may increase intracellular calcium, activate protein kinases, stimulate DNA synthesis mitogens and activate transcription factors such as NF-κB and thereby controlling gene expression and vascular response to injury (Berk 1999).

An increase in NAD(P)H oxidase subunit expression is demonstrated in diabetic patients, which partly normalizes after lowering of plasma glucose (Avogaro et al. 2003). Generation of $O_2^{\cdot}$ and its metabolites H_2O_2, $ONOO^-$ and OH^- are responsible for many detrimental consequences of vascular hyperglycemia, such as endothelial dysfunction, oxidative modification of protein and lipids and the regulation of gene expression (Sheetz & King 2002). High glucose as well as thrombin are known to activate the vascular NAD(P)H oxidase through PKC mediated phosphorylation of the subunit p47phox (Inoguchi et al. 2003). Antioxidant treatment has been reported to reduce atherothrombosis and attenuate neointima formation by inhibiting ROS in the vascular SMCs (De Rosa et al.; Won et al. 2009; Wu et al. 2009). There is some evidence that PAR-1 can be regulated in ROS dependent manner, but whether ROS participate in regulation of PAR-4 has not been reported (Capers et al. 1997; Nguyen et al. 2001). In present study, inhibition of NAD(P)H oxidase with the combination of DPI and apocynin prevented the high glucose stimulated PAR-4 in human vascular SMCs.

In addition to generation of intracellular O_2^- by redox enzymes, extracellular stimuli including Ang-II, exogenous H_2O_2, cytokines, growth factors and lipophilic substrates may modulate cellular redox state. Ang-II, a vasoactive peptide is a classical activator of vascular NAD(P)H oxidase and contributes to bulk of ROS produced in vascular cells (Griendling et al. 2000). It is also involved in the development of cardiovascular diseases such as hypertrophy via superoxide and H_2O_2 production and perhaps through mechanisms independent of hypertension (Sleight et al. 2001; Zafari et al. 1998). Both Ang-II and exogenous H_2O_2 mimicked the regulatory action of high glucose on PAR-4 expression in vascular SMCs. Thus PAR-4 expression is clearly regulated in a

redox dependent manner, and this mechanism likely to contribute to the thrombotic, proliferative and inflammatory events after vascular injury.

4.4. Clinical relevance and future prospects

Given the ability of high glucose to directly or specifically augment PAR-4 expression and function, this thrombin receptor may represent a central player in cardiovascular remodeling and atherosclerosis in diabetic patients (Lytle et al. 1985; Scheen & Warzee 2004). Particularly since PAR-4, unlike PAR-1, appears to be subject of an auto-regulatory feedback mechanism leading to enhanced expression in response to thrombin or selective receptor-activating peptide. The high local levels of thrombin on the vicinity of the lesion could serve to maintain high expression of PAR-4 in addition to effects of high glucose. In this context, the demonstration of PAR-4 immunoreactivity in atherosclerotic plaques from diabetic vs healthy vessels, in near-intimal regions strongly suggests high level of PAR-4 expression in atherosclerotic plaques. Co-localization of PAR-4 with SMCs migrated to form a cellular cap in near-intimal regions of diabetic plaques supports an important role of PAR-4 in diabetes related vascular pathologies.
The potential role of PAR-4 as a therapeutic target in this regard remained to be defined. While a recent study in apo-$E^{-/-}$/PAR-$4^{-/-}$ mice showed that PAR-4 does not alter development and progression of early atherosclerosis, (Hamilton et al. 2009) these findings do not preclude a central role of PAR-4 in diabetic vascular pathology. The distribution and particularly the function of individual PARs differ greatly between mouse and human and make it difficult to directly correlate the results of animal models to human vascular pathologies. The observations that increased PAR-4 expression in association with enhanced functions in response to thrombin via PAR-4 in human saphenous vein SMCs exposed to high glucose and that PAR-4 is highly abundant in human diabetic plaque support a central role of this thrombin receptor in diabetic vascular pathophysiology.

Thrombin receptors have been attracted much attention during the last few years in the search for safer and novel treatments for atherothrombosis and other vascular disorders (Angiolillo et al.; Oestreich 2009). A PAR-1 antagonist, SCH 530348, may prove promising with reduced risk of bleeding and less hemostatic disturbances. It is currently undergoing clinical trials to investigate its clinical benefit over other antithrombotic agents. In a phase II clinical trial of patients undergoing percutaneous coronary intervention, SCH-530348 added to standard therapy with aspirin did not enhance bleeding, and demonstrated a trend towards decreased major adverse cardiovascular events versus placebo (Oestreich 2009). Given the findings of present study that PAR-4 rather than PAR-1 influences thrombin mediated responses in smooth muscle cells under high glucose condition, this approach to use PAR-1 antagonist alone may be inadequate to treat thrombosis in diabetic patients, and may need to inhibit PAR-4 as well.

A few PAR-4 antagonists are now available to inhibit PAR-4 responses *in-vivo*. Peptide PAR-4 inhibitor trans-cinnamoyl-YPGKF-amide (tc-Y-NH(2)) and a cell penetrating pepducin- palmitoyl-SGRRYGHALR-amide (P4pal10), and a synthetic PAR-4 inhibitor YD-3 [1-benzyl-3-(ethoxycarbonylphenyl)-indazole] have been characterized *in-vitro* and *in-vivo* systems (Strande et al. 2008; Wu et al. 2002), however unlike PAR-1 antagonist SCH-530348, none of the PAR-4 antagonists could reach the clinical trials. P4pal10 (10 µg/kg) treatment significantly decreased infarct size in rat model of ischemia- reperfusion injury. Tc-Y-NH(2) (5 µmol/L) treatment before ischemia decreased infarct size by 51% *in-vitro* and increased recovery of ventricular function by 26% (Strande et al. 2008). Synthetic inhibitor YD-3 showed inhibition in platelet aggregation induced via PAR-4 (Quinton et al. 2004; Wu et al. 2002). Wu et al. showed that inhibition of both PAR-1 and PAR-4 receptors is necessary for effective inhibition of platelet activation (Wu 2006). Further research in thrombin receptors' regulation and function in different pathological settings is required to better understand the pathophysiology of vascular disorders and to reveal new targets for anti-restenotic therapy, besides inhibition of PAR-1.

In this study differential regulation of thrombin receptors by high glucose may explain the enhanced thrombin activity and vascular dysfunctions in diabetic patients. It shows an ability of high glucose to activate diverse signaling pathways including PKC, NF-κB and ROS generation leading to selective, rapid and sustained upregulation of PAR-4 in human vascular SMCs. Enhanced expression of PAR-4 leading to enhanced calcium signaling, SMC migration and inflammatory gene expression mediated in response to thrombin via PAR-4. The current study sights the possibility of targeting only PAR-1 may not be sufficient, especially in thrombosis or restenosis associated with diabetes, rather PAR-4 may be an appropriate target for novel anti- restenotic therapies in diabetes associated vascular pathologies.

5. SUMMARY

Diabetes is clinically associated with enhanced thrombin generation, atherothrombosis and vascular remodeling. Clotting factor thrombin could modify these pathologies via protease-activated receptors- PAR-1, PAR-3 and PAR-4, exerting pleiotropic effects on vascular SMCs. This study investigates the possible regulation of thrombin receptors by high glucose in SMCs

Human saphenous vein SMCs were incubated under normal (5.5 mmol/L) or elevated (25 mmol/L) glucose conditions to investigate the influence on thrombin receptor expression, signaling and function. High glucose treatment selectively up regulated PAR-4 mRNA, protein and surface expression but not PAR-1 and PAR-3 expression in vascular SMCs. This regulation of PAR-4 was found to be independent of osmolar changes. Specific inhibitors of PKC-β or -δ or NF-κB prevented effect of high glucose on PAR-4 expression. Similar results were obtained from siRNA mediated silencing of PKC-δ or NF-κB in vascular SMCs. High glucose treatment induced NF-κB activation and translocation to the cell nucleus (maximal at 3 h pretreatment), where it could bind to the PAR-4 promoter, as demonstrated by the ChIP assay. Luciferase reporter assay indicated the possible transcriptional regulation of PAR-4 by high glucose.

Beside PKC and NF-κB activation, high glucose mediated-ROS generation via NAD(P)H oxidase was also found to contribute as selective inhibitors to NAD(P)H oxidase prevented the high glucose regulation of PAR-4 in human vascular SMCs. Like high glucose, other pro-oxidants such as Ang-II or exogenous H_2O_2 could enhance PAR-4 expression, probably via PKC dependent ROS generation and NF-κB activation (Mohan et al. 2007; Rask-Madsen & King 2005).

This selective PAR-4 upregulation by high glucose was associated with enhanced SMC migration, intracellular calcium signaling and inflammatory gene expression in responses to thrombin or PAR-4AP but not to PAR-1AP in human vascular SMCs. The positive immunoreactivity to PAR-4 and its colocalization with SMCs was seen in the vicinity of the intimal plaque region of diabetic atherosclerotic plaques.

The present study provides the first evidence of a direct regulatory action of high glucose on expression and function of vascular thrombin receptors. Findings of the study highlight a unique role of PAR-4 in settings of hyperglycemia, which is likely to contribute to the enhanced cellular effects of thrombin and exaggerated cardiovascular complications of diabetes. This study suggests that targeting only PAR-1 may not be sufficient to treat diabetes associated atherothrombotic complications and sights that PAR-4 may be a novel and potential target for antithrombotic and antirestenotic therapeutics, specially in diabetic patients.

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7. PUBLICATIONS

7.1. Research Papers

- Dangwal S, Rauch BH, Gensch T, Dai L, Bretschneider E, Schrör K, Rosenkranz AC, '**High glucose upregulates human vascular protease-activated receptor-4 via protein kinase C and nuclear Factor kappa-B**' (submitted to Atherosclerosis, thrombosis and Vascular biology).

- Balaraman R., Dangwal S., Mohan M. '**Antihypertensive effect of *Trigonella foenum-graecum* seeds in experimentally induced hypertension in rats**'; Pharmaceutical Biology 2006; 44(8): 568- 575.

7.2. Abstracts (Proceeding of scientific conferences)

- '**High Glucose Upregulates Protease Activated Receptor-4 in Human Vascular Smooth Muscle Cells**', S. Dangwal, B. Rauch, K. Schrör, AC Rozenkranz, Poster in Atherosclerosis thrombosis vascular biology, San Francisco, CA (April 2010).

- **'High glucose induces protease-activated receptor-4 (PAR-4) upregulation in human vascular smooth muscle cells via Protein kinase C-δ and nuclear factor kappa B**', S. Dangwal, B. Rauch, K. Schrör, AC Rozenkranz, oral pesentation in 50th annual meeting- German Society of Experimental and Clinical Pharmacology and Toxicology, Mainz, Germany (February 2010).

- '**Transcriptional regulation of protease activated receptor-4 in human vascular smooth muscle cells**', S. Dangwal, B. Rauch, K. Schrör, AC. Rozenkranz; oral presentation in 'New Drugs in Cardiovascular Research' a joint meeting of 'British Pharmacological Society', 'German Society of Pharmacology' 'German society of Clinical Pharmacology and Therapy', Dresden, Germany (May 2009).

- '**Increased expression of protease-activated receptor-4 in human vascular smooth muscle cells in response to high glucose',** S. Dangwal, K. Jobi, B. Rauch, K. Schror, AC Rozenkranz; oral presentation in 50th annual meeting- German Society of Experimental and Clinical Pharmacology and Toxicology, Mainz, Germany (March 2009).

- '**High glucose up-regulates protease-activated receptor-4 (PAR-4) in human vascular smooth muscle cells**', S. Dangwal, B. Rauch, K. Schrör, AC Rozenkranz oral presentation in annual meeting- Society of Thrombosis and Hemostasis Research, Vienna, Austria (February 2009).

- '**Upregulation of PAR-4 thrombin receptors in high glucose-treated human vascular smooth muscle cells'**, S. Dangwal, B. Rauch, K. Schrör, AC Rozenkranz ; **Best oral presentation** in Joint International Conference of the International Society for Heart Research and the International Academy of Cardiovascular Sciences, Indian section, Surat, India (December 2008).

- '**Elevated glucose transcriptionally upregulates protease-activated receptor-4 (PAR-4) thrombin receptor in human vascular smooth muscle cells**', S. Dangwal, B. Rauch, K. Schrör, AC Rozenkranz; poster in International Conference of Translational Pharmacology and 41st annual conference of Indian Pharmacological Society, AIIMS, New Delhi, India (December 2008).

- '**Modulation of NAD(P)H oxidase subunit mRNA expression by activated factor X and high glucose in human vascular smooth muscle cells**' K. Jobi, S. Dangwal, B. Rauch, K. Schrör, AC Rozenkranz; poster in annual meeting of German Society of Thrombosis and Hemostasis Research, Wiesbaden, Germany (May 2008).

8. ACKNOWLEDGEMENTS

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9. OFFICIAL LEAGALLY BINDING STATEMENT (EIDESSTTATLICH ERKLAERUNG)

Ich versichere an Eides statt, dass die vorliegende Doktorschrift selbstständig und ohne fremde Hilfe angefertig worden ist, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt worden sind, den benutzten Werken wörtlich oder inhaltlich entnommene Stellen als solche kenntlich gemacht zu haben, und dass diese Doktorschrift an keiner anderen Facultät eingereicht worden ist.

Düsseldorf, den 16. Juni 2010

10. CURRICULUM-VITAE

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- '**High glucose regulation of human vascular thrombin receptors**' in German Diabetes Centre, Düsseldorf, Germany (January 2010).

- '**High glucose upregulates human vascular protease-activated receptor-4 via protein kinase C and Nuclear Factor kappa-B**' in Institute of Structural Biology-1 and Structural Biophysics, Jülich Research Centre, Germany (November 2009).

- '**Regulation of vascular thrombin receptor by high glucose**' in Cardiology and Haemostasis Workshop- (German Society of Cardiology, German society of Thrombosis and Haemostasis Research, Austrian Society of cardiology), Solingen, Germany (November 2009).