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# **Diabetic Vascular Complications & Inflammation**

**- Role of NFAT and TNF $\alpha$**

Jenny Nilsson-Öhman

AKADEMISK AVHANDLING

som med vederbörligt tillstånd av Medicinska fakulteten vid Lunds universitet för avläggande av doktorsexamen i Medicinsk Vetenskap i ämnet Molekylär och cellulär fysiologi, kommer att offentligen försvaras i Medicinklinikens Aula, Ingång 35, UMAS, Malmö, fredagen den 16 oktober 2009, kl. 9.15.

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Professor Holger Nilsson

Sektionen för fysiologi

Göteborgs Universitet



*Till min familj*

*You are all I long for  
All I worship and adore*

*Ur "Fly me to the Moon" av Bart Howard*

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# TABLE OF CONTENTS

TABLE OF CONTENTS	5
LIST OF PAPERS	6
ABBREVIATIONS	8
BACKGROUND	11
Diabetes	11
Diabetes and Inflammation	15
The Diabetic Vascular Wall	15
Diabetic Atherosclerosis	17
Diabetic Retinopathy	19
Ca <sup>2+</sup> Signaling	19
NFAT	21
AIMS	26
METHODS	27
RESULTS & DISCUSSION	40
Glucose Activates NFAT in Native Arteries	40
Role of Extracellular Nucleotides	42
Role of GSK-3 $\beta$ and PKA	44
Hyperglycemia Induces NFAT Activity <i>in vivo</i>	47
NFAT Regulates Vascular Osteopontin Expression	48
Hyperglycemia Activates NFAT in Retinal Vessels	51
Role of TNF $\alpha$ in Diabetes-Induced Vascular Inflammation	55
CONCLUSIONS	59
POPULÄRVETENSKAPLIG SAMMANFATTNING	60
ACKNOWLEDGEMENTS	64
REFERENCES	66

# LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numerals:

- I. **J. Nilsson**, L.M. Nilsson, Y.W. Chen, J.D. Molkenkin, D. Erlinge & M.F. Gomez. High glucose activates Nuclear Factor of Activated T-cells (NFAT) in native vascular smooth muscle. *Arterioscler Thromb Vasc Biol.* 2006 Apr;26(4):794-800.
- II. L.M. Nilsson-Berglund, Anna V. Zetterqvist, **J. Nilsson-Öhman**, M. Sigvardsson, L.V. Gonzalez-Bosc, M.L. Smith, A.S. Salehi, E. Agardh, G. Nordin-Fredriksson, C.-D. Agardh, J. Nilsson, B.R. Wamhoff, A. Hultgårdh-Nilsson & M.F. Gomez. Nuclear Factor of Activated T-cells (NFAT) c3 regulates osteopontin expression in arterial smooth muscle in response to diabetes-induced hyperglycemia. *Arterioscler Thromb Vasc Biol*, 2009. (submitted)
- III. **J. Nilsson-Öhman\***, A.V. Zetterqvist\*, S. de Frutos Garcia, P.G. McGuire, L.V. Gonzalez Bosc & M.F. Gomez. Hyperglycemia activates the Ca<sup>2+</sup>/calcineurin-dependent transcription factor NFAT (Nuclear Factor of Activated T Cells) in retinal microvessels in vivo. 2009. (manuscript, \* indicates equal contributions).
- IV. **J. Nilsson-Öhman**, G. Nordin Fredrikson, L.M. Nilsson, C. Gustavsson, E. Bengtsson, M.-L. Smith, C.-D. Agardh, E. Agardh, S. Jovinge, M.F. Gomez & J. Nilsson. Tumor necrosis factor-alpha does not mediate diabetes-induced vascular inflammation in mice. *Arterioscler Thromb Vasc Biol*, 2009. (accepted)

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List of papers not included in the thesis:

L.M. Nilsson, Z.W. Sun, **J. Nilsson**, D.-W. Swensson, Yung-Wu Chen, Stevan W. Djuric, M.L. Lydrup & M.F. Gomez. Novel blocker of NFAT activation inhibits interleukin-6 production in human myometrial arteries and reduces vascular smooth muscle cell proliferation. *Am J Physiol Cell Physiol*. 2007 Mar;292(3):C1167-78.

D. Jönsson, **J. Nilsson**, M. Odenlund, G. Bratthall, J. Broman, E. Ekblad, M-L. Lydrup & B-O. Nilsson. Demonstration of mitochondrial estrogen receptor  $\beta$  and estrogen-induced attenuation of cytochrome c oxidase subunit I expression in human periodontal ligament cells. *Arch Oral Biol*. 2007 Jul;52(7):669-76.

L.M. Nilsson, **J. Nilsson-Öhman**, A. V. Zetterqvist & M. F. Gomez. NFAT (Nuclear Factor of Activated T-cells) transcription factors in the vasculature: The Good Guys or the Bad Guys? *Curr Opin Lipidol*. 2008 Oct;19(5):483-90. Review.



# ABBREVIATIONS

[Ca <sup>2+</sup> ] <sub>i</sub>	intracellular calcium concentration
ADP	adenosine 5' diphosphate
AGEs	advanced glycation end products
AIF	allograft inflammatory factor
Ang II	angiotensin II
AP-1	activator protein-1
ApoE	apolipoprotein E
ATP	adenosine 5' triphosphate
BK	large conductance Ca <sup>2+</sup> activated K <sup>+</sup> channels
BMP-2	bone morphogenic protein-2
BSA	bovine serum albumin
BTP	bis(trifluoromethyl)pyrazole
ChIP	chromatin immunoprecipitation
CK1 $\alpha/2$	casein kinase 1 $\alpha/2$
COX-2	cyclooxygenase 2
CRAC	Ca <sup>2+</sup> -release activated Ca <sup>2+</sup> channels
CREB	Ca <sup>2+</sup> -cyclic AMP response element binding protein
CsA	cyclosporine A
DAG	diacylglycerol
DCCT	diabetes control and complications trial
DSCR1	down's syndrome critical region-1
DYRK1a	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A
EDIC	epidemiology of diabetes interventions and complications
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
Et-1	endothelin-1
FBS	fetal bovine serum
FOXP3	forkhead box protein-3
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC/MS	gas chromatography/mass spectrometry
GLP-1	glucagon-like peptide
GM-CSF	granulocyte/macrophage-colony stimulating factor
GSK-3 $\beta$	glycogen synthase kinase-3beta
HDL	high-density lipoprotein
20-HETE	20-hydroxyeicosatetraenoic acid
HUVEC	human umbilical vein endothelial cells
ICAM	intercellular adhesion molecule
IFN- $\gamma$	interferon gamma
IL	interleukin
IP <sub>3</sub>	inositol triphosphate
IP <sub>3</sub> R	IP <sub>3</sub> receptor
IP-GTT	intraperitoneal glucose tolerance test
IRT	interferon responsive transcript
JNK	c-Jun N-terminal kinase
KC/GRO	keratinocyte-derived chemokine/ growth-regulated oncogene

LDL	low-density lipoprotein
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
MEF	myocyte enhancer factor
mTOR	mammalian target of rapamycin
NES	nuclear export signal
NFAT	nuclear factor of activated T-cells
NF $\kappa$ B	nuclear factor kappa-B
NLS	nuclear localization signal
NO	nitric oxide
NOS	nitric oxide synthase
OPN	osteopontin
PAI-1	plasminogen activator inhibitor-1
PARP	poly(ADP-ribose) polymerase
PDGF	platelet-derived growth factor
PE	phenylephrine
PI3K	phosphoinositide-3 kinase
PIP2	phosphatidylinositol 4,5-biphosphate
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PMCA	plasma membrane Ca <sup>2+</sup> ATPase
PPAR $\gamma$	proliferator-activated receptor gamma
ROS	reactive oxygen species
RyR	ryanodine receptor
SERCA	sarcoplasmic-endoplasmic reticulum Ca <sup>2+</sup> ATPase
SM- $\alpha$ actin	smooth muscle alpha actin
SM-MHC	smooth muscle myosin heavy chain
SOCE	store-operated Ca <sup>2+</sup> entry
Sp-1	specificity protein-1
SR	sarcoplasmic reticulum
SRF	serum response factor
STIM-1	stromal interaction protein-1
STZ	streptozotocin
TF	tissue factor
TGF $\beta$	transforming growth factor beta
TNF $\alpha$	tumor necrosis factor-alpha
TRPC	transient receptor potential channel
UDP	uridine 5' diphosphate
UKPDS	United Kingdom prospective diabetes study
USF	upstream stimulating factor
UTP	uridine 5' triphosphate
VCAM-1	vascular cell adhesion molecule-1
VDCC	voltage-dependent Ca <sup>2+</sup> channel
VEGF	vascular endothelial growth factor
VLDL	very low-density lipoprotein
VSMC	vascular smooth muscle cell
vWF	von Willebrand factor



# BACKGROUND

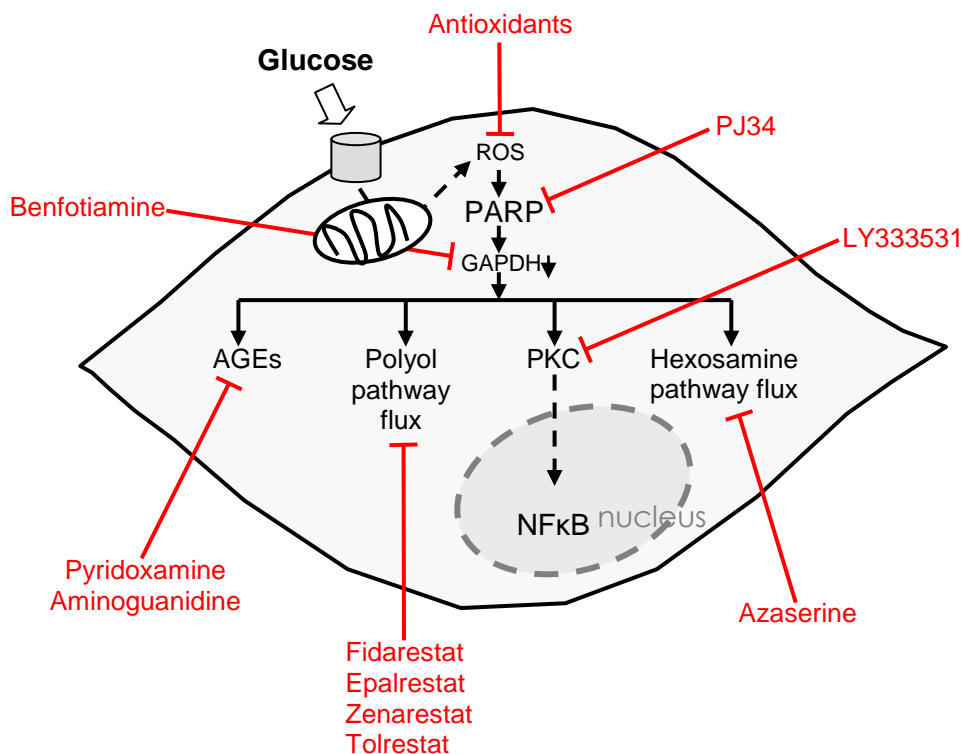
I first came in to contact with diabetes when I was working as a domestic help nursing assistant. One of my patients was a man in his early 50's who had suffered from diabetes almost all his life. When I met him he was recovering from a recent myocardial infarction. He was bound to his wheelchair because of his severely ulcerated feet and he had nearly no eye sight left. Past and current insulin treatment is far from optimal and this patient was living proof of the severe consequences of diabetes.

## Diabetes

In 2006, the global prevalence of diabetes mellitus reached 180 millions, and it has been estimated that it will close to double within 20 years from now [6]. As illustrated by the patient mentioned in the first paragraph, a person diagnosed with diabetes will come to develop complications of the disease later in life. This applies to the two major types of diabetes. Type I diabetes is an autoimmune disease primarily affecting children and adolescents. Autoantibodies directed towards insulin-producing beta cells destroy the endocrine pancreas and leave the patient unable to produce insulin. The result of insufficient insulin production is a life-threatening increase in blood glucose concentration that can only be reversed by subcutaneous or intravenous administration of exogenous insulin. Patients who are diagnosed with type 2 diabetes are typically middle-aged or older and do not immediately require insulin treatment. Instead type 2 diabetes is characterized by insulin resistance, meaning the inability of cells to respond to insulin. Since the pancreas of a type 2 diabetic patient is unaffected it will continue to produce insulin in response to continuously raised blood glucose concentrations, which in turn renders the patient not only hyperglycemic but also hyperinsulinemic. However, with age pancreatic function will decline and also the type 2 patient will eventually require insulin for blood sugar control. Contrary to most type 1 patients, type 2 patients also suffer from dyslipidemia with high levels of triglycerides and low-density lipoprotein (LDL) particles and low levels of high-density lipoprotein (HDL) [7].

Complications of diabetes include microvascular complications (diabetic nephropathy, polyneuropathy, and retinopathy) and macrovascular complications (ischemic heart disease, stroke, and peripheral vascular disease). Microvascular disease also contributes to reduced myocardial vascularization and poor wound healing [8]. In addition, diabetic patients are more susceptible to infectious disease

compared to non-diabetic patients [9]. Diabetic complications result in poor outcome for patients after interventions within all medical disciplines, thereby posing a major challenge for today's health care. Although micro- and macrovascular complications have some common characteristics, they also differ significantly in pathogenesis and distribution between patient groups. This thesis work presents data that may be relevant for both processes, since it focuses on diabetic atherosclerosis and diabetic retinopathy.



**Fig. 1** Known molecular mechanisms of glucose-induced damage include increased flux through the polyol pathway, formation of AGEs, PKC activation and increased flux through the hexosamine pathway. These pathways have been proposed by Brownlee and colleagues to be activated via ROS production and decreased GAPDH activity. (modified from Brownlee 2005 [3])

### *Molecular mechanisms of glucose-induced cell damage*

The molecular basis of glucose-induced damage has been suggested to be similar in all complications of diabetes [3]. Up until now, four different mechanisms have been established to play a role in the process: increased flux through the polyol pathway, formation of advanced glycosylation end-products (AGEs), activation of protein kinase C (PKC), and increased flux through the hexosamine pathway [2-4]. Figure 1 shows an overview of the mechanisms as reviewed by Brownlee [3] and possible pharmacological ways of interfering with them.

Increased flux through the polyol pathway leads to degradation of glucose to sorbitol catalyzed by aldose reductase and subsequent conversion of sorbitol to fructose, catalyzed by sorbitol dehydrogenase [10]. This process consumes NADPH which in turn results in a substrate deficit for glutathione reductase, thus rendering the cell more vulnerable to intracellular oxidative stress [11]. Extensive studies have been conducted to assess aldose reductase as a potential drug target in the treatment of diabetic complications. Clinical trials devoted to the effect of aldose reductase inhibitors have however shown little or no effect on the development of diabetic retinopathy and polyneuropathy [12, 13] [14] [15] [16].

AGEs alter the function of intracellular proteins and modify plasma proteins in a way that enables them to bind to AGE receptors on the surface of endothelial cells and macrophages, inducing oxidative stress and changes in gene transcription [10]. In addition, AGEs modify extracellular matrix proteins which changes vessel permeability and remodeling capacity [10]. The functional role of AGEs in diabetic complications has been demonstrated by the use of AGE inhibitors (for example aminoguanidine and pyridoxamine) in diabetic animal models of nephropathy and retinopathy [17-19]. There is currently an ongoing Phase 2 clinical trial investigating the effect of pyridoxamine in diabetic nephropathy [20].

PKC activation has been shown to induce a range of vascular modifications including blood flow abnormalities, increased vessel permeability, capillary occlusion, inflammation, and oxidative stress [10]. Hyperglycemia-induced activation of PKC via *de novo* synthesis of diacylglycerol (DAG) has been reported in vessels, kidney and retina of diabetic animals [21]. Elevated glucose has been shown to increase the expression of vascular endothelial growth factor (VEGF) in aortic smooth muscle cells via activation of PKC. VEGF is a mediator of angiogenesis and vascular permeability, with strong implications in the pathogenesis of diabetic retinopathy. Intraocular anti-VEGF therapy is investigated as a new mean of treating active proliferative diabetic retinopathy, alone or in addition to photocoagulation, which is the only currently available treatment of this complication [22]. PKC inhibitors have also attracted interest as potential new drugs for treatment of diabetic complications. Clinical trials have

revealed modest beneficial effects of the PKC- $\beta$  inhibitor LY333531 in non-proliferative diabetic retinopathy [23] and neuropathy [24]. In addition, LY333531 has been shown to improve endothelial function in type 2 diabetics [25].

The contribution of hexosamine pathway flux is less known. It has been suggested to change transcriptional activity via activation of specificity protein 1 (Sp1) in VSMCs, leading to increased expression of the pro-coagulative protein plasminogen activator inhibitor 1 (PAI-1) [26]. Increased levels of PAI-1 may contribute to the hypercoagulability seen in diabetic individuals [27].

The influence of hyperglycemia at the level of transcriptional regulation is less well characterized, but the transcription factors nuclear factor  $\kappa$ B (NF $\kappa$ B) and activator protein a (AP-1) are known to respond to hyperglycemia. Increased glucose concentrations have been shown to alter the expression of a number of target genes in endothelial cells [28, 29], vascular smooth muscle cells (VSMCs) [30-32] and macrophages [33] via NF $\kappa$ B and AP-1.

Brownlee and colleagues have demonstrated that the polyol, AGE, PKC, and hexosamine pathways are all activated by an excess of mitochondria-derived reactive oxygen species (ROS), such as superoxide, and subsequent activation of the key glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) via the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) [3]. Increased intracellular ROS induces strand breaks in the DNA, which activates PARP, thereby inducing the formation of ADP-ribose polymers. The polymers accumulate on the GAPDH enzyme, leading to a decrease in its activity [3]. These findings have revealed additional possible drug targets for the treatment of diabetic complications. Benfotiamine, a thiamine derivative, acts to redirect glucose metabolites from the detrimental GAPDH-repressing pathway to the more harmless pentose phosphate pathway [34]. In a recent study, combined oral treatment of a small group of type 1 diabetic patients with benfotiamine and the antioxidant  $\alpha$ -lipoic acid was shown to reduce the activity in the hyperglycemia-induced pathways described in this section [35]. Inhibitors of PARP (for example PJ34) have been evaluated in animal models of diabetic endothelial dysfunction [36], retinopathy [37], neuropathy [38], and nephropathy [39] with positive results, but there are no results available from clinical trials.

Research investigating glucose-induced mechanisms has so far contributed with a wealth of knowledge concerning diabetic complications, but despite all efforts, no established therapies have yet emerged from this research. Even though a few studies have shown positive results on the progress of diabetic retinopathy and neuropathy with PKC and aldose reductase inhibition, the effects have been moderate and limited to a subset of patients. Further studies are necessary to

elucidate more details concerning the mechanisms behind glucose-induced vascular disease, in order to find new potential drug targets for prevention and treatment of diabetic vascular complications.

## **Diabetes and Inflammation**

Inflammation is the body's response to trauma, tissue injury or infection involving the immune system and the vasculature. The primary task of inflammation is to assess if the body has been invaded by microorganisms, and if so, to rapidly eradicate them [40]. This is an evolutionarily conserved life-preserving system that needs to be tightly regulated in order not to cause damage to the body itself. The challenge of overcoming the threat of invasion by organisms able to multiply at a high rate, has however forced the body to take some chances. One is the risk of not recognizing self, another is the risk of not being able to terminate the accelerating inflammatory response [40]. Chronic inflammation has been suggested to result from an inability to shut down the inflammatory response. This is supported by findings indicating spontaneous inflammation in patients and animals carrying certain mutated or disrupted genes. Paradoxically, some of these genes code for proteins which are known to be highly pro-inflammatory such as the transcription factor NF- $\kappa$ B and the potent pro-inflammatory cytokine tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), testifying to the complexity of immune regulation [40].

Atherosclerosis is established as an inflammatory disease of the vessel wall. Diabetic vascular complications also seem to depend on vascular inflammation, but the mechanisms are not fully established. The endothelium, VSMCs, and immune cells collectively contribute to the inflammatory response in vascular complications of diabetes. TNF $\alpha$  and NF- $\kappa$ B are canonical mediators of vascular inflammation in atherosclerosis and also appear to play significant roles in diabetic complications. In this thesis, a novel hyperglycemia-responsive transcription factor – nuclear factor of activated T-cells (NFAT) is introduced in the context of vascular complications of diabetes. In addition, we present evidence suggesting an unexpected role for TNF $\alpha$  in diabetic macroangiopathy.

## **The Diabetic Vessel Wall**

The vessel wall of large arteries such as the aorta is composed of endothelium, VSMCs, and adventitia, as well as extracellular matrix and elastic laminae. The number of VSMC layers progressively decreases with vessel size and capillaries are simply endothelial tubes surrounded by basement membrane. Because of their



role in blood pressure control, resistance arteries contain larger amounts of VSMCs relative to lumen compared with larger arteries that have primarily a conductive function. Very small vessels, especially those located in the brain and retina, contain cells called pericytes, which among other functions support the endothelium and regulate endothelial proliferation and angiogenesis [41]. All constituents of the vessel wall are affected by diabetes but contribute in different ways to the pathogenesis of diabetic vascular complications.

The endothelium lines the inside of the vasculature and regulates leukocyte extravasation, platelet adhesion, and blood vessel patency. Diabetes, as well as a number of other vascular conditions, is associated with endothelial dysfunction characterized by increased inflammatory response, impaired endothelium-dependent relaxation, and increased blood coagulability [42]. Hyperglycemia has been shown to promote endothelial expression of inflammatory molecules such as IL-6, IL-8, vascular cell adhesion molecule 1 (VCAM-1) and monocyte chemoattractant protein 1 (MCP-1), and to decrease nitric oxide (NO) availability leading to defective endothelium-dependent vessel relaxation [42, 43]. Other endothelial changes that are associated with diabetes are increased expression of potent vasoconstrictors such as angiotensin II (Ang II), endothelin-1 (Et-1), and increased expression of adhesion molecules facilitating the adhesion of platelets and monocytes to the endothelium [42]. Activated endothelial cells recruit monocytes into the vascular tissue where they are transformed into macrophages. Macrophages have been shown to express several pro-inflammatory molecules (IL-1 $\beta$ , IL-6, CD36 and MCP-1) in response to diabetes [42].

VSMCs of arteries function to regulate blood flow and pressure by contracting or dilating in response to vascular mediators. Healthy VSMCs are highly contractile and are not prone to proliferate or migrate. However, if the vessel wall is injured, the phenotype of the VSMCs transforms from a contractile into a synthetic phenotype which, in addition to synthesizing cytokines and extracellular matrix, easily proliferates and migrates [44]. The phenotypic switch is characterized by loss of differentiation markers such as smooth muscle specific  $\alpha$ -actin (SM  $\alpha$ -actin), smooth muscle myosin heavy chain (SM-MHC) and SM22 $\alpha$ , and occurs both in vascular disease and in culture [45].

VSMCs have been demonstrated to increase proliferation and migration rates in response to glucose. Cultured VSMCs and intact mouse aortas exposed to high glucose have been shown to express elevated levels of cytokines (TNF $\alpha$ , IL-1 $\beta$  and IL-6), adhesion molecules (intercellular adhesion molecule 1 (ICAM-1) and VCAM-1), and chemokines (MCP-1) [46-48]. In addition, high glucose has been demonstrated to increase the expression of growth factors such as vascular endothelial growth factor (VEGF) [49]. Genes involved in the regulation of

calcification and bone formation, including Cbfa1, bone morphogenic protein 2 (BMP2)[50], and osteopontin (OPN) [51], have also been demonstrated to be upregulated in response to high glucose.

## Diabetic Atherosclerosis

Lipid depositions in the vascular wall occur already early in life and form precursor lesions called “fatty streaks”. In the vasculature there are sites which are predisposed for atherosclerotic lesion formation because of turbulent blood flow, for example the carotid bifurcation and the aortic root. The fatty streaks can eventually evolve into atherosclerotic plaques containing a lipid-rich necrotic core and phenotypically altered VSMCs. The necrotic core is protected from the blood stream by a layer of VSMCs and extracellular matrix termed the fibrous cap. Myocardial infarction and stroke usually result from rupture of the fibrous cap, exposing the inside of the lesion to the blood, which in turn leads to thrombus formation and acute occlusion of the vessel [52].

From a cellular point of view, the endothelium seems to be the initiator of the atherosclerotic process followed by involvement of VSMCs, macrophages and lymphocytes. The endothelium acts as a protective barrier between blood and tissue, and expresses molecules involved in coagulation, inflammation and vascular tone. Studies have also demonstrated a regulatory function of endothelium over VSMC, since denudation of vessels results in VSMC migration and proliferation [53]. Endothelial dysfunction leading to decreased production of NO, increased expression of adhesion molecules and cytokines, and increased vascular permeability is thought to be the initial step in the atherosclerotic process, followed by entrapment of LDL particles in the subendothelial matrix. Endothelial cells express VCAM-1 in response to lipid accumulation, which recruits monocytes and lymphocytes to the site of the lesion. Stimulated by the secretion of stimulating chemokines from VSMCs and endothelium, the monocytes are activated into macrophages [54]. LDL particles trapped beneath the surface of the vascular wall are subsequently oxidized to oxLDL. These and other modified lipoprotein-containing particles are engulfed by recruited macrophages, which are then transformed into foam cells. Macrophages actively secrete apoE, thereby promoting lipid and cholesterol extraction from the vessel wall [55]. The apoE<sup>-/-</sup> mouse is an extensively used animal model for atherosclerosis research, because of its ability to rapidly develop atherosclerotic lesions. In advanced lesions, the majority of plaque cells are synthetic VSMCs that deposit extracellular matrix and produce cytokines. Transition back to a contractile phenotype has been proposed to induce plaque stabilization, thus reducing the risk of ruptures leading to detrimental cardiovascular events [56].

Diabetic patients have been shown to develop premature atherosclerosis [57]. Burke et al investigated the morphologic characteristics of diabetic atherosclerotic plaques in a post-mortem study of subjects of cardiac sudden death [58]. Total plaque burden, lipid core size, calcification, macrophage infiltration, lymphocyte infiltration, apoptosis and signs of thrombosis were assessed. Atherosclerotic plaques from both type 1 and type 2 patients demonstrated increased lipid core size, increased macrophage infiltration and apoptosis of VSMCs and macrophages compared to plaques in non-diabetic subjects. T-lymphocyte accumulation was increased in type 1 diabetes patients, but not in type 2 patients. Plaques matrix calcification was increased in type 2 subjects, but rather decreased in type 1 subjects. The results of the Burke et al post-mortem study point out that there are characteristics typical of diabetic atherosclerosis, such as increased inflammation and enhanced plaque vulnerability. It also indicates that the pathology of atherosclerosis in type 1 and type 2 diabetes differs in some aspects, including plaque calcification. Diabetic plaques are also known to display increased expression of certain genes, for example the pro-coagulatory molecule PAI-1 and the matrix cytokine OPN [59].

### *Role of hyperglycemia, dyslipidemia and insulin resistance*

Both type 1 and type 2 diabetic patients suffer from accelerated atherosclerosis, but the cardiovascular risk of type 2 diabetic patients is considerably larger than that of type 1 diabetic subjects. This difference is attributed to the prominent dyslipidemia of the type 2 patient compared to type 1, and to the effects of insulin resistance. The role of dyslipidemia in diabetic atherosclerosis development is thought to be similar in non-diabetic atherosclerosis. On top of the effects of dyslipidemia, insulin resistance can independently contribute to accelerated atherosclerosis in type 2 diabetes patients [60]. The role of hyperglycemia in atherosclerosis of type 2 diabetes patients is on the other hand a matter of controversy. The United Kingdom Prospective Diabetes Study (UKPDS), which sought to investigate the benefit of intensive glucose-lowering treatment for risk of vascular complication development in type 2 diabetes, showed for example no significant effect of intensive blood glucose control in reducing the risk of atherosclerosis in type 2 diabetes patients [61]. On the other hand, a corresponding study for type 1 diabetes, the Diabetes Control and Complications Trial (DCCT), showed a risk reduction of any cardiovascular event by 42% when type 1 diabetes patients were given intensive treatment [62]. It has been proposed that the effect of hyperglycemia on atherosclerosis development in type 2 diabetic subjects is masked by the effects of lipids, and that lipids and glucose affect the vascular wall through similar mechanisms [63]. Confirming this notion, studies using animal

models of diabetes have demonstrated an independent effect of hyperglycemia and lipids in the initiation and progression of diabetic atherosclerosis [64, 65].

## Diabetic Retinopathy

The DCCT [66] and UKPDS [61] trials revealed the importance of hyperglycemia in the pathogenesis of microvascular complications, including diabetic retinopathy, in both type 1 and type 2 diabetes. The sites of development for microvascular complications mirror the inability of cells in those organs to protect themselves from overwhelming intracellular concentrations of glucose when extracellular glucose concentrations are high. Intracellular high glucose levels cause abnormal blood flow and increased vascular permeability, which initiates the pathophysiological process leading to microangiopathy [10]. Early diabetic retinopathy is characterized by loss of pericytes by apoptosis and subsequent loss of endothelial cells, producing acellular capillaries. In late stage retinopathy the endothelium starts proliferating into dysfunctional bleeding capillaries, with the consequence of blurred vision and ultimately blindness [67]. Inflammation has been suggested to contribute to the development of diabetic retinopathy, but the full picture and relevance of the inflammatory response is not known [67].

## Ca<sup>2+</sup> Signaling

Ca<sup>2+</sup> channels are the phylogenetically oldest ion channels and the plasma membrane of ancient prokaryotes was penetrated by Ca<sup>2+</sup> pumps, Na<sup>+</sup>/Ca<sup>2+</sup> exchangers and Ca<sup>2+</sup>/H<sup>+</sup> exchangers, maintaining the intracellular Ca<sup>2+</sup> level ([Ca<sup>2+</sup>]<sub>i</sub>) 10 000-20 000 times lower than that in the extracellular space [68]. With the continuous evolution of cells, Ca<sup>2+</sup> signaling has developed into a complex regulatory system involved in various functions of our body.

Ca<sup>2+</sup> channels involved in Ca<sup>2+</sup> signaling are present both in the plasma membrane and in the membrane of intracellular stores such as the endoplasmic/sarcoplasmic reticulum (ER/SR). Plasma membrane Ca<sup>2+</sup> channels include voltage-dependent, receptor-operated, store-operated, and mechanically regulated channels. Voltage-dependent Ca<sup>2+</sup> channels (VDCCs) couple changes in membrane potential with changes in [Ca<sup>2+</sup>]<sub>i</sub>, making them important players in processes related to excitation, such as pacemaking, contraction, secretion, and transcription [69]. VDCC activity can be studied using specific blockers such as verapamil and nifedipine.

Cells contain several proteins that function as  $\text{Ca}^{2+}$  buffers by binding  $\text{Ca}^{2+}$  ions with high affinity. The very strong buffering capacity of the cytosol creates local  $\text{Ca}^{2+}$  microdomains able of spatial control of  $\text{Ca}^{2+}$  signals, but with the disadvantage of hampering long distance propagation of  $\text{Ca}^{2+}$  signals within cells. This problem is overcome by intracellular stores in which high concentrations of free  $\text{Ca}^{2+}$  ions reach most parts of the cell without interfering with biological function [68]. The SR  $\text{Ca}^{2+}$  channels inositol 1,4,5-triphosphate receptors ( $\text{IP}_3\text{R}$ ) and ryanodine receptors (RyR), act by releasing  $\text{Ca}^{2+}$  from the intracellular stores into the cytosol.  $\text{IP}_3\text{R}$  are activated by G-protein-coupled and tyrosine kinase linked receptors that activate phospholipase C (PLC), which in turn generates  $\text{IP}_3$  and DAG by cleavage of phosphatidylinositol 4,5-biphosphate (PIP2).  $\text{IP}_3\text{R}$  are opened in response to  $\text{IP}_3$  and to small increases in cytosolic  $[\text{Ca}^{2+}]_i$ . RyR generate local release of  $\text{Ca}^{2+}$  into the cytosol when global  $[\text{Ca}^{2+}]_i$  is raised, for example during muscle contraction. Together, RyR and  $\text{IP}_3\text{R}$  contribute to  $\text{Ca}^{2+}$  oscillations by propagating  $\text{Ca}^{2+}$  waves throughout the cell [68]. In VSMCs,  $\text{IP}_3\text{R}$ -mediated  $\text{Ca}^{2+}$  release contributes to contraction while RyR-sensitive  $\text{Ca}^{2+}$  release events ( $\text{Ca}^{2+}$  sparks) cause relaxation by hyperpolarizing the membrane via activation of large conductance  $\text{K}^+$  (BK) channels [70, 71].

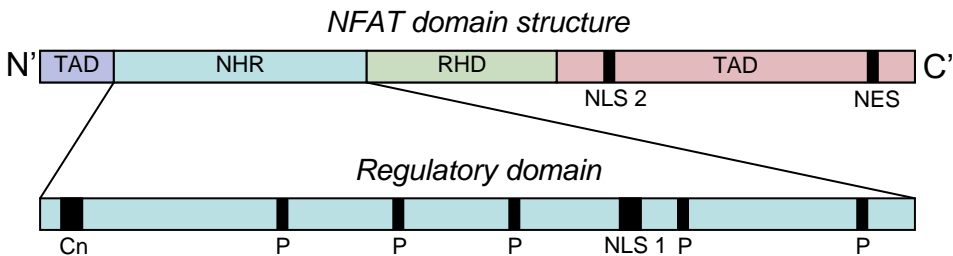
$[\text{Ca}^{2+}]_i$  elevations are terminated by the plasma membrane  $\text{Ca}^{2+}$  ATPase (PMCA) and the ER/SR  $\text{Ca}^{2+}$  ATPase (SERCA) that rapidly pump  $\text{Ca}^{2+}$  ions out of the cell and into the ER/SR [72]. Depletion of intracellular stores activates store-operated channels (SOCs) in the plasma membrane, which function to refill the ER/SR of the cell. Events related to SOCE can be studied by using SERCA pump inhibitors such as thapsigargin, which cause depletion of the ER/SR.

VSMC  $\text{Ca}^{2+}$  handling has been investigated in response to AGEs. A four day incubation of cultured VSMC with AGE-modified albumin resulted in an agonist-induced rise in  $[\text{Ca}^{2+}]_i$  that was of increased duration but equal amplitude compared to non-treated VSMC. The enhanced duration of the  $\text{Ca}^{2+}$  response was suggested to be mediated by a reduction in RyR channel activity or expression [73]. Endothelial cells on the other hand respond to high glucose with attenuated agonist-induced  $\text{Ca}^{2+}$  response, which has been shown to contribute to endothelial dysfunction and impaired endothelium-dependent relaxation [74-77]. These reports show that diabetes affect vascular  $\text{Ca}^{2+}$  handling, which in turn may underlie the observed endothelial dysfunction, VSMC phenotypic modulation, changes in contractility, and altered  $\text{Ca}^{2+}$ -dependent transcription.

# NFAT

## *The NFAT Family*

NFAT is a  $\text{Ca}^{2+}$ /calcineurin-dependent transcription factor that was originally identified from nuclear extracts of activated T cells and demonstrated to be involved in the regulation of early response genes of antigen-exposed T cells [78, 79]. The NFAT family consists of four  $\text{Ca}^{2+}$ -dependent isoforms (NFATc1-c4) and one fifth constitutively nuclear isoform (NFAT5) [80]. Alignment of the different isoforms reveals two conserved regions; one DNA-binding domain and one NFAT homology region [80]. Figure 2 shows the domain structure of NFAT family proteins.



**Fig. 2** The domain structure of NFAT proteins includes two transactivation domains (TAD), a Rel homology domain (RHD), and a NFAT homology region (NHR). The RHD is the DNA-binding part of the proteins, while the NHR is the regulatory domain. The regulatory domain contains a number of serine-rich regions (here depicted P) that serve as phosphorylation sites for different kinases. The proteins have two nuclear localization signals (NLS1/2) and a nuclear export signal (NES). (modified from Hill-Eubanks et al [4])

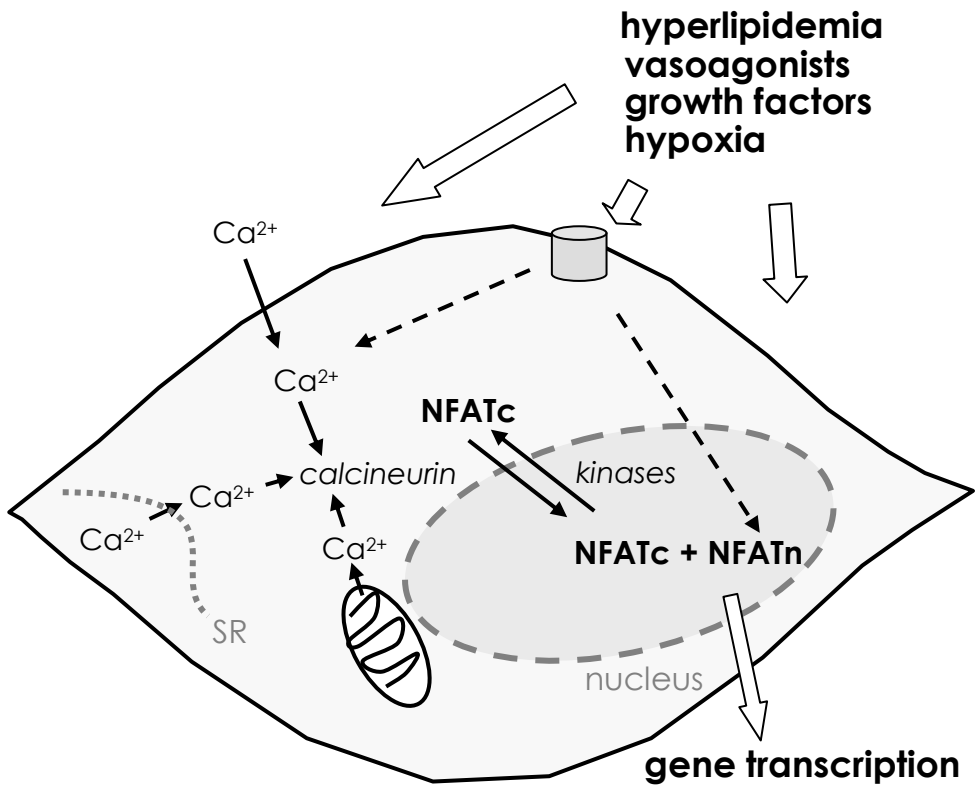
The NFAT homology region is the regulatory domain of NFAT and contains a region of heavily phosphorylated serine residues, interaction sites for the  $\text{Ca}^{2+}$ /calmodulin-activated serine phosphatase calcineurin, and the nuclear localization signal (NLS) [81]. Upon activation by elevated intracellular  $\text{Ca}^{2+}$ , calcineurin dephosphorylates NFAT thus unmasking the NLS, allowing NFAT to translocate to the nucleus [80]. In the nucleus, NFAT often binds to DNA together with a partner (NFATn) such as AP-1 (T-cells) [82], MEF2 (skeletal muscle) [83] and GATA4 (cardiac muscle) [84].

NFAT has been shown to participate in a range of developmental processes including lymphangiogenesis [85], cardiac valve formation, vasculogenesis, axonal outgrowth, muscle differentiation, bone formation, lung maturation, gastrointestinal tract development and immune system maturation [86]. In

addition, NFAT has been demonstrated to play a significant role in the pathogenesis of pathological cardiac hypertrophy [87] and postnatal angiogenesis [88].

*NFAT Regulation: Points of control*

The canonical pathway of NFAT includes elevation of  $[Ca^{2+}]_i$  and subsequent activation of calcineurin, which dephosphorylates NFAT rendering it susceptible to nuclear import. In addition to these crucial steps several other points of control have been identified, which vary between cell types [81]. A sketch of general features of NFAT regulation is found in Figure 3.



**Fig. 3** Simplified sketch featuring NFAT regulation. Cytoplasmic NFATc is dephosphorylated by the  $Ca^{2+}$ /calmodulin-dependent phosphatase calcineurin. In the nucleus, NFATc combines with a partner, NFATn, and binds to DNA in order to induce gene transcription. Kinases counteract NFAT nuclear accumulation by rephosphorylating the protein. (modified from Nilsson LM et al [2])

NFAT has an unprecedented ability to decode different  $\text{Ca}^{2+}$  signaling modalities. NFAT activation is sensitive to mode of entry, amplitude, duration, and localization of  $\text{Ca}^{2+}$  changes. Interestingly,  $\text{Ca}^{2+}$  signal requirements for NFAT activation differ between cell types. In T cells, NFAT activation is dependent on a sustained  $[\text{Ca}^{2+}]_i$  plateau created by SOCE in response to cell stimulation, whereas a short transient spike is insufficient for activation [89]. In neurons on the other hand, a short transient induced by depolarization is an effective stimulus for NFAT activation [90]. Depolarization is also an effective stimulus for NFAT in cardiac and skeletal muscle where rapid  $\text{Ca}^{2+}$  oscillations lead to increased NFAT activity [91, 92]. In contrast, depolarization is not a sufficient stimulus for activation of the calcineurin/NFAT pathway in smooth muscle [93]. Instead, NFAT activation depends on influx of  $\text{Ca}^{2+}$  through L-type VDCCs in the plasma membrane, and through  $\text{IP}_3\text{R}$  channels in the SR [94]. In addition,  $\text{Ca}^{2+}$  sparks from RyR of arterial smooth muscle have been shown to inhibit NFAT nuclear accumulation [95]. Endothelial cells have the same  $\text{Ca}^{2+}$  requirements as T cells [96], but in endocardial endothelial cells during embryogenesis it has also been found that  $\text{Ca}^{2+}$  flowing from adjacent cells through gap junctions activates NFATc1 [97].

Calcineurin activates NFAT by both unmasking the NLS and masking the NES, and hence, is also able to dephosphorylate NFAT in the nucleus [81]. When the NESs of NFAT are uncovered, the export protein Crm-1 will translocate NFAT back to the cytoplasm [98]. Rephosphorylation of specific serine residues on NFAT after calcium signaling termination is necessary for export to occur [99]. Several NFAT phosphorylating kinases have been identified, including protein kinase A (PKA), glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ) [100], c-Jun N-terminal kinase (JNK) [101], p38 MAPK [102], casein kinase 1 $\alpha$  (CK1 $\alpha$ ) [103], casein kinase 2 (CK2) [104], extracellular signal-regulated kinase 5 (ERK5), mammalian target of rapamycin (mTOR) [105] and dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (DYRK1a) [106]. In T-cells, PKA and DYRK1a primes for subsequent phosphorylation by GSK-3 $\beta$  but is not able to induce NFAT export on its own [100, 106]. Similarly, ERK5 primes for CK1 $\alpha$  but can also induce export individually [105]. Additionally, kinases that are not able to phosphorylate NFAT directly can still aid in the regulation, for example by stabilizing the association of another kinase with NFAT [103]. It has been suggested that constitutive kinases cooperate to phosphorylate NFAT in unstimulated cells, and that inducible kinases act to rephosphorylate NFAT when cells are stimulated [81]. NFAT exporting kinases vary between isoforms and cell types, further adding to the complexity of this signaling pathway. In VSMCs, the export mechanism of NFATc3 has previously been shown to involve JNK2 and Crm-1 [94].



### *NFAT in the Vasculature*

In the adult vasculature, NFAT has been identified in both the endothelium and in VSMCs. Several studies report an involvement of NFAT in the proliferation [107-109] and motility [110] of VSMCs. Proliferation of cultured rat VSMCs has been shown to be induced by PDGF-BB and thrombin in an NFATc1-dependent manner, at least in part via induction of cyclin A expression [111]. In another study, suppression of NFAT activity in rat VSMCs led to decreased expression of cyclin D1 and retinoblastoma protein (Rb) phosphorylation [112]. Very low-density lipoproteins (VLDL) have also been shown to induce VSMC proliferation via NFATc3 activation [108].

In cultured rat aortic VSMC, motility was shown to involve the expression and release of IL-6 [110]. In an earlier paper from our laboratory, not included in this thesis, we demonstrated that chronic inhibition of NFAT with A-285222 reduced IL-6 production in human myometrial arteries and inhibited proliferation of human VSMCs. On the other hand, inhibition of NFAT had no effect on the synthesis rates of contractile proteins or on force generation in response to depolarization or vasoagonist stimulation [109].

Roles for NFATc3 have been implicated in VSMC differentiation and pulmonary hypertension. Santana and colleagues have shown that NFATc3 down-regulates the  $\beta_1$  subunit of large conductance  $\text{Ca}^{2+}$  activated potassium channels in VSMCs and hence contributes to Ang II-induced hypertension. They also showed that NFATc3 regulates expression of  $\text{K}_v2.1$  potassium channel subunit, leading to increased VSMC excitability and hypertension in rat *in vivo* [113, 114]. Using cerebral arteries from mouse, Gonzalez-Bosc et al showed that NFATc3 was translocated to the nuclei of VSMC in response to increased intraluminal pressure via a NO/PKG-dependent mechanism [115]. NFATc3 activity has also been demonstrated to mediate intermittent hypoxia-induced hypertension, a condition associated with sleep apnea [116]. NFATc3, and even more so NFATc2, has been implicated in the development of pulmonary arterial hypertension by contributing to  $\text{K}_v1.5$  down-regulation [117].

Data from several studies support a role for NFAT in vascular response to injury including neointima formation and restenosis after balloon injury. *In vitro* studies of VSMC using the NFAT inhibitory peptide VIVIT showed significant inhibition of PDGF-BB and thrombin induced proliferation and PDGF-BB induced expression of inflammatory molecules (IL-6, IL-8, MCP-1, and stromal cell-derived factor-1 $\alpha$ ), but did not affect VSMC apoptosis or endothelial wound healing capacity [118]. Two studies performed in rat carotid injury models have shown a reduction in neointima formation after delivery of the NFAT inhibitory

peptide VIVIT. These studies also demonstrated a role for NFAT in the expression of cyclooxygenase 2 (COX-2), CD45 and cyclin A in VSMC [110, 111].

In endothelial cells, inhibition of NFAT with the inhibitory peptide VIVIT has been reported to disrupt VEGF-mediated proliferation in human pulmonary valve endothelial cells [119]. Identified target genes for the calcineurin/NFAT pathway in endothelial cells are granulocyte/macrophage-colony stimulating factor (GM-CSF), E-selectin [96], tissue factor (TF) [120, 121], IL-8 [122], VCAM-1 [123] and the endogenous calcineurin/NFAT inhibitor DSCR1 [124, 125]. DSCR1, which is induced by VEGF and thrombin in an NFAT-dependent manner, functions as a calcineurin/NFAT autoinhibitor and represses the expression of molecules related to inflammation, proliferation, coagulation homeostasis and angiogenesis [123-125].

## AIMS

- to investigate if hyperglycemia activates the  $\text{Ca}^{2+}$ -dependent transcription factor NFAT in the vasculature
- to explore the mechanisms behind high glucose induced NFAT activation in vascular smooth muscle
- to investigate the role of NFAT in the regulation of the pro-inflammatory cytokine osteopontin in the vascular wall in the context of diabetes
- to study the involvement of NFAT in the pathogenesis of diabetic retinopathy
- to elucidate the role of  $\text{TNF}\alpha$  in hyperglycemia-induced vascular inflammation

## METHODS

The aim of this section is to highlight and discuss some of the techniques employed in this thesis, rather than giving a comprehensive description of all methods used. For more detailed information about the conducted experiments, please refer to the Material and Methods sections in the individual papers.

### Quantification of NFAT Localization

The activity of NFAT is dependent on its subcellular localization. Therefore, quantification of NFAT nuclear accumulation is a useful way of evaluating if a certain stimulus is able to activate the calcineurin/NFAT pathway. The subcellular localization of a protein can be established by using either biochemical or imaging techniques. Originally, NFAT subcellular localization in immune cells was demonstrated using an immune based biochemical technique: Western blot of nuclear fractions [126]. NFAT nuclear accumulation has also been estimated with imaging techniques such as non-immune based tagging of NFAT [99] and immunohistochemistry [127]. Imaging using immunohistochemistry has the advantage that it is possible to distinguish one cell type from another when working with intact tissue such as arteries, but relies on the quality of the antibodies used.

As in earlier work [94, 95], in Paper I, II and III, we used immunohistochemistry and confocal microscopy to quantify NFATc3 nuclear accumulation in intact arteries in response to high glucose and other previously recognized stimuli. After treatment and dissection, cerebral arteries, cryosections of aorta (10  $\mu\text{m}$ ) or retinas were briefly fixed using formaldehyde or Histochoice™® and subsequently permeabilized with the detergent Triton-X-100 and blocked using bovine serum albumin (BSA). Fixation is essential in order to maintain the ultrastructure of non-living tissue, but at the same time it can be a coarse treatment that may disrupt the integrity of proteins and hide epitope sites targeted by antibodies. As opposed to formaldehyde, Histochoice™® is a non-toxic and non-cross-linking fixative, which maintains a fresher appearance of the tissue and allows improved visualization with immunohistochemical techniques due to the preservation of epitope sites. Permeabilization further improves epitope exposure by partially denaturing fixed proteins. Blocking with BSA reduces non-specific binding since albumin will compete with the antibodies for binding sites of irrelevant proteins.

In our experience a slow incubation with the primary antibody at a low temperature decreases non-specific binding compared to a shorter one at room temperature and we therefore incubated with the primary rabbit anti-NFATc3 antibody over night at 4°C. Several wash steps after fixation and incubation further ensured specific binding of the antibodies. Specificity control of the staining was obtained by omitting the primary or the secondary antibody from the staining protocol. The secondary Cy5-anti-rabbit IgG antibody was on the other hand applied for one or two hours at room temperature. Cy5 is a fluorescent moiety that emits light in the far red region of the wavelength spectrum (excitation wavelength of 633 nm and emission wavelength of >650 nm). This fluorophore was chosen because the emission wavelength does not overlap with autofluorescence originating from the elastic laminae of the arteries that otherwise may interfere with measurements. Finally, nuclei were visualized by staining with the fluorescent nucleic acid dye SYTOX Green and the preparations were mounted onto glass slides using a glycerol-based medium which preserves tissue structure and opposes photo bleaching. Photo bleaching reduces the quality of imaging but can be avoided by working in darkness when preparing, working with and storing the samples.

NFATc3 and nuclear staining was visualized using a Zeiss LSM 510 laser scanning confocal microscope. The term confocal refers to the use of two pinholes with the same focus that the light passes on its way to and from the specimen being examined. The second pinhole prevents the passage of light originating from above or below the plane of focus thereby creating an optical section of the tissue (0.5-1  $\mu\text{m}$ ). Optical sectioning with confocal microscopy allowed us separate visualization of endothelium and vascular smooth muscle layer in whole-mounted retina and cerebral arteries, and improved resolution of NFAT subcellular distribution.

The actual quantification of NFAT nuclear accumulation after imaging is what poses the largest challenge and it has been a matter of discussion and continuous development over the period of time that the method has been employed. For quantification, Cy5 and SYTOX green fluorescence was monitored and pseudocoloured for visualization. NFAT appears red and nuclei green in the confocal images, but when they colocalize pixels are colored white or yellow.

In the first papers published examining NFAT nuclear accumulation in intact vessels, percentage of NFAT nuclear accumulation was determined by manually counting the total numbers of cells, identified by their nuclei, and the number of NFAT-positive nuclei defined by the presence of colocalized yellow pixels [94, 95, 109] (Paper 1). Multiple fields for each vessel were imaged and counted by two independent observers under blinded conditions. This method was reliable but

time-consuming and susceptible to inter-observer variation. In experiments using cultured VSMCs [109], we instead used the colocalization tool of the Zeiss LSM 510 laser scanning confocal microscope. Each pixel was plotted in a scattergram where the axes represented the intensity (range 0 to 255 grayscale values) of NFATc3 and nuclear staining, respectively. Using a cross-hair function we defined areas of interest by dividing the scattergram into four quadrants. Each quadrant represented a certain population of pixels: one quadrant represented colocalized pixels which demonstrated high intensities of both NFATc3 and nuclear fluorescence, one represented nuclear fluorescence only, one represented NFATc3 fluorescence only, and one represented background fluorescence for both NFATc3 and nuclear staining. We calculated the number of pixels in the colocalized quadrant relative the number of pixels displaying nuclear staining above background, that is, the sum of pixels in the colocalized quadrant and that representing nuclear fluorescence only. This software-based analysis generated comparable results and proved less time-consuming than manual counting, and was therefore employed in Paper II. Nuclear accumulation of NFAT in endothelial cells of retinal vessels was more difficult to evaluate due to the small size of the vessels and the abundance of other cell types, such as neurons and glia cells, present in the whole-mounted preparations. To make sure that only the nuclei of endothelial cells were included in the analysis, in Paper III, we manually defined the nuclei and then measured the mean fluorescence intensity of NFAT.

## **Quantification of NFAT Transcriptional Activity**

Increased NFAT nuclear accumulation does not necessarily translate into increased NFAT transcriptional activity since availability of co-factors and insufficient exposure of DNA may limit the possibility for NFAT to induce transcription. To investigate whether glucose could affect NFAT transcriptional activity in the vessel wall, we used a luciferase reporter assay.

Luciferase is an enzyme used by organisms featuring bioluminescence, for example the firefly. It catalyzes the reactions of luciferin and ATP to produce light. In 1987, de Wet et al were the first to express the luciferase protein in mammalian cells [128]. One year later DiLella et al used the firefly luciferase gene to monitor promoter activity in transgenic mice. The luciferase gene was cloned downstream of a small DNA fragment of the chicken alpha-skeletal actin promoter and the hybrid gene was used to generate transgenic mice expressing the luciferase gene product [129].

In Paper I, II and III, we used an NFAT-luciferase (NFAT-luc) transgenic mouse expressing nine copies of an NFAT binding site from the IL-4 promoter positioned

5' to a minimal promoter from the  $\alpha$ -MHC gene (-164 to +16) and inserted upstream of a luciferase reporter gene. The NFAT-luc mice are bred on a FVBN background and have been reported to be phenotypically normal. They display detectable luciferase activity in most tissues surveyed at three weeks of age, with highest expression occurring in the brain, kidney, and heart [130]. For our experiments, aortas, portal veins and retinal vessels were homogenized in lysis buffer. An aliquot of supernatant, obtained by centrifugation, was added to luciferase substrate reagent containing ATP and luciferin. Optical density was measured, normalized to protein concentration and the result expressed as relative luciferase units.

Luciferase reporter assay reflects transcriptional activity of all NFATc isoforms, since they all have the ability to bind to the inserted NFAT binding site. To assess the contribution of NFATc3 to glucose-induced transcriptional activity, NFAT-luc mice were backcrossed with NFATc3<sup>-/-</sup> mice, and their response to glucose compared to that of NFAT-luc mice (Paper II).

## Evaluation of NFAT DNA-Binding Capacity

In paper II, our aim was to investigate the role of NFAT in OPN gene expression of vascular smooth muscle. Since the effect of NFAT on OPN expression levels may not be a direct effect, we wanted to find out if NFAT actually can bind to the OPN promoter and if this occurs in living cells. EMSA was performed on nuclear extracts of VSMCs treated with UTP, and ChIP on intact aortas.

### *Electrophoretic mobility shift assay (EMSA)*

EMSA is an *in vitro* technique for investigating the ability of a protein to bind to DNA. The DNA-binding assay using mobility shift polyacrylamide gel electrophoresis is based on the observation that DNA-protein complexes migrate through polyacrylamide gels more slowly than unbound DNA fragments [131]. Basically, radioactively labeled DNA probes containing particular protein binding sites are prepared and added to a protein mix (typically a nuclear fraction). The migrating speed of the unbound DNA probe is compared to that of the DNA-protein complex. If a protein has bound to the DNA-probe, the band will be “shifted” upwards on the gel compared to unbound DNA-probe. The sequence specificity of protein-DNA interactions can be evaluated using a competition binding assay [132]. Unlabeled non-specific or specific DNA fragments are added prior to electrophoresis. Successful competition will prevent the mobility shift of the DNA-protein complex. A specific DNA fragment is typically the same unlabeled oligonucleotide as the DNA probe, while a nonspecific competitor is a

fragment with an unrelated sequence. The optimal competitor is an oligonucleotide identical to the DNA probe except for a mutation in the binding site that is known to disrupt function. To identify proteins present in the protein-DNA complex an antibody supershift assay can be performed, where a specific antibody is added to the reaction. If the protein recognized by the antibody is involved in complex formation, the antibody can either block complex formation, or it may form an antibody-protein-DNA ternary complex and thereby further reduce the mobility of the protein-DNA complex (supershift) [133].

In paper II, EMSA was performed using nuclear extracts of VSMCs treated with UTP for NFAT translocation. Purity of nuclear fractions was demonstrated by lack of immunoreactivity for the cytosolic marker GAPDH. Nuclear extracts were incubated with an NFAT consensus probe labeled with  $\gamma$ [ $^{32}$ P] ATP. Samples were separated on a polyacrylamide gel, which was dried and subjected to autoradiography. A mutated NFAT binding site oligonucleotide was used as a control and for competition assays we used competitor oligonucleotides for three different NFAT-binding sites. Antibodies directed against NFATc1, NFATc2, NFATc3 and NFATc4 were used for supershift assays. Oligonucleotides were as follows:

NFAT site 1-2 sense: 5'-

TAAACA**ACTGGAAA**ATCCCACTTAGGGAAAATGTCAGCAAC

NFAT site 1-2 antisense: 5'-

GTTGCTGAC**ATTTTCC**CTAAGTGGGATTTTCCAGTTGTTA

NFAT site 3 sense: 5'-GTGATCTACTCTT**CCTTCC**TTATGGATCC

NFAT site 3 antisense: 5'-GGATCCATA**AGGAAAG**GGAAGAGTAGATCAC

NFAT site 4 sense: 5'-TTGTGTGT**GTTTCC**TTTTCTTC

NFAT site 4 antisense: 5'-GAAGAAA**AGGAA**ACACACAA

NFAT consensus sense: 5'-CGCCCAAAG**AGGAAA**ATTTGTTTCATA

NFAT consensus antisense: 5'-TATGAAACAA**ATTTCC**TCTTTGGGCG

NFAT binding sites [80] are indicated in bold and underlined sequences were mutated to AA (sense strand) in NFATmut.

### *Chromatin immunoprecipitation (ChIP)*

ChIP is used to determine if a protein can bind to DNA *in vivo*. The protein is cross-linked when interacting with DNA in living cells and the complex is subsequently immunoprecipitated with the use of an antibody directed at the DNA-binding protein [134]. After immunoprecipitation, the complexes are resolved by heating and the DNA fragments extracted. The identity of the DNA, as well as the amount, is determined with real-time PCR. In paper II, we performed ChIP using intact aortas because we wanted to demonstrate that NFAT binds to the



OPN promoter in vascular smooth muscle *in situ*. Arteries were treated *ex vivo* and transferred to 1% formaldehyde to cross-link protein-DNA and protein-protein interactions within intact chromatin. After purification and shearing, the chromatin was immunoprecipitated with NFATc3 antibody or no antibody. Real-time PCR was performed on immunoprecipitated DNA and on input DNA using primers designed to flank the NFAT site 3 in the OPN promoter. The NFATc3 antibody increased the number of immunoprecipitated chromatin fragments compared to no antibody control. The relative increase in NFATc3 binding to the OPN promoter was calculated using the comparative threshold method ( $\Delta\Delta C_t$ ) with input DNA as the reference.

## NFAT Inhibition

Inhibition of NFAT activity is a powerful tool for examining the involvement of NFAT in vessel physiology and pathology. There are four groups of NFAT inhibitors including clinically used immunosuppressants, endogenous inhibitors of calcineurin and NFAT, peptide inhibitors and small interfering organic molecules. Traditionally, the calcineurin/NFAT pathway has been blocked by using immunosuppressant drugs such as cyclosporine A (CsA), FK506, and rapamycin. These were developed to stop transplanted organs from being rejected by interfering with the NFAT pathway in T-cells. However, these drugs target interactions upstream of NFAT making them unspecific and thereby crude tools in the search of NFAT functions. From a clinical point of view CsA, FK506, and rapamycin are effective, but they also cause a range of adverse effects including nephrotoxicity, neurotoxicity, dyslipidemia, and diabetes. VIVIT is a selective peptide inhibitor of NFAT that was created to suppress NFAT dephosphorylation by blocking the NFAT docking site of calcineurin. VIVIT inhibits activation of NFATc1, c2 and c3 without interfering with calcineurin and it does not affect NF $\kappa$ B activity. When we first started working with NFAT, VIVIT was not cell-permeable and therefore not suitable for our aims. Since then, cell-permeable derivatives of VIVIT (11R-VIVIT) have been created [7].

### A-285 222

The bis-trifluoromethyl-pyrazole (BTP) compound A-285 222 was designed in an attempt to create a new, more specific immunosuppressant drug with less adverse effects than the presently used. BTPs are small organic molecules that were found to inhibit human T-cell IL-2 synthesis in a high-throughput screen. In efficacy studies for the inhibition of IL-2 production when the drug was administered orally, A-285 222 (ED<sub>50</sub> ~100 nM) was ten times more potent than CsA. Surprisingly, the compound was at least four times less potent in immune cells

from rodents [135]. A-285 222 blocks the nuclear localization of NFAT without affecting the activity of calcineurin, NFκB, AP-1 or the exporting kinases GSK-3, JNK and p38. The mechanism of action for this compound is still unknown [136]. Since A-285 222 had poor potency in rodent cells, the first *in vivo* pharmacokinetic study was carried out using cynomolgus monkeys. Monkeys were treated orally and the effect of the compound on intracellular T-cell cytokine production was assessed with flow cytometry. Neurotoxicity was observed when the plasma concentration of A-285 222 exceeded 4 µg/ml (9.6 µM), but at 1-2 µg/ml (2.4-4.8 µM) the drug was well tolerated. This concentration was obtained with 7.5 mg/kg twice a day after a starting bolus dose of 10 mg/kg on day 1. A 70% inhibition of cytokine production was achieved already after one day and persisted for the rest of the 14 days that the experiments lasted, and recovered to baseline values in about 5 days after the last given dose [137].

We first used A-285 222 in a study aiming to investigate the function of NFAT in the vasculature. Human myometrial vessels were used and proliferation, cytokine production (IL-6) and contractility were assessed after treatment of vascular smooth muscle cells and native vessels respectively, with A-285 222. Concentrations of 0.1 and 1 µM were used for these *ex vivo* experiments resulting in successful inhibition of NFAT [109]. In paper I we treated murine tissue with 1 µM A-285 222 and obtained a similar effect on NFAT activity compared to what we had observed in human tissue. For paper II we wanted to extend our studies of NFAT activity to include the *in vivo* situation. A-285 222 was administered intraperitoneally to one group of Balb/c mice receiving STZ-treatment and one group receiving vehicle-treatment for four weeks. Based on our experience from using A-285 222 in murine tissue and on the Birsan T et al study [137], we aimed at a plasma concentration of 10 µM (4.17 µg/ml), taking into account a suboptimal absorption of the drug when administered intraperitoneally. Assuming a blood volume of 7% of the body weight, we calculated a daily dose of 0.29 mg/kg body weight. Animals receiving STZ-treatment as well as A-285 222-treatment developed more severe hyperglycemia and weight loss than STZ-treated animals injected with vehicle. To avoid further weight loss in the group receiving A-285 222, we adjusted the dose to 0.15 mg/kg body weight after two weeks with the desired effect.

## ***In vivo* Hyperglycemia and Animal Models of Diabetes**

Despite an extensive search for suitable animal models of atherosclerosis and other vascular complications of diabetes, research has failed to create a model that sufficiently reflects human pathology. Therefore we have used different models of hyperglycemia and diabetes to answer specific questions in each particular study.

For *in vitro* and acute *in vivo* experiments we have used a simple approach of raising the glucose level from low/normal to high for a short time period in an otherwise physiological environment. For sustained hyperglycemia over weeks and months streptozotocin (STZ)-induced hyperglycemia in normal strains as well as in genetically altered mice was used. In this chapter different animal models for studying the pathogenesis of diabetic vascular disease will be reviewed and our choices discussed.

### *In vivo hyperglycemia*

In paper I, we showed that high glucose activates NFAT *ex vivo*. In paper II we wanted to examine whether this is true also *in vivo*. At this point we only had experience from acute experiments, and we therefore also wanted an acute *in vivo* experiment. The intraperitoneal glucose tolerance test (IP-GTT) measures the clearance of an intraperitoneally injected glucose load. Animals were fasted overnight and a solution of glucose (2 g per kg body weight) was administered by intraperitoneal injection. When used as a traditional tolerance test, blood glucose is measured at different time-points during 2 hours after glucose administration, however, we used it as a model for acute hyperglycemia and the animals were euthanized already after 18 minutes (paper II) or 30 minutes (paper III). Blood glucose levels were measured from whole venous blood at time 0 minutes and at end-point. The shorter time period in Paper II was based on the observation in Paper I that NFATc3 nuclear accumulation response to high glucose reached its maximum at 20 mM glucose after 20 minutes. Moreover, we anticipated a faster response due to the higher temperature of a living animal compared to the *ex vivo* experiments that were executed at room temperature. The relative increase of NFATc3 nuclear accumulation did however not match the results from the *ex vivo* experiments in Paper I. In Paper III we used 30 minutes to obtain greater changes in NFATc3 nuclear accumulation.

In paper II, we also wanted to investigate if NFAT activity was withheld during a longer period of hyperglycemia. Persistent hyperglycemia was induced with injections of STZ (further described below) and the effect on NFAT activity was evaluated after 8, 12 or 16 days. In paper II our aims were to investigate if NFAT is activated by hyperglycemia *in vivo* and if NFAT is involved in the regulation of OPN gene expression in response to hyperglycemia. OPN expression was assessed in animals subjected to STZ-induced hyperglycemia for 4 and 8 weeks. To be able to compare results from experiments in which we used the NFAT blocker A-285 222 with results from experiments using NFATc3<sup>-/-</sup> mice we primarily used Balb/c for most mechanistic experiments in the paper, since the NFATc3<sup>-/-</sup> mice are bred on a Balb/c background.

### *Animal models of diabetes*

Over the years many different species have been used for diabetic research including rabbit, pig, rat, and non-human primates. There are several advantages of using mouse models compared to other species including the small size, short generation time, the ease of inducing diabetes, and finally cost effectiveness [138]. Diabetes can be induced mice by islet cell destruction, diets and genetics. Islet cell destruction produces models for type 1 diabetes, diet-induced diabetes on the other hand typically reflects type 2 diabetes. Genetically altered mice can be used for both purposes.

Chemical destruction of the endocrine pancreas using the glucosamine-nitrosurea antibiotic STZ is an established model of hyperglycemia. STZ is a compound that is structurally similar to glucose and preferentially taken up by the GLUT2 transporters on beta-cells [139]. The toxic effect on the beta-cells results in death of the cells accompanied by a T-cell mediated immune response [139] leading to insulinopenic diabetes. Even though STZ-induced diabetes is a well accepted model, it does not exactly mimic type 1 diabetes since most mice are not ketotic and do not require insulin for their survival. Since STZ-treated mice are neither obese nor insulin resistant, this method is not suitable for modeling type 2 diabetes, at least not when working with macrovascular disease [140]. A major disadvantage of using STZ is that it is a toxin with potential confounding effects on the target cells apart from pancreatic beta-cells. Islets can also be destroyed by using transgenic mice expressing a viral antigen in the  $\beta$  cell using the rat insulin promoter. These mice will, when infected with the lymphocytic choriomeningitis mouse virus, develop an immune insulinitis and islet cell destruction [64].

Type 1 diabetes can also be modeled with the use of genetically modified mice. One example is the Akita mouse which spontaneously develops severe hyperglycemia, hypoinsulinemia and polydipsia at age 3-4 weeks. It has been shown to develop diabetic retinopathy, neuropathy and nephropathy [59], hence representing a good alternative to the chemically induced diabetes. Chemically induced diabetes in atherosclerosis prone strains such as apoE<sup>-/-</sup>, LDL receptor deficient (ldlr<sup>-/-</sup>) and human apoB-expressing mice also serve as models for type 1 diabetes.

Mice with characteristics of type 2 diabetes such as hyperglycemia, obesity and insulin resistance can be obtained by feeding C57BL/6, apoE<sup>-/-</sup> or ldlr<sup>-/-</sup> mice with high-fat diet, or by crossing apoE<sup>-/-</sup> and ldlr<sup>-/-</sup> mice with leptin-deficient mice. Unfortunately these models develop severe dyslipidemia, making it difficult to evaluate the contribution of hyperglycemia to vascular complications.

We have used diabetes models for several purposes, in paper III we wanted to study the involvement of NFAT in early stage diabetic retinopathy, in paper II we focused on the involvement of NFAT in diabetic macroangiopathy, and in paper IV the role of TNF $\alpha$  in diabetic macroangiopathy and microangiopathy was examined. These purposes imposed different demands on the experimental approach and when choosing a model of diabetic vascular complications, two major questions must be addressed: do we want a model of type 1 or type 2 diabetes? And do we want a model of micro- or macroangiopathy?

#### *Mouse models of diabetic retinopathy*

To date there is no evidence of any differences in the pathogenesis of diabetic retinopathy between type 1 and type 2 diabetes, and studies show that hyperglycemia is sufficient to initiate the development of retinopathy in mouse, rat, and man, though hypertension and dyslipidemia, often associated with type 2, have been shown to increase the rate of progression [141]. STZ-induced hyperglycemia in mice is a sufficient model for diabetic retinopathy in the early stage, but it does not recreate the proliferative phase of diabetic retinopathy, partly owing to the limitations in the duration of diabetes in laboratory animals. In paper III we studied the involvement of NFAT in early stage diabetic retinopathy by using changes in inflammatory markers in the retina of STZ-induced hyperglycemic mice.

#### *Mouse models of diabetic macroangiopathy*

There are two major limitations of the use of mouse models in diabetic macroangiopathy research: mouse models do not mimic human disease and diabetic wild-type mice do not develop more advanced atherosclerotic lesions unless it is associated with more severe dyslipidemia [138]. FVB mice develop diabetes and insulin resistance but not atherosclerosis and C57Bl/6 are atherosclerosis prone but do not exhibit more advanced lesions when made diabetic [59]. Several mouse strains will develop an atherogenic lipoprotein profile on high-fat diet, but their highly effective lipoprotein clearance system makes genetic modification necessary. Diabetes type 1 and 2 differ in their effects on plasma lipids and insulin levels. Patients with type 1 diabetes are hypoinsulinemic and generally have subtle alterations in their lipid profile, whereas patients with type 2 are hyperinsulinemic and often demonstrate an atherogenic lipoprotein profile. The challenge is to create a model where diabetes accelerates atherosclerosis and where the effect of hyperglycemia can be differentiated from the effect of hypercholesterolemia. Optimally, the lesions should also share characteristics with diabetic atherosclerotic lesions formed in man, such as increased inflammation depicted either by increased number of immune cells or

increased expression of cytokines, increased expression of certain genes such as OPN and PAI-1, and, in the case of type 2 diabetes, increased calcification [59].

There are three strains of transgenic mice commonly used in atherosclerosis research: apoE<sup>-/-</sup>, ldlr<sup>-/-</sup>, and human apoB-expressing mice. All of these have been used as models for diabetic macroangiopathy and can either be induced by diet or STZ. They exhibit accelerated atherosclerosis compared to non-diabetic mice, but the disadvantage of using them is that they often are severely hypercholesterolemic, leading to the effects of dyslipidemia overshadowing those of diabetes [138]. This problem can be diminished by feeding the mice with chow instead of western diet and there are studies showing hyperglycemia-dependent increases in atherosclerotic lesions of the aorta in STZ-induced hyperglycemic apoE<sup>-/-</sup> mice [142]. Normal Balb/c mice also develop atherosclerotic lesions, though moderate in size, when made diabetic with STZ. In 1996 Kunjathoor published a paper describing the first murine model using STZ. It was concluded that diabetes could increase lesion size in Balb/c but not in C57BL/6 mice [143]. The above mentioned studies use plaque size in aorta as the end-point for assessment of atherosclerosis, but as mentioned before, increased inflammation, increased expression of proteins, and calcification are also associated with human pathogenesis of diabetic macroangiopathy. Therefore models that do not increase lesion size can still be of value when investigating early changes of the vascular wall.

In paper IV our aim was to investigate the role of TNF $\alpha$  in hyperglycemia-induced vascular inflammation. We chose an approach based on STZ-induced hyperglycemia in two different strains: C57BL/6 and apoE<sup>-/-</sup> mice (bred on a C57BL/6 background). Also, hyperglycemia was induced with STZ in the tnfa<sup>-/-</sup> and apoE<sup>-/-</sup>/ tnfa<sup>-/-</sup> mice, both bred on C57BL/6 background. At 22 weeks of age, 60 mg of STZ per kg body weight was injected intraperitoneally once a day for 5 days, consistent with a low-dose regimen [144]. The mice were kept for 8 weeks after the induction of hyperglycemia, and blood glucose levels and body weight of the mice were assessed once a week. Because of the catabolic state of the diabetic animal, dramatic weight loss may occur. Animals that lost more than 10% of their original body weight were excluded from the study. These mice had severe hyperglycemia and were typically male, consistent with the often reported greater sensitivity to STZ of male mice compared to female [59]. The animals were fed chow in order to avoid high levels of cholesterol that might interfere with the study by making it more difficult to distinguish the effects of hyperglycemia from the effects of dyslipidemia. Still, the apoE<sup>-/-</sup> and apoE<sup>-/-</sup>/ tnfa<sup>-/-</sup> mice had significantly higher levels of cholesterol when made hyperglycemic, while STZ-treatment had no effect on the cholesterol levels of the C57BL/6 and tnfa<sup>-/-</sup> mice. The significance of this will be further discussed in the Results and Discussion

section. Apart from differences in cholesterol levels, another drawback of our model was that there was no increase in atherosclerosis lesion size seen in any of the STZ-treated strains. However, since vascular inflammation, another hallmark of diabetic atherosclerosis, could be assessed and differences demonstrated, the model still proved valid in relation to our aim.

### *Mouse models of cerebral angiopathy*

Diabetes increase the risk of cognitive decline, a problem that is increasing in significance as the diabetic patient population grows older. Cerebral vessels of all sizes are subjected to the detrimental effects of hyperglycemia [145]. Patients with cerebral intracranial macroangiopathy do not always suffer from cerebral microangiopathy, and vice versa [146]. In paper IV we examine the role of TNF $\alpha$  in hyperglycemia-induced vascular inflammation of cerebral vessels, using the same model as described above. We examined vessels of all sizes ranging from ~5-300  $\mu\text{m}$ . In man the definition of a cerebral microvessels is <100  $\mu\text{m}$  which include capillaries, arterioles and venules [147], but in mouse there is no true consensus on such a precise limit. However, measurements of cerebral microvessels in mice have shown arteriole and venule sizes of ~50  $\mu\text{m}$  [148]. In our studies, the basilar artery which is the largest one typically measures 250-300  $\mu\text{m}$  in diameter. There is little experience of using animal models for studying hyperglycemia-induced cerebral vessel pathology. Most of the current knowledge on cerebral microangiopathy of the diabetic patient has been obtained through clinical studies [145], and very little is known regarding diabetes-induced intracranial macroangiopathy. Hopefully, our model will provide experience that can be used in future studies of diabetic cerebral angiopathy.

## **Evaluation of Plaques and Vascular Inflammation**

Quantification of atherosclerotic plaques and evaluation of vascular inflammation are of fundamental importance when working with animal models of atherosclerosis and diabetes. Oil-Red-O staining of the descending aorta and determination of cross-sectional plaque area of the aortic root are commonly used methods for measuring lesion size based on the original work by Paigen et al [149] and Tangirala et al [150]. In paper IV, *en-face* preparations of the descending aorta were stained using the fat-soluble dye Oil-red-O [151]. Stained area (representing lesion area) and total aortic area were quantified by microscopy and computer aided morphometry under blinded conditions. The plaque area of aortic root cross-sections (10  $\mu\text{M}$  frozen subvalvular sections) were also assessed using computer morphometry. The combination of these two methods give a precise and reliable quantification of the atherosclerotic lesions of murine aorta, especially since mice

with mild lesion development often only display atherosclerotic changes in the aortic root [150].

As discussed in the previous chapter, quantifying the size of lesions is not sufficient to fully evaluate changes in atherosclerotic pathology, especially not in diabetic models. The inflammatory qualities of plaques are at least as important to understand the pathogenic process. Plaque inflammation can be investigated by staining for immune cells and autoantibodies against oxLDL. In paper IV, frozen sections of 10  $\mu\text{m}$  were collected from the subvalvular region and stained with rat anti-mouse MOMA-2 antibodies for monocyte/macrophage detection. For detection of IgM and IgG autoantibodies, slides were incubated with biotinylated anti-mouse IgM or IgG antibodies. Slides were counter-stained with hematoxylin and omission of primary or secondary antibodies were used as controls. Stained area was quantified by microscopy and computer aided morphometry under blind conditions.



## RESULTS & DISCUSSION

Research concerning the influence of diabetes on the vessel wall has in the past been focused around four oxidative stress-induced pathways: the polyol pathway, the hexosamine pathway, the PKC pathway and the AGE pathway [10]. Even though studies conducted in diabetic animal models have shown promising results, data from clinical trials targeting these pathways in diabetic patients have been disappointing [152]. The main object of this thesis was to elucidate novel molecular mechanisms, which may underlie the pathogenesis of diabetic vascular complications.

### Glucose Activates NFAT in Native Arteries

Characteristics of vascular dysfunction and VSMC phenotypic modulation associated with vascular disease include altered  $\text{Ca}^{2+}$  signaling, suggesting a possible involvement of  $\text{Ca}^{2+}$ -dependent transcription. Studies showing a role for NFAT in the differentiation of cardiac and skeletal muscle sparked an interest for this transcription factor in the field of vascular physiology. It was shown that NFAT is present in the vascular wall and that it is activated by vasoagonists such as Et-1 and UTP [95]. Apart from a significant role in the development of the heart and vascular tree during embryogenesis [153, 154], the function of NFAT in the vessel wall was unknown.

In 2002, Lawrence et al published data showing that NFAT could promote insulin gene transcription in beta-cells in response to glucose and GLP-1 [155]. Inspired by the Lawrence et al paper, we formulated the aim of Paper I: to investigate whether changes in extracellular glucose levels activate NFAT in the vascular smooth muscle of native arteries. Figure 4 summarizes the findings of Paper I. In Paper II, we followed up data from Paper I, and tested if hyperglycemia could activate NFAT also *in vivo*.

NFAT needs to translocate to the nucleus in order to function as a transcription factor. Using immunofluorescence and confocal microscopy, we evaluated the subcellular localization of NFAT in the vascular smooth muscle of mouse cerebral arteries. Previously it was shown that NFATc3 is the predominant isoform in murine cerebral arteries [95], therefore we focused on this isoform. Raising the glucose concentration from 11.5 mM to 20 mM for 30 minutes resulted in a 2.5-

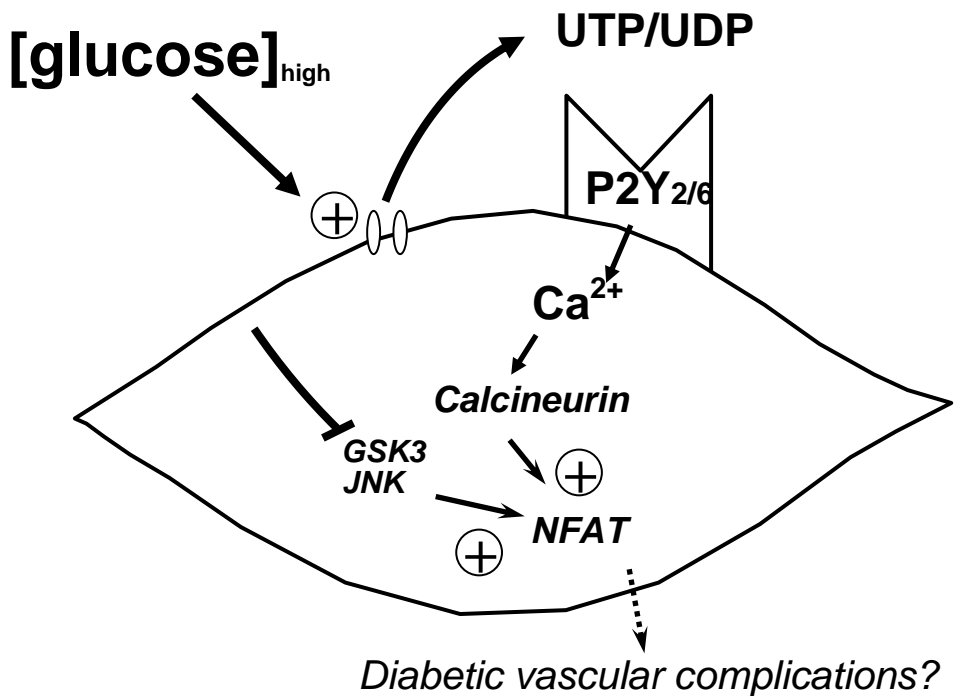


Fig. 4 Cartoon depicting the principal findings of Paper I. Exposure to HG leads to activation of NFAT in vascular smooth muscle via at least two mechanisms: 1) Release of extracellular nucleotides acting on P2Y receptors, leading to increased  $[Ca^{2+}]_i$ , and activation of calcineurin and 2) inhibition of GSK-3 $\beta$  and JNK, resulting in decreased kinase activity and hence, down-regulation of NFAT nuclear export activity. (modified from Nilsson J et al 2006 [1])

fold increase in the number of NFATc3-positive VSMC nuclei. The response of NFAT to high glucose in cerebral arteries was comparable to that of UTP and Et-1 [95] both concerning duration and amplitude [94]. The maximal time used for stimulation, 30 minutes, was chosen based on earlier work using the same experimental setup [94, 95]. When studying the time-dependency of the response, a significant increase was detected after 8 minutes of stimulation. This time frame is consistent with import studies of NFAT in T-cells [98]. Dose-response experiments showed that the effect of glucose is dose-dependent, with a stepwise increase of NFATc3 nuclear accumulation when raising the extracellular glucose concentration above the control concentration of 11.5 mM. Although a basal glucose level of 11.5 is typical for *in vitro* experiments, in which the only source of energy is the glucose in the surrounding solution, this concentration would be considered hyperglycemic *in vivo*. In Paper I, many of the experiments were performed using 11.5 mM as the control concentration since all previous work on NFAT had been performed at this concentration; but we also performed experiments using 2 mM glucose as basal level, to provide evidence for the responsiveness of this pathway at physiologically relevant levels.

Nuclear accumulation of NFATc3 does not always result in significant changes in transcriptional activity. Therefore, NFAT-luc mice were used to evaluate NFAT-dependent transcriptional activity after stimulation with 20 mM glucose for 30 minutes. Luciferase activity was measured 6 hours later, showing a 2-fold increase in glucose-induced NFAT-dependent transcription in aorta and portal vein. When aortas from NFAT-luc mice were equilibrated in 2 mM glucose over night and subsequently treated with 7, 11.5 or 20 mM glucose, a significantly increased NFAT-dependent transcription was measured. It is notable that the levels of NFATc3-positive nuclei and NFAT transcription from arteries incubated over night are higher than in non-cultured arteries, possibly reflecting a response of NFAT to culture itself. The results of these experiments indicate that NFAT may equilibrate during status quo conditions in order to again stand ready to respond to alterations in extracellular glucose concentration. In conclusion, our findings suggest that NFAT is a novel glucose-responsive transcription factor of the vessel wall, which may contribute to the development of diabetic complications.

## **Role of Extracellular Nucleotides**

UTP is an extracellular nucleotide that previously has been demonstrated to activate NFATc3 in the vascular smooth muscle of cerebral arteries [95]. Based on studies showing that glucose increases the release of ATP from human umbilical vein endothelial cells (HUVECs) and pancreatic  $\beta$ -cells [156, 157], we hypothesized that release of UTP may be a mechanism for glucose-induced NFAT

activation in vascular smooth muscle. To test this, we used the ectonucleotidase apyrase which effectively cleaves extracellular nucleotides. Addition of apyrase to high glucose treatment completely prevented NFATc3 nuclear accumulation and NFAT transcriptional activity. Resting non-excitatory cells release low amounts of nucleotides such as UTP and ATP, thereby maintaining steady state nanomolar concentrations of extracellular nucleotides. Changes in this relationship, either by increasing the rate of nucleotide release or repressing the activity of ecto-nucleotidases, lead to higher levels of extracellular nucleotides [158]. Our experiments suggest that high glucose disrupts this balance and increase the level of nucleotides available for auto-/paracrine signaling.

It is not evident from what cells the nucleotides are released, but there are a number of possibilities. Several vascular cell types have been shown to release nucleotides including vascular smooth muscle, endothelium, erythrocytes and platelets in response to mechanical or pharmacological stimuli [158]. In our experimental setting the possible sources of extracellular nucleotides are primarily endothelial cells and VSMC, but platelets and erythrocytes may also contribute.

Extracellular nucleotide concentrations are precisely regulated by ecto-enzymes which cleave nucleotide tri-/diphosphates. In our experiments we added apyrase in order to increase extracellular nucleotide hydrolysis, leading to an abolishment of NFAT response to glucose. Apyrase is abundantly expressed in vascular smooth muscle and endothelium and acts to regulate the ligand availability for P2Y receptors.

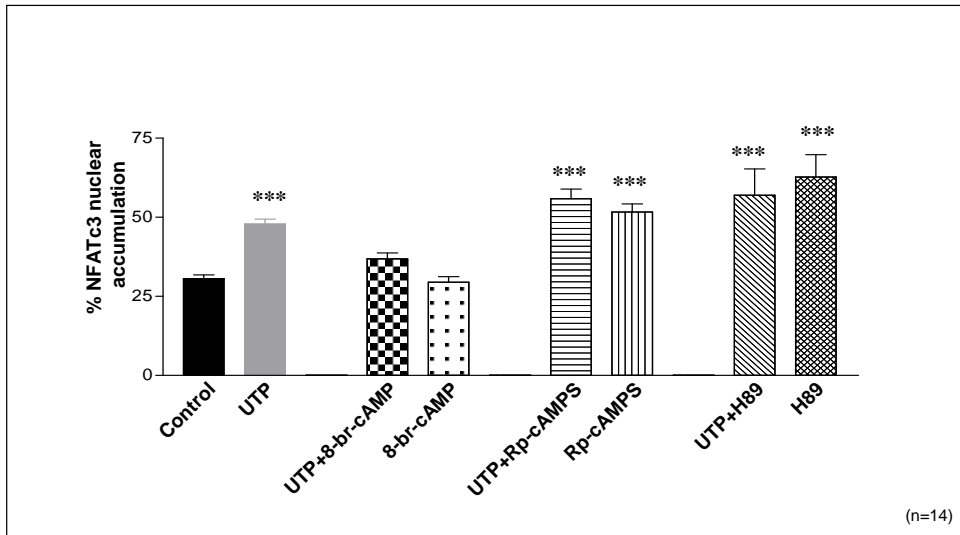
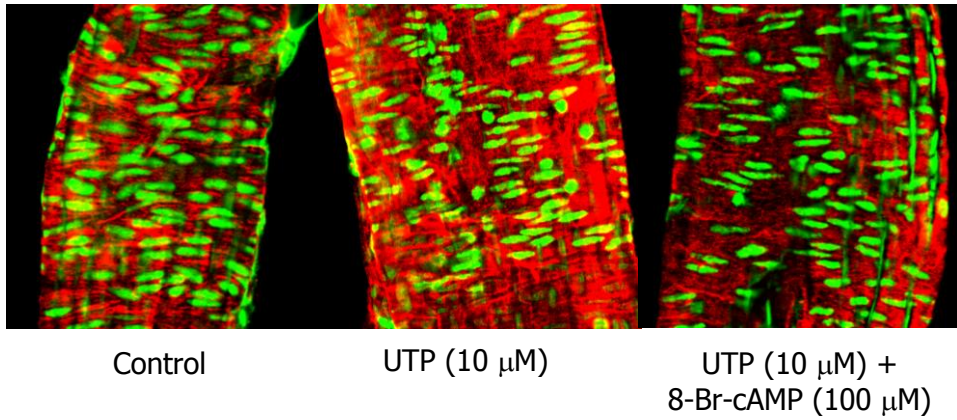
Extracellular nucleotides exert their auto-/paracrine signaling by binding to P2 receptors present at the cell surface. The P2 receptor family is divided into two groups: P2X receptors which are ligand-activated ion channels and P2Y receptors which are G-protein coupled receptors (reviewed in [159]). Each receptor is characterized by its affinity pattern for different nucleotides and different cell types display varying receptor profiles. Human and rat cerebral arteries have been shown to contract primarily in response to the UDP-receptor P2Y<sub>6</sub> with a lesser contribution of P2Y<sub>2/4</sub> (UTP and ATP receptors) and P2X<sub>1</sub> (ATP receptor) [160, 161]. To further investigate what receptors are involved in NFAT activation, we used the stable analogues UDPβS and UTPγS, selective agonist for P2Y<sub>6</sub> and P2Y<sub>2/4</sub> receptors, respectively and MRS2578, a selective antagonist of the UDP receptor P2Y<sub>6</sub>. UTP, UDPβS and UTPγS all increased NFATc3 nuclear accumulation but UTP was slightly more effective, suggesting that the less stable UTP may act both on P2Y<sub>2/4</sub> receptors, and after degradation to UDP, on P2Y<sub>6</sub> receptors. Inhibition of the P2Y<sub>6</sub> receptor decreased the NFATc3 response to high glucose, providing further evidence for the involvement of extracellular nucleotides in this response.

ATP, ADP, UTP and UDP increase global  $[Ca^{2+}]_i$  in arterial smooth muscle [95, 162, 163]. Addition of high glucose to cerebral arteries increased the intracellular  $Ca^{2+}$  concentration of the VSMC. This response could be prevented by apyrase, demonstrating that the glucose-stimulated increase in extracellular nucleotides is responsible for the observed increase in  $[Ca^{2+}]_i$ . Previous data showed that NFATc3 nuclear accumulation in smooth muscle depends on influx of  $Ca^{2+}$  from both intracellular stores and through VDCCs [95]. Dependence on VDCC activation for glucose-induced NFATc3 nuclear accumulation was shown by adding the VDCC inhibitors verapamil and nifedipine to the high glucose treatment, which resulted in a significant decrease in the response. However, as opposed to UTP-induced NFATc3 nuclear accumulation, high glucose-induced NFATc3 activity was not completely abrogated by adding verapamil or nifedipine. One possible explanation is that the  $Ca^{2+}$  signaling pathways of UTP and glucose are not identical. Studies devoted to examining the  $Ca^{2+}$  response to glucose and UTP in VSM have shown that UTP induces a transient peak followed by a plateau phase for at least 30 minutes, while glucose caused a progressive accumulation over the course of one hour [162, 164].

## **Role of GSK-3 $\beta$ and PKA**

NFATc3 nuclear accumulation reflects a dynamic balance between  $Ca^{2+}$ /calcineurin-dependent import and export mediated by kinases that rephosphorylate NFAT. In vascular smooth muscle, UTP has previously been shown to induce NFATc3 nuclear accumulation partly through JNK2 inhibition [94]. We showed that stimulation with 20 mM glucose for 30 min decreased the phosphorylation level, and thereby activity, of JNK in mouse aorta, providing further support for the notion that glucose induces NFAT activation via extracellular nucleotides such as UTP. Our data suggest a mechanism where acutely elevated glucose induces release of extracellular nucleotides, which in turn reduces JNK2 activity, leading to increased NFAT nuclear accumulation in VSMCs.

GSK-3 $\beta$  was originally demonstrated to inhibit glycogen synthesis in muscle cells by phosphorylating glycogen synthase. Endogenous inhibition of GSK-3  $\beta$  is achieved by phosphorylation of a single serine residue [165]. Insulin inhibits GSK-3 $\beta$  via the  $PIP_3$ -Akt pathway, in order to induce storage of glycogen [165]. GSK-3 $\beta$  has been demonstrated to inhibit NFAT nuclear accumulation in



**Fig. 5** PKA is tonically active in cerebral arteries and suppresses NFAT activity by phosphorylation (unpublished data, Nilsson-Öhman J. & Gomez M. F.).

transfected cells [100] and to counteract cardiac and skeletal muscle hypertrophy through inhibition of NFAT signaling [166, 167]. In Paper I, we demonstrated that high glucose increased the phosphorylation level of GSK-3 $\beta$  in cerebral arteries. Considering the ability of high glucose to inhibit the activity of GSK-3 $\beta$ , we wanted to test whether high glucose could induce increased NFATc3 nuclear accumulation through inhibition of GSK-3 $\beta$ . Indeed, 30 min incubation with the cell permeable GSK-3 $\beta$  inhibitor Myr-N-GKEAPPAPPQSpP-NH<sub>2</sub> induced NFATc3 nuclear accumulation to a level similar to that induced by glucose. Also, simultaneous administration of the GSK-3 $\beta$  inhibitor and high glucose did not result in any further increase of NFATc3 nuclear accumulation. These data show that GSK-3 $\beta$  is constitutively active in the VSM of cerebral arteries and that glucose activates NFAT partly via the inhibition of GSK-3 $\beta$ . A similar mechanism has previously been demonstrated for VLDL, which was shown to increase NFAT activity in vascular smooth muscle via inhibition of GSK-3 $\beta$  by PI3-kinase [108].

GSK-3 $\beta$  phosphorylation has been reported to be dependent on “priming” by another kinase, since it only phosphorylates proteins at serine/threonine residues located four amino acids N-terminal to another already phosphorylated site [168]. Results from studies performed in T-cells have shown that PKA primes for subsequent phosphorylation by GSK-3 $\beta$  but is not able to induce NFAT export on its own [100]. In relation to the work presented in Paper I, we wanted to investigate whether PKA is involved in the regulation of NFAT in vascular smooth muscle. Inhibition of PKA increased NFATc3 nuclear accumulation, whereas the cAMP analog 8-Br-cAMP prevented NFATc3 activation induced by UTP (Fig. 5). Hence, PKA is constitutively active in VSM of murine cerebral arteries, counteracting NFAT nuclear accumulation. Treatment with PKA inhibitors (Rp-cAMPS and H89) resulted in a significant increase relative control (Fig. 5). The PKA inhibitors could not further increase UTP-induced NFATc3 nuclear accumulation (Fig. 5), suggesting that UTP in part acts via inhibition of PKA. PKA has previously been shown to be tonically active in pressurized vessels [169] and it has been implicated to be an important inhibitor of smooth muscle proliferation [170].

## **Hyperglycemia Induces NFAT Activity *in vivo***

To investigate whether high glucose activates NFATc3 *in vivo*, we performed IP-GTT in mice. Blood glucose values reached 18 mM 18 minutes after injection of the glucose bolus. Hyperglycemia acutely induced NFATc3 nuclear accumulation in VSM of cerebral arteries demonstrating that this signaling pathway is engaged also *in vivo*. The basal level of NFATc3 nuclear accumulation in perfusion fixed arteries was higher than the control level measured in prior *ex vivo* experiments in

which arteries were fixed under non-pressurized conditions, possibly reflecting stimulation of NFATc3 by intraluminal pressure [115]. Since a quick rise in the blood glucose levels of healthy animals leads to increased levels of insulin, we wanted to exclude a possible effect of insulin on NFATc3 nuclear accumulation. *Ex vivo* treatment of cerebral arteries with insulin showed no effect on NFATc3 nuclear accumulation.

To further evaluate the effects of hyperglycemia on NFAT activity, NFAT-luc mice were treated with STZ. Luciferase activity of the aorta was assessed at day 8, 12 and 16 after the first STZ-treatment. At day 16, NFAT transcriptional activity was significantly higher in STZ-treated mice compared to vehicle-treated mice and significantly correlated to blood glucose concentrations. Similar experiments were performed in NFAT-luc mice that had been backcrossed for at least 4 generations with NFATc3<sup>-/-</sup> mice, yielding NFAT-luc/NFATc3<sup>+/+</sup>, NFAT-luc/NFATc3<sup>+/-</sup> and NFAT-luc/NFATc3<sup>-/-</sup> mice. NFAT-dependent transcriptional activity was significantly increased in diabetic NFATc3 competent mice when compared to non-diabetic controls, but no changes in luciferase activity were detected in heterozygous or NFAT-deficient mice, demonstrating that the response to hyperglycemia in mouse aorta is dependent on the NFATc3 isoform.

From these findings we propose a role for NFAT as a metabolic sensor of the vessel wall. NFAT has previously been identified as a regulator of several genes in the vasculature, which are of considerable interest regarding the pathogenesis of diabetic complications including COX-2 [88, 171], VCAM-1 [172], and TF [120, 173]. Interestingly, NFAT was recently found to be ADP-ribosylated in T-cells by PARP (previously demonstrated to mediate glucose-induced cell damage in several cell types), leading to enhanced NFAT DNA binding and transcriptional activity [174]. In the future, it would be attractive to investigate if NFAT DNA binding activity is increased by PARP also in the diabetic vessel wall.

#### *In vivo administration of A-285 222*

*In vivo* inhibition of NFAT and evaluation of end-points known to be associated with the pathogenesis of diabetic retinopathy are necessary in order to understand the involvement of NFAT in this process. We wanted to investigate how well the NFAT inhibiting drug A-285 222 was absorbed when administered intraperitoneally (see data in Paper III). This was achieved by comparing plasma concentrations of A-285 222 from animals receiving intracardiac delivery of the compound, to plasma concentrations from animals receiving intraperitoneal injection. Gas chromatography/mass spectrometry (GC/MS) was used to analyze plasma samples from different time points after drug administration. Quantification of A-285 222 plasma concentration was achieved by adding a



known concentration of the analogous compound A-216 491 to all plasma samples. The concentration of A-285 222 in plasma was calculated using a response factor determined from extraction and GC/MS analysis of plasma samples spiked with a known concentration of both A-285 222 and A-216 491. Mice subjected to intracardiac injection of 0.15 mg/kg body weight displayed plasma concentrations of 3.8  $\mu$ M after one minute and 3.2  $\mu$ M after five minutes. The plasma concentration of A-285 222 five minutes after intraperitoneal injection of the same dose reached a similar concentration (3.4  $\mu$ M), indicating sufficient drug absorption with this form of administration.

## NFAT Regulates Vascular Osteopontin Expression

After showing that NFAT is activated by increased levels of extracellular glucose *in vivo*, we were interested in possible target genes for NFAT in vascular smooth muscle, particularly genes involved in the pathogenesis of vascular complications of diabetes. In our laboratory, a cDNA microarray study using human myometrial arteries had previously been performed, which identified a number of genes that were differentially affected by NFAT inhibition with A-285 222. These genes could be divided into distinct groups involved in: a) immune and inflammatory responses, b) cellular growth, differentiation and division, c) metabolism (in particular mitochondrial ribosomal proteins), d) vasculogenesis and e) general repressors. The matrix cytokine OPN caught our interest, since it was strongly regulated by NFAT inhibition, and served as a starting point in the search of potential downstream targets of NFAT in vascular smooth muscle. In Paper II, we investigated whether OPN could be a possible downstream target of hyperglycemia-induced NFAT activity.

OPN acts both as a matrix protein facilitating adhesion and migration, and as a soluble cytokine [175]. Recently, it has also been demonstrated as an intracellular adaptor protein of antigen presenting cells [176]. OPN is a potent inhibitor of bone mineralization and is highly expressed in autoimmune and chronic inflammatory diseases, such as atherosclerosis [177]. High glucose and UTP have been shown to induce OPN expression in vascular smooth muscle [178, 179]. Arteries of diabetic patients display increased expression of OPN [180, 181] and plasma levels of OPN have been shown to correlate to the progression of diabetic nephropathy and retinopathy [182, 183]. In addition to evaluating glucose-induced NFAT activation *in vivo*, the second aim of Paper II was to investigate the involvement of NFAT in the regulation of OPN gene expression in response to hyperglycemia.

### *NFAT signaling regulates hyperglycemia-induced OPN expression*

NFAT involvement in the regulation of OPN expression was assessed ex-vivo and in vivo in cerebral arteries and aorta from Balb/c mice. Culture of intact cerebral arteries and aortas from Balb/c mice in 25 mM glucose for two days resulted in increased OPN expression compared to control (5 mM), as assessed by confocal immunofluorescence. This effect was prevented by apyrase and A-285 222, suggesting a role for extracellular nucleotides and NFAT in the regulation of hyperglycemia-induced OPN expression in native arteries. UTP has previously been found to induce OPN expression in cultured VSMCs [178]. We showed that UTP-induced OPN expression in intact arteries and that this is prevented by A-285222. The involvement of the NFATc3 isoform was established in similar experiments performed on cerebral arteries and aortas from NFATc3<sup>-/-</sup> mice, where UTP failed to induce OPN expression.

*In vivo*, OPN was found to be upregulated in plasma and in subvalvular aortic sections after eight weeks of STZ-treatment. The level of expression correlated significantly with blood glucose concentrations. The involvement of NFAT was examined in a four-week experiment where STZ-treated mice were injected intraperitoneally with A-285 222 once a day for the entire study period and compared to STZ-treated mice injected with NaCl (vehicle), non-STZ-treated mice injected with A-285 222 and non-STZ-treated mice injected with NaCl. STZ-treated animals developed hyperglycemia (>13.9 mM glucose) after two weeks and showed a significant increase in aortic OPN expression after four weeks. *In vivo* NFAT inhibition with A-285 222 significantly reduced aortic OPN expression, revealing that OPN expression in the aorta of hyperglycemic mice is NFAT-dependent. Interestingly, NFAT inhibition also reduced OPN expression in non-STZ-treated mice, suggesting a role for NFAT also in the constitutive expression of OPN.

Patient OPN plasma levels positively correlate with the presence and extent of coronary artery disease [184] and with restenosis after balloon angioplasty [185]. OPN has also been reported to be a component of the extracellular matrix of human atherosclerotic lesions [186-188]. Moreover, OPN is not only a marker of disease, but it takes part in driving the process of atherosclerosis [189]. Studies using balloon angioplasty in rat arteries have also demonstrated an upregulation of OPN expression in VSMCs [186, 190]. OPN have been suggested to play an important part in VSMC dedifferentiation [191], proliferation [192-194] and migration [178, 195, 196]. Experiments using OPN transgenic mice have demonstrated increased atherosclerosis [197], while OPN deficient mice develop less atherosclerotic lesions [198, 199]. Our results suggest that hyperglycemia induces OPN expression via NFAT in the vessel wall of diabetic individuals, possibly contributing to diabetic vascular complications.

### *NFAT binds to the OPN promoter region*

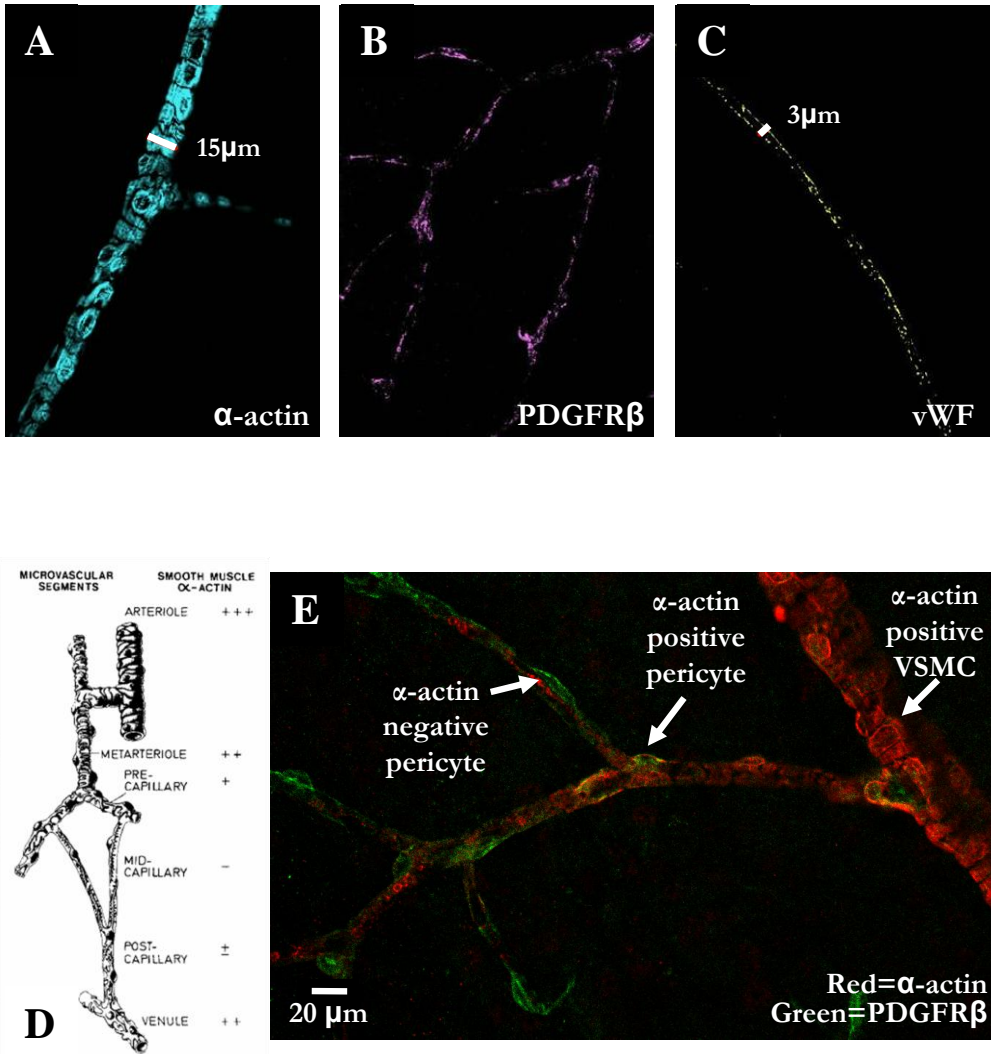
To demonstrate a potential direct effect of NFAT on OPN gene expression through binding to the OPN promoter region, we performed EMSA and a quantitative ChIP assay on mouse VSMCs and intact aortas, respectively. A computer analysis of the OPN gene promoter region revealed four potential NFAT binding sites of which two (site 1 and 2) were located close together in the distal region (-2405 to -2371), and two (site 3 and 4) were located in the proximal region (-270 to -152). Using EMSA and ChIP we demonstrated that NFAT binds to site 3 in OPN gene promoter region. Only NFATc3 was demonstrated to bind to the OPN promoter region, further supporting the notion that out of the four NFAT isoforms, NFATc3 is indeed involved in the regulation of OPN expression. High glucose as well as UTP has been shown to induce OPN gene expression in rat VSMCs and induction seems to be dependent on USF-1 and AP-1 [179, 200-203]. Renault and colleagues have suggested a coordinate action of NF $\kappa$ B, AP-1 and USF-1 for UTP-induced OPN expression in VSMCs [202]. Results from our ChIP experiments together with our data showing reduced glucose-induced OPN expression after pharmacological or genetic NFAT inhibition, suggest that NFAT binds to the OPN gene promoter under these conditions. From our data it can be concluded that glucose-induced OPN expression in intact mouse arteries is dependent on the activity of NFATc3.

OPN as well as other key elements of our story (UTP, JNK and GSK-3 $\beta$ ) have all been implicated to take part in the pathogenesis of neointima and/or restenosis formation [185, 186, 190, 204-207] and one report of NFAT participation in neointima/restenosis development in rat carotid artery had been published already in 2005 [171]. Over the years the evidence for NFAT involvement in the vascular response to injury has been piling up and at this point NFAT has been shown to mediate VSMC proliferation [111, 112, 118], cytokine production [118] and wound repair [208]. A parallel project at our laboratory has provided evidence for the involvement of NFATc3 in the regulation of alternative splicing of allograft inflammatory factor-1 (AIF-1) and interferon responsive transcript-1 (IRT-1), which are two human MHC class III transcripts of importance for neointima formation (Nilsson-Berglund LM, 2009; submitted). The study shows that overexpression of AIF-1 stimulates migration and proliferation of VSM cells, whereas its splicing variant IRT-1 exerts the opposite effect. Also, adenoviral infection of angioplasty injured rat carotid arteries with AdAIF-1 exacerbates intimal hyperplasia, whereas infection with AdIRT-1 reduces neointima. Pharmacological inhibition of NFAT or targeting of NFATc3 with siRNA results in lowered AIF-1/IRT-1 ratio and favors an anti-proliferative outcome. This data, together with data from other studies, strongly suggests a role for NFAT in the pathogenesis of restenosis. NFAT may thus be a potential target for the treatment of proliferative vascular disease.

## Hyperglycemia Activates NFAT in Retinal Vessels

The retina is a highly specialized tissue with particular demands for its supply of oxygen and nutrients [209]. The retinal vasculature consists of endothelium, vascular smooth muscle and pericytes. Vascular smooth muscle encircles arterioles, while pericytes are located adjacent to the endothelium of retinal capillaries. Histological studies have revealed that pericytes/VSMCs follow a sliding scale from highly contractile, SM  $\alpha$ -actin containing VSMCs in arterioles to stretched-out, non-contractile cells in the true capillaries of the retina [5] (Fig. 6). These features also seem to reflect the function of pericytes throughout the retinal vasculature tree. While pericytes with a contractile phenotype serves a similar role as VSMCs, pericytes with a non-contractile phenotype is believed to act as a regulator of endothelial function and as a mediator of signals between the blood stream and the neuronal part of the retina [41]. The blood retina barrier is made up by functional units of endothelial cells, pericytes, and glia cells (astrocytes and Müller cells).

The contribution of different cell types in the retina, as well as the chronology of events in the pathogenesis of diabetic retinopathy, is not fully understood. Early changes include increased leukocyte adhesion throughout the retinal vascular tree, increased endothelial permeability, endothelial dysfunction, neurodegeneration and alterations in glia cell morphology and reactivity [67]. Molecular changes include increased expression of adhesion molecules, cytokines and growth factors. *In vivo* animal models of diabetes have revealed an upregulation of NF- $\kappa$ B, VEGF, COX-2, prostaglandin E2, iNOS, ICAM-1, caspase-1, IL-1 $\beta$ , TNF $\alpha$ , and Fas in early stage retinopathy [67]. In humans, diabetic retinopathy is associated with elevated serum concentrations of NO, soluble IL-2 receptor (sIL-2R), IL-8, and TNF $\alpha$  which also correlate with severity of the disease [210]. In addition, studies investigating cytokine levels in the vitreous from patients with proliferative diabetic retinopathy have reported elevated concentrations of macrophage-specific colony-stimulating factor (M-CSF), MCP-1, TNF $\alpha$ , soluble ICAM-1, soluble VCAM-1, IL-6 receptor, IL-6, IL-8, and OPN [183, 211-215]. The serum level of TNF $\alpha$  has been shown to be an independent marker of proliferative retinopathy in type 1 diabetes patients [216]. Considering the ability of NFAT to sense and act upon changes in extracellular glucose levels, in Paper III, we wanted to investigate if NFAT is expressed in retinal microvessels and if it is activated by hyperglycemia. Further we wanted to study possible downstream targets and events of hyperglycemia-induced NFAT activation in retinal microvessels, linking this signaling pathway to the pathogenesis of diabetic retinopathy.



**Fig. 6** The retinal vasculature consists of endothelium, VSMCs, and pericytes. **A**, Retinal arteriole stained with SM  $\alpha$ -actin. **B**, Capillary network stained with the pericyte marker PDGF receptor  $\beta$  (PDGFR $\beta$ ). **C**, Capillary stained with the endothelial marker von Willebrand factor (vWF). **D**, Contractility and SM  $\alpha$ -actin expression vary throughout the retinal vascular tree (modified from Nehls & Drenckhahn 1991 [5] with permission). **E**, Double staining with the pericyte marker PDGFR $\beta$  and SM  $\alpha$ -actin, showing negative or positive SM  $\alpha$ -actin expression in pericytes depending on location.

### *Expression of NFAT isoforms in retinal microvessels*

Our studies have revealed expression of NFATc1, NFATc2 and NFATc3 at mRNA level in intact mouse retina. Isolated retinal vessels showed mRNA expression of NFATc2 and NFATc3, but not NFATc1. This suggests that NFATc1 is present only in the neuronal part of the retina, which is in agreement with earlier studies in neuronal tissue [217]. The vasculature showed an enrichment of NFATc2 relative to whole retina preparations. With confocal imaging it is possible to distinguish NFAT isoform expression in different cell types. We found that NFATc3 protein was expressed in VSMCs of arterioles and in endothelial cells, but it was not detected in pericytes.

### *Hyperglycemia activates NFAT retinal microvessels*

Based on the results of Paper I and II and the presence of NFAT in retinal microvessels, we wanted to test whether or not NFAT is able to respond to raised glucose levels also in these vessels. We incubated whole retinas in 2 mM and 20 mM glucose *ex vivo* for 30 minutes. This resulted in a significant increase in mean fluorescence intensity of nuclear NFATc3 in the endothelial cells. As in previous studies, the nuclear accumulation could be prevented by apyrase, demonstrating the involvement of extracellular nucleotides. Interestingly, addition of A-285222 or apyrase to glucose treatment decreased NFATc3 nuclear accumulation below control levels, indicating a higher level of constitutively active NFAT in retinal endothelium compared with previously studied VSMCs in other vascular beds.

As in Paper II, we used IP-GTT as an *in vivo* model of acutely induced hyperglycemia. 30 minutes after injection of a glucose bolus the blood glucose concentration averaged 16 mM, which resulted in a 64% increase in mean fluorescence intensity of nuclear NFATc3 in retinal endothelium compared to vehicle (NaCl). NFATc3 nuclear accumulation significantly correlated with blood glucose concentrations.

Also chronic hyperglycemia resulted in elevated NFAT activity *in vivo*. Hyperglycemia was induced in NFAT-luc mice by treating them for five days with STZ (corresponding to a low-dose regimen), resulting in a mean blood glucose concentration of 26 mM 12 days after the initial STZ-injection. At this time point, NFAT-dependent transcriptional activity in isolated retinal vessels from STZ-treated animals was 86% higher when compared to vessels from non-diabetic mice. This change correlated significantly with blood glucose levels. No significant differences were observed when NFAT-luciferase activity was measured in whole retinas from STZ-treated and control mice, suggesting that the effect of glucose on NFAT-dependent transcriptional activity takes place most likely only in the vasculature of the retina.

### *Hyperglycemia leads to increased expression of OPN in retinal vessels*

In Paper II, we demonstrated glucose-induced OPN expression by direct binding of NFATc3 to the OPN promoter. OPN has been reported to be increased in the vitreous of patients with diabetic retinopathy compared to non-diabetic patients [183]. In an in vitro study using retinal endothelial cells, high glucose was shown to induce endothelial cell migration accompanied by an increase in OPN expression [218]. The implication is that OPN contributes to the pathogenesis of diabetic retinopathy by promoting inflammation, cell adhesion, migration, and angiogenesis.

Even though vitreous fluids from diabetic patients have been demonstrated to contain elevated amounts of OPN, no study so far has investigated the presence of OPN in the vessels of the diabetic retina. Using immunofluorescence, we showed an elevated level of OPN protein expression in retinal vessels from STZ-treated rats. The immunofluorescence experiments were carried out one month after the induction of hyperglycemia. Our data show that OPN is present in the retinal vasculature of diabetic rats as early as after one month of diabetes. We are currently investigating the involvement of NFAT in the regulation of retinal OPN in response to diabetes.

### *Effect of hyperglycemia and inhibition of NFAT activity on plasma cytokine levels*

Using intraperitoneal administration of A-285 222, we wanted to elucidate the potential role of NFAT in diabetic retinopathy by evaluating established endpoints known to be associated with the disease. Mice were randomized into two groups receiving either vehicle or STZ. Mice were further divided into two groups given either vehicle or A-285 222 intraperitoneally once a day for the duration of the experiment. After four weeks the experiment was terminated and plasma was collected and eyes dissected out from the animals. So far we have acquired data from plasma samples, but not from retinas. Ongoing experiments investigate retinal vessels regarding vascular permeability using the Evan's blue technique, adhesion molecule mRNA expression using PCR, and cytokine protein levels using a multiplex cytokine assay.

For cytokine level quantification we chose a multiplex assay, which is designed to measure the concentrations of interferon  $\gamma$  (IFN $\gamma$ ), IL-10, IL-12p70, IL-1 $\beta$ , IL-6, TNF $\alpha$ , and keratinocyte-derived chemokine (KC)/growth-regulated oncogene (GRO)- $\alpha$  (CXCL1). Except for KC, all of these cytokines have been implicated in the pathogenesis of diabetic retinopathy [213, 219-229]. In our study there was no change in plasma cytokine concentrations for any of these proteins. The failure of this model to display the same changes as seen by others may relate to differences

between species, experimental design and disease duration. We also performed a separate set of experiments where NFATc3<sup>-/-</sup> and NFATc3<sup>+/+</sup> littermates were made hyperglycemic by injecting STZ. Four weeks of hyperglycemia did not cause any significant changes in plasma cytokine levels, nor did the lack of NFATc3. NFAT inhibition with A-285 222 significantly increased the level of KC, but had no effect on the concentration of any of the other cytokines. KC is a murine functional homologue of the human chemokine IL-8 [230]. IL-8 is a known angiogenic mediator that has been shown to be produced by retinal endothelial and glial cells in relation to ischemic angiogenesis [231]. Serum and vitreous levels of IL-8 have been demonstrated to correlate with the presence and activity of proliferative diabetic retinopathy [210-213, 232, 233]. In atherosclerotic lesions of apoE deficient mice, KC has been shown to protect arteries from vascular injury by improving vascular healing [234]. The source of KC in our experiments and the mechanism by which the NFAT signaling pathway may influence its expression is still unclear. On-going experiments measuring changes in cytokine expression upon diabetes and NFAT inhibition specifically in the retina will help us elucidate these questions.

## **Role of TNF $\alpha$ in Diabetes-Induced Vascular Inflammation**

Activation of innate immunity with the subsequent development of a chronic low-grade inflammatory response is recognized as a critical factor in the pathogenesis of diabetic complications. TNF $\alpha$  is one of the central pillars of innate immunity and has been implicated to play a role in important features of diabetic vascular complications including endothelial dysfunction, oxidative stress and inflammation [3, 42, 235]. TNF $\alpha$  is one of the most abundant cytokines expressed by macrophages and it exerts a range of tasks in inflammation, including regulation of cytokines, adhesion molecules and matrix metalloproteinases [236, 237]. After the discovery of the pivotal role of inflammation in atherosclerosis, numerous studies have been performed to better understand the involvement of TNF $\alpha$  in the disease process. It was discovered that TNF $\alpha$  is expressed in atherosclerotic plaques [238, 239] and that it is associated with atherosclerosis and cardiovascular events in epidemiological studies [240-242]. Experimental studies have demonstrated that TNF $\alpha$ -deficient hyper-cholesterolemic mice, as well as mice treated with TNF $\alpha$  inhibitors, develop less atherosclerosis [243, 244].

Considering the emerging evidence for a prominent influence of inflammation in diabetes, a similar role could be expected for TNF $\alpha$  in diabetic vascular complications. It has been shown that plasma levels of TNF $\alpha$  are increased in patients with both type 1 and type 2 diabetes and positively correlate with elevated fasting glucose levels, glycated hemoglobin (HbA<sub>1c</sub>) and markers of oxidative



stress [245, 246]. To investigate the role of TNF $\alpha$  in diabetic vascular changes we studied the development of early atherosclerotic alterations in large arteries, as well as endothelial activation in resistance cerebral arteries, in C57BL/6 wild type, apoE $^{-/-}$ , TNF $\alpha^{-/-}$ , apoE $^{-/-}$ /TNF $\alpha^{-/-}$ , and infliximab-treated apoE $^{-/-}$  mice made hyperglycemic with STZ. Infliximab is an inhibitory anti-TNF $\alpha$  antibody, which is currently in clinical use for the treatment of rheumatoid arthritis and other autoinflammatory diseases.

#### *TNF $\alpha$ and hyperglycemia-induced atherosclerotic plaque development*

Contrary to earlier reports [247-249], hyperglycemia had no significant effect on atherosclerotic plaque size in apoE $^{-/-}$ , apoE $^{-/-}$ /TNF $\alpha^{-/-}$  or infliximab-treated apoE $^{-/-}$  mice. This inconsistency may result from differences in diet and duration of the study. No plaques were observed in control and hyperglycemic C57BL/6 mice, but several of the TNF $\alpha^{-/-}$  mice developed lesions in the descending aorta. There was no difference between TNF $\alpha^{-/-}$  control and hyperglycemic mice, but hyperglycemic TNF $\alpha^{-/-}$  mice developed significantly more aortic atherosclerosis than hyperglycemic C57BL/6 mice. In agreement with previous studies [243, 244] the plaque area in the descendent aorta of apoE $^{-/-}$ /TNF $\alpha^{-/-}$  mice tended to be reduced compared with apoE $^{-/-}$  mice. Due to considerable variation of Oil-Red-O staining in the apoE $^{-/-}$  group, this difference was however not significant.

#### *TNF $\alpha$ and hyperglycemia-induced inflammation*

Macrophage inflammation and accumulation of autoantibodies against oxLDL were evaluated in the aortic root of all groups. Hyperglycemia significantly increased macrophage accumulation in atherosclerotic plaque of apoE $^{-/-}$  mice, but not accumulation of autoantibodies against oxLDL. These findings confirmed previous reports showing that diabetes aggravates the inflammatory response of large vessels in hyperlipidemic mice [63, 247, 248]. VCAM-1 expression was also evaluated in aortic root plaques of control and hyperglycemic apoE $^{-/-}$  and apoE $^{-/-}$ /TNF $\alpha^{-/-}$  mice, but was unaffected by hyperglycemia.

Surprisingly, hyperglycemic apoE $^{-/-}$ /TNF $\alpha^{-/-}$  and infliximab-treated apoE $^{-/-}$  mice had a more pronounced accumulation of macrophages, indicating a protective rather than aggravating effect of TNF $\alpha$  on macrovascular inflammation. In addition, hyperglycemic apoE $^{-/-}$ /TNF $\alpha^{-/-}$  mice also demonstrated increased accumulation of autoantibodies in aortic root plaques. Plasma levels of glucose and cholesterol significantly correlated with macrophage accumulation in both control and hyperglycemic apoE $^{-/-}$  mice. The association between plasma glucose and plaque macrophage accumulation was even stronger in apoE $^{-/-}$  mice lacking

TNF $\alpha$ , while the association between plasma cholesterol and macrophage accumulation was weaker.

#### *TNF $\alpha$ and hyperglycemia-induced endothelial activation in cerebral arteries*

Hyperglycemia increased VCAM-1 expression in cerebral arteries  $>150\ \mu\text{m}$  in apoE $^{-/-}$  mice and to an even greater extent in apoE $^{-/-}$ /TNF $\alpha^{-/-}$  mice. In the latter group an increased VCAM-1 expression was evident also in smaller arteries. Interestingly, blood glucose levels correlated with endothelial VCAM-1 expression in TNF $\alpha^{-/-}$ /apo E $^{-/-}$  and TNF $\alpha^{-/-}$  mice, but not in TNF $\alpha$ -competent mice. These data are consistent with the above suggested protective role of TNF $\alpha$  in diabetes-induced vascular inflammation. However, in cerebral arteries, hyperglycemia failed to increase VCAM-1 expression in infliximab-treated apoE $^{-/-}$  mice. These data suggest that the increased VCAM-1 expression in TNF $\alpha$  deficient mice is not a direct effect of the lack of TNF $\alpha$ , but rather a consequence of the transgenic model as a whole. Nevertheless, these data support the notion that TNF $\alpha$  is not a driving force of diabetic vascular inflammation in cerebral arteries.

#### *Protective role of TNF $\alpha$*

In conclusion, our findings demonstrate that hyperglycemia enhances vascular inflammation in the aorta and augments endothelial activation in cerebral resistance arteries of hyperlipidemic mice. We also conclude that diabetes-induced inflammation is not dependent on TNF $\alpha$ . Instead, our data rather suggest a protective role for TNF $\alpha$  in diabetic vascular complications.

TNF $\alpha^{-/-}$  mice exhibit defects in the development and organization of secondary lymphoid tissue such as spleen and lymph nodes. The primary effect of TNF deficiency is failure to organize the marginal zone of the spleen, and malformation of germinal centers [250, 251]. These alterations provide insufficient support for follicular dendritic cell migration and function, in turn leading to a deficiency in B-cell maturation and clonal expansion [252]. It is thus possible that the unexpected effect of TNF deficiency in this model is attributed to a fundamental dysregulation of the immune system. However, data acquired from the infliximab-treated mice clearly speaks against this possibility. There are in the literature several reports of TNF $\alpha$  having a protective role against severe injury, especially in autoimmune disease. TNF $\alpha$  has been found to have a protective function in autoimmune demyelination [253], autoimmune lupus nephritis [254], and type I diabetes [255]. The failure of mice to resolve late-phase inflammation when deficient of TNF $\alpha$  has previously been demonstrated [256, 257]. Proposed mechanisms for the anti-inflammatory effects of TNF $\alpha$  include trans-activation of

NF $\kappa$ B leading to the induction of anti-apoptotic genes improving cell survival [258] and induction of macrophage expression of alternative cytokine signaling including cytokines involved in anti-inflammatory responses such as IL-10 and transforming growth factor  $\beta$  (TGF $\beta$ ) [259, 260]. TNF $\alpha$  has also been shown to induce the release of IL-12 from macrophages that in turn stimulates lymphocyte secretion of IFN $\gamma$ , which acts to reduce macrophage chemokine production during late-phase inflammation [256]. Further studies are necessary in order to elucidate the mechanism behind the suggested protective role of TNF $\alpha$  in diabetic vascular inflammation.

## CONCLUSIONS

- High glucose activates NFAT in vascular smooth muscle *in vitro* and *in vivo* via release of extracellular nucleotides and inhibition of GSK-3 $\beta$  and JNK activity
- NFAT regulates OPN expression in arterial smooth muscle in response to hyperglycemia
- Hyperglycemia increases NFAT activity in retinal microvessels *in vivo*
- Diabetes enhances macrovascular inflammation in hyperlipidemic mice
- Diabetes-induced inflammation is not dependent on TNF $\alpha$ , which may exert a protective role in mice

# POPULÄRVETENSKAPLIG SAMMANFATTNING

Diabetes är en sjukdom som kan drabba människor i alla åldrar. Människor som drabbats av diabetes saknar förmåga att reglera sitt blodsocker. Hos friska människor producerar bukspottskörteln hormonet insulin som via blodet når kroppens alla muskler och organ. När insulin binder till sin receptor på cellernas yta, berättar det för cellen att den ska ta upp mer socker från blodet. Sockret kan sedan användas direkt eller lagras beroende på cellens behov. Unga människor drabbas framförallt av typ I diabetes som uppstår på grund av att bukspottskörteln attackeras av kroppens egna immunförsvar. Anledningen till att sjukdomen uppkommer är inte känd. När bukspottskörteln förstörs kan kroppen inte längre producera insulin och cellerna i kroppens muskler och organ förstår inte att de ska ta upp socker från blodet. Konsekvensen blir att det ansamlas stora mängder socker i blodet, vilket leder till ett livshotande tillstånd. Insulin kan tillföras kroppen genom att spruta in det antingen direkt i blodet eller i underhuds fett, en behandling som är livräddande för diabetespatienten. Eftersom det inte finns någon bot för diabetes måste diabetiker fortsätta behandlingen med insulin resten av livet. Äldre människor drabbas sällan av typ I diabetes, men kan istället få den närbesläktade sjukdomen typ II diabetes. En person med typ II diabetes kan fortfarande producera insulin, men cellerna förstår inte det insulinet försöker berätta för dem, de är insulinresistenta.

Både typ I och typ II diabetes leder till förhöjt blodsocker hos den drabbade individen. Trots att det finns blodsockersänkande mediciner för både typ I och typ II diabetiker, så drabbas majoriteten av diabetespatienter av svåra komplikationer ett eller två decennier efter sjukdomens debut. Komplikationerna uppstår på grund av att blodkärlen blir sjuka. Det är både små och stora kärl som drabbas och man delar därför in diabeteskomplikationer i små- och storkärlssjukdom. Det är framförallt små kärl i ögats näthinna, i njuren och i hjärnan som blir sjuka, vilket leder till synnedläggning, njursvikt och försämrad kognitiv förmåga. Även de små kärl som försörjer nerver drabbas vilket leder till känslnedläggning i bland annat

fötterna. Stora kärl drabbas lättare av åderförkalkning, vilket i förlängningen medför ökad risk för hjärtinfarkt och stroke hos diabetiker. Man tror att en del av förklaringen ligger i att diabetikers blodkärl blir inflammerade på grund av den ändrade sammansättningen av socker och fetter i blodet.

I vår forskning har vi undersökt rollen av två proteiner: nuclear factor of activated T-cells (NFAT) och tumor necrosis factor alpha (TNF $\alpha$ ). I våra kroppar finns tiotusentals olika proteiner som vart och ett har en särskild roll att fylla. För att ta reda på mer om vilken roll ett protein spelar behöver vi först av allt något att undersöka: en bit vävnad (till exempel en liten bit av ett blodkärl), ett djur (till exempel en mus), eller en människa. Utifrån de resultat som vi och andra forskare fått från tidigare studier skapar vi en hypotes. Hypotesen testas genom ett experiment där vi på något sätt manipulerar vår vävnadsbit, till exempel tillsätter en hög koncentration socker eller ett läkemedel, och sedan studerar vad som händer med det vi är intresserade av, till exempel ändrad aktivitet hos NFAT eller att en mus utvecklar mildare eller svårare diabetiska komplikationer efter behandling.

NFAT är ett protein som vi sedan tidigare vet är involverat i regleringen av inflammatoriska gener. En gen kan liknas vid en mall för ett protein. NFAT fungerar som en kontrollör av vilka mallar som ska användas för tillfället. Inflammatoriska gener är mallar för de proteiner som hjälper kroppen att försvara sig mot bakterier, virus och även andra skador. Vi vet sedan tidigare att diabetiker lider av en lågmäld inflammation i blodkärlen som med tiden skadar dem så svårt att det leder till komplikationer. Vår hypotes var att högt blodsocker ändrar aktiviteten hos NFAT, vilket skulle kunna leda till ökad inflammation i blodkärlens väggar. För att testa hypotesen använde vi möss som vi behandlade med ett ämne som förstör bukspottskörteln och därmed gör dem diabetiska. Efter en viss tid avlivade vi mössen och tog ut kroppspulsådern samt blodkärl från ögat och hjärnan. Genom att mäta aktiviteten hos NFAT kunde vi visa att det höga blodsockret hos mössen hade lett till att NFAT nu tillät blodkärlens celler att tillverka många fler kopior från de inflammatoriska mallarna. Vi drog från detta

slutsatsen att vår hypotes var riktig: NFAT påverkas av högt blodsocker vilket skulle kunna leda till ökad inflammation i blodkärlen hos diabetiker.

Vi satte nu upp en ny hypotes: vi tror att ökad aktivitet hos NFAT till följd av högt blodsocker leder till att en av de inflammatoriska mallarna, genen för osteopontin, kopieras i mycket större utsträckning än i den friska kärlväggen. Vi undersökte först att högt blodsocker leder till att det bildas mer osteopontin i kärlväggen, vilket det gjorde. För att bevisa att NFAT är nödvändig för kopiering av osteopontingenen, så använde vi oss av två verktyg: dels ett ämne som vi vet kan hämma NFAT's aktivitet, samt möss som helt saknar proteinet NFAT. När vi hämmade NFAT's aktivitet kunde högt blodsocker inte längre förmå blodkärls celler att producera mer osteopontin, vilket är ett bevis för att NFAT är nödvändig för att högt blodsocker ska kunna förändra produktionstakten av osteopontin. På samma sätt kunde blodkärl från musen som saknar NFAT inte bilda mer osteopontin när musen hade högt blodsocker.

I ett försök att förstå om aktivering av NFAT verkligen leder till sjukdom i ögats blodkärl, diabetisk retinopati, har vi arbetat med att karakterisera tidiga förändringar i dessa kärl hos diabetiska möss, bland annat vad gäller inflammation. Till exempel har vi sett att produktionen av osteopontin ökar vid diabetes även i dessa kärl. Vi har också genomfört ett djurförsök där diabetiska möss behandlats antingen med NFAT-hämmare eller med placebo under en månads tid. Vi har mätt koncentrationen av NFAT-hämmaren i blodet och sett att kroppen tar upp ämnet på ett tillfredsställande sätt. Vidare har vi samlat in vävnadsprover för framtida analys. Det vi vet hittills är att NFAT aktiveras av högt blodsocker även i dessa kärl och att vi kan minska denna aktivitet genom att behandla mössen med NFAT-hämmaren. Nu återstår mer arbete med att undersöka vävnadsproverna och få en första indikation på om NFAT-hämmaren kan förhindra utvecklingen av sjukdom i ögats blodkärl i samband med diabetes.

TNF är ett protein som är känt som ett av de viktigaste i kroppens immunförsvar. TNF produceras i stora mängder i samband med infektion och bidrar till att ge oss

feber samt lokal rodnad och svullnad kring infektionen. Det har också visat sig att TNF spelar en viktig roll i inflammatoriska sjukdomar som inte orsakas av virus och bakterier, till exempel reumatisk värk och inflammatorisk tarmsjukdom. För patienter med dessa sjukdomar har behandling med hämmare av TNF visat sig vara till stor hjälp. På experimentell nivå har man också tidigare visat att TNF bidrar till utvecklingen av åderförkalkning. Eftersom diabetes ger inflammation i kärlväggen och ger upphov till ökad risk för åderförkalkning, ställde vi upp hypotesen: TNF är viktig även för inflammation i kärlväggen orsakad av diabetes. För att testa vår hypotes använde vi oss av möss som helt saknar proteinet TNF. Vi gjorde dessa möss diabetiska och studerade utvecklingen av inflammation och åderförkalkning i kroppspulsådern, samt förekomst av inflammation i hjärnans kärl. Till vår stora förvåning stämde inte vår hypotes, resultaten från vårt försök visade istället att TNF snarare skyddar mot diabetessjukdomens effekt på blodkärlen. De möss som saknade TNF hade drabbats av mer inflammation i sina blodkärl än normala möss. Behandling med anti-TNF-behandling gav oss samma resultat: inflammationen ökade hos diabetiska möss som erhållit anti-TNF-behandling. Anledningen till den skyddande effekten av TNF vet vi inget om, men det finns andra studier som visat att TNF inte bara påskyndar den inflammatoriska processen utan också är den del av att bromsa den när den inte längre behövs.

I framtiden hoppas vi kunna undersöka om hämning av NFAT kan fungera som en behandling av diabeteskomplikationer. Vidare hoppas vi lära oss mer om hur olika proteiner bidrar till den inflammatoriska processen och förhoppningsvis också nya sätt att förhindra och/eller behandla diabetiska kärlkomplikationer.



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