Insulin resistance in platelets and monocytes

Anja Gerrits

Printed by Gildeprint Drukkerijen, Enschede
Cover art: ‘Obese figures’ by Joost Rompen

ISBN/EAN: 978-94-901-2294-2

Insulin resistance in platelets and monocytes

Insuline resistentie in bloedplaatjes en monocyten
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. J.C. Stoof, ingevolge het besluit
van het college voor promoties in het openbaar te verdedigen
op donderdag 7 januari 2010 des middags te 2.30 uur

door

Janna Gerrits

geboren op 24 september 1980, te Hardenberg

Promotor: Prof. dr. J.W.N. Akkerman
Co-promotor: Dr. T.W. van Haeften

The study described in this thesis was supported by a grant from the Dutch Diabetes Research Foundation (2004.00.029).

Financial support by the Netherlands Heart Foundation and the Dutch Diabetes Research Foundation for the publication of this thesis is gratefully acknowledged.

Additional financial support for the publication of this thesis by J.E. Jurriaanse Stichting is gratefully acknowledged.

Contents

Abbreviations		7
Chapter 1	General introduction	11
Chapter 2	The adipokines resistin, leptin, PAI-1 and RBP4 induce degradation of IRS-1 in megakaryocytes and production of insulin-resistant platelets	39
Chapter 3	Enhanced platelet tissue factor synthesis in type 2 diabetes patients	63
Chapter 4	Insulin inhibits tissue factor expression in monocytes	83
Chapter 5	Increased tissue factor expression in type 2 diabetes monocytes caused by insulin resistance	101
Chapter 6	Insulin inhibition of platelet-endothelial interaction is mediated by insulin effects on endothelial cells without direct effects on platelets: a rebuttal	113
Chapter 7	General discussion	119
Nederlandse samenvatting		137
Dankwoord		143
Curriculum Vitae and List of Publications		147

Abbreviations

ACRP30	Adipocyte Complement Related Protein of 30 kD
ADP	adenosine diphosphate
AGEs	advanced glycation end products
ASF	alternative splicing factor
ASP	acylation-stimulating protein
Bcl-3	B-cell lymphoma 3
BMI	body mass index
BSA	bovine serum albumin
CA	cantharidin
$[Ca^{2+}]_i$	intracellular Ca^{2+} level
cAMP	cyclic adenosine monophosphate
CBP	CREB-binding protein
CHX	cycloheximide
Clk1	Cdc2-like kinase 1
COX-1/2	cyclo-oxygenase-1/2
CREB	cAMP response element-binding protein
CRP	C-reactive protein
CVD	cardiovascular diseases
DM	diabetes mellitus
F	Factor
HDL	high-density lipoprotein
HT	HEPES–Tyrode
IBMX	3-isobutyl-1-methylxanthine
ICAM-1	intracellular adhesion molecule-1
IGF-1 R	insulin-like growth factor-1 receptor
IL-1β	interleukin-1β
IL-6	interleukin-6
INS-R	insulin receptor
IRS-1	insulin receptor substrate-1
ISI	insulin-sensitivity index
JAK2	Janus kinase 2
JNK	Jun NH_2-terminal kinase
LPS	lipopolysaccharide
LRP	lipoprotein-related protein

MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
MetS	metabolic syndrome
MODY	Maturity Onset Diabetes of the Young
$NaVO_3$	sodium vanadate
NCEP-ATPIII	National Cholesterol Education Program's Adult Treatment Panel III
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PAI-1	plasminogen activator inhibitor-1
PI3-K	phosphoinositide-3 kinase
PKB	protein kinase B
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
PRP	platelet-rich plasma
PTB	polypyrimidine-tract binding
PTP1B	protein-tyrosine phosphatase 1B
RBP4	retinol binding protein-4
rFVIIa	recombinant Factor VIIa
Ser	serine
serpin	serine protease inhibitor
SF2	splicing factor 2
SOCS	Suppressor Of Cytokine Signaling
SR	serine/arginine dipeptide
STAT	Signal Transducers and Activators of Transcription
T2DM	type 2 diabetes mellitus
TBS	tris-buffered saline
Thr	threonine
TF	Tissue Factor
TFPI	tissue factor pathway inhibitor
TLR4	Toll-like receptor 4
TNF-α	tumor necrosis factor-α
tPA	tissue plasminogen activator
TPO	thrombopoietin
TxA_2	thromboxane A_2
Tyr	tyrosine

VCAM-1	vascular cell adhesion molecule-1
VWF	von Willebrand factor
WAT	white adipose tissue

Chapter 1

General introduction

Disturbances of the haemostatic balance in type 2 diabetes

In the following part we review the pathogenesis of obesity leading to the prothrombotic situation in the metabolic syndrome and in type 2 diabetes. Obesity is a major risk factor for insulin resistance, type 2 diabetes and cardiovascular diseases (CVD). Increased levels of adipokines -the following are studied in this thesis: leptin, Plasminogen Activator Inhibitor-1 (PAI-1), resistin, Retinol Binding Protein4 (RBP4), visfatin, Tumor Necrosis Factor-α (TNF-α), Interleukin-6 (IL-6)- might induce insulin resistance. Insulin resistance leads to hypersensitive platelets, monocytes and endothelial cells resulting in easier activation of these cells. This might explain the prothrombotic situation in the metabolic syndrome and type 2 diabetes.

Obesity

The prevalence of obesity in adults has increased rapidly in western countries in the last decades [1,2]. In adults, overweight is defined as a body mass index (BMI) of 25 to 29.9 kg/m^2 and obesity as BMI ≥ 30 kg/m^2. Currently nearly 70% of adults in the US are classified as overweight or obese compared to 25% in the early 1960s [3,4]. Obesity alone was present in 15% of the adult population in the US in 1980 and has increased to 33% in 2004 [5]. The World Health Organization estimates that there are at least 400 million obese adults worldwide and this number will increase to more than 700 million in 2015. Obesity results from an excess energy intake and/or too little energy expenditure. Evidence indicates that obesity is associated with greater morbidity and poorer health-related quality of life than smoking, alcoholism or poverty [6]. If current trends continue, obesity will soon overtake smoking as the primary preventable cause of death [7]. Obesity is associated with numerous co-morbidities such as CVD, type 2 diabetes, hypertension, hypercholesterolemia, hypertriglyceridemia, nonalcoholic fatty liver disease, osteo-arthritis, asthma, sleep apnea/sleep-disordered breathing and certain cancers. Growing evidence suggests that a state of chronic inflammation associated with obesity provides a molecular link to some of these pathologic conditions.

Adipose tissue

In contrast to the idea that the only function of adipose tissue is to serve as an efficient storage depot for triglycerides, nowadays adipose tissue is considered an endocrine organ, which has a central role in lipid and glucose metabolism and

plays a substantial role in the pathogenesis and complications of obesity. In addition to adipocytes, adipose tissue contains a connective tissue matrix in which numerous immune cells including macrophages are present together with fibroblasts, endothelial cells and pre-adipocytes and which is well vascularized and innervated [8,9]. White adipose tissue (WAT) is the storage depot for triglycerides and when other tissues require energy, lipids are mobilized from here for systemic utilization. WAT is subdivided into subcutaneous and abdominal fat, which may have different (patho)physiological functions. Adipose tissue dysfunction results in several pathological processes such as inflammation, insulin resistance, hypertension and dyslipidemia [10]. These are caused by the active secretion of cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) [11,12], and other proteins and hormones expressed by adipose tissue together called adipokines. These adipokines or adipocytokines help to regulate metabolic homeostasis such as glucose metabolism, lipid metabolism, inflammation, coagulation, blood pressure and feeding behaviour, and include leptin, adiponectin, resistin, TNF-α, IL-6, monocyte chemoattractant protein-1 (MCP-1), plasminogen activator inhibitor-1 (PAI-1), angiotensinogen, visfatin, retinol-binding protein-4 (RBP4), serum amyloid A and others [13]. When adipose tissue and adipocyte volume increase, plasma adipokine levels rise, except for plasma adiponectin, which is lower in obesity [14]. Leptin and adiponectin are considered true adipokines, because they are primarily expressed by adipocytes [15,16], while other adipokines are more generally expressed. Resistin is produced by mononuclear cells such as macrophages [17] and TNF-α, IL-6, MCP-1, visfatin and PAI-1 are expressed in adipocytes as well as activated macrophages and other immune cells [18]. The relative contribution of the adipocyte versus the macrophage present in the adipose tissue is still unclear. Thus, adipose tissue is a complex and active secretory organ that both sends and receives signals that modulate energy expenditure, appetite, insulin sensitivity, endocrine and reproductive functions, bone metabolism, inflammation and immunity[19].

Adipokines

Leptin is a 167-amino acid adipokine of the *ob* gene with a molecular weight of about 16 kD and circulates in plasma in a concentration of ~30 ng/mL. It is mainly produced by adipocytes and has important effects on metabolism and feeding behavior [20]. Administration of recombinant leptin reduces food intake and increases energy expenditure in mice deficient for leptin, the *ob/ob* mice. In addition, leptin regulates inflammation, insulin secretion and angiogenesis [21]. Obesity is charac-

terized by elevated leptin levels, suggesting resistance to leptin in obese individuals [22]. Resistance to leptin might be tissue-specific and therefore several actions of leptin might be preserved in obese subjects, i.e. selective leptin resistance [23]. Elevated plasma leptin concentrations are an independent risk factor for CVD [24]. The direct link between leptin and the risk for thrombotic complications in obese individuals appeared in studies with *ob/ob* mice, which show delayed thrombotic occlusion and unstable thrombi, which more frequently embolize. Thrombus formation was restored to the phenotype of wild-type mice after leptin administration. Similar effects were observed with leptin-neutralizing antibodies [25]. Leptin potententiates human platelet aggregation *in vitro* in response to adenosine diphosphate (ADP) and thrombin in a dose-dependent manner [26]. Leptin activation occurs via activation of leptin receptors of which two subtypes, designated as leptin receptor Ob-Ra and Ob-Rb are known. Indeed, both leptin receptor Ob-Ra and Ob-Rb have been detected in platelets and signaling involves Janus kinase 2 (JAK2), insulin receptor substrate-1 (IRS-1), phosphoinositide-3 kinase (PI3-K) and protein kinase B (PKB), which cause elevation of cytosolic Ca^{2+}, activation of phospholipase C and protein kinase C (PKC) [27].

Adiponectin, also known as adipoQ or Adipocyte Complement Related Protein of 30 kD (ACRP30), is an abundantly present adipokine with a plasma concentration of around 5-10 µg/mL. It is mainly expressed by adipocytes and is a member of the complement factor Clq family of proteins [16,28,29]. It consists of a 244-amino-acid-long polypeptide that aggregates into multimeric complexes of various molecular mass. In plasma it circulates in three different full-length isoforms (trimer, hexamer, and multimers) and as globular form. In abdominal obesity and type 2 diabetes, plasma levels are decreased. Hotta *et al* described a 1.2- and 1.5-fold reduction of adiponectin levels in diabetic men and women respectively compared to nondiabetic subjects [14]. Adiponectin improves insulin sensitivity through inhibition of hepatic glucose production, and via enhancement of glucose uptake in muscle. It increases fatty acid oxidation in both liver and muscle and augments energy expenditure *in vitro*, presumably by enhanced uncoupling of ATP generation in mitochondria. Furthermore, it has anti-atherosclerotic and anti-inflammatory properties [10,30,31]. The association between adiponectin levels and platelet activation was studied in adiponectin-knockout mice. These mice showed accelerated thrombus formation, thereby demonstrating the antithrombotic effect of adiponectin. Opposite effects were observed in an overexpression model, which resulted in diminished thrombus formation [32]. Receptors for adiponectin have been identi-

fied in several tissues; AdipoR1, AdipoR2 and T-cadherin, which affect the downstream target AMP kinase. The presence of transcripts for adiponectin receptors in mouse platelets and human megakaryocytes suggests that adiponectin may have direct effects on these cells [32].

Plasminogen activator inhibitor-1 (PAI-1) is a single chain glycoprotein serine protease inhibitor (serpin) with a molecular weight of 50 kD consisting of 379 amino acids and has a plasma concentration of around 26 ng/mL. It binds and inactivates tissue plasminogen activator (tPA) and urokinase plasminogen activator inhibiting plasmin formation and degradation of fibrin clots. Thus, it thereby plays an important role in the regulation of fibrinolysis. It is expressed by hepatocytes, adipocytes, fibroblasts, mononuclear cells [33,34] and detected in α-granules of platelets, megakaryocytes, endothelial cells and smooth muscle cells [33,35]. PAI-1 circulates in its active form in complex with the glycoprotein vitronectin, which stabilizes the active conformation and increases its biological half-life. Plasma PAI-1 levels are elevated in patients with coronary artery disease [36-38]. Plasma PAI-1 activity, adipose tissue PAI-1 secretion and adipose PAI-1 mRNA levels were 7-fold, 6-fold and 2-fold increased respectively, in the obese group compared to healthy subjects [39]. Elevated plasma PAI-1 levels in the obese state correlated with increased PAI-1 mRNA and protein in adipose tissue [40]. Of all different haemostatic factors, PAI-1 shows the strongest association with insulin resistance. PAI-1 levels are strongly correlated with factors that define the metabolic syndrome such as BMI, blood pressure, plasma triglycerides and insulin level [41,42]. Elevated PAI-1 levels were an independent risk factor for the development of type 2 diabetes in healthy subjects [43], suggesting that they may be a very early risk marker for the development of the metabolic syndrome and type 2 diabetes. The receptor for PAI-1 that leads to these effects remains unknown. However, Degryse *et al* demonstrated that PAI-1 binds to low density lipoprotein-related protein (LRP), thereby activating the JAK2/Signal Transducers and Activators of Transcription (STAT) signaling pathway and stimulating cell migration [44].

Resistin is an inflammatory cytokine produced by adipocytes and immune cells and is upregulated by pro-inflammatory cytokines and lipopolysaccharide (LPS) [45-47]. The length of the pre-peptide is 108 amino acids, the molecular weight is around 12.5 kD and it circulates in a concentration of ~15 ng/mL. Resistin stimulates intracellular signaling through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation, which in turn promotes the synthesis of other

proinflammatory cytokines, including TNF-α, IL-6, MCP-1, and IL-12 and the surface adhesion molecules intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). However, the resistin receptor has not yet been identified. Various proinflammatory stimuli, such as TNF-α, IL-6, IL-1β and LPS, in turn induce the expression of resistin. In addition to its potential roles in insulin resistance, resistin may play a role in the inflammation associated with the pathogenesis of CVD, although this has not been established in humans.

Retinol Binding Protein 4 (RBP4) consists of 183 amino acid residues, has a molecular weight of 21 kD and its plasma concentration is ~50 ng/mL. RBP4 was discovered in an adipose-specific GLUT-4 knockout mouse, where the production of an adipocyte-derived protein that contributed to the effects on systemic metabolism was altered [48]. Serum levels of RBP4 were ~ 1.9-fold increased in obese and type 2 diabetic humans compared with lean controls and 2.5-fold elevated in GLUT-4 knockout mice compared to control mice. A direct role for RBP4 in insulin sensitivity was demonstrated by overexpression and administration of RBP4, which caused insulin resistance, whereas deletion of the gene reduced insulin resistance and blood glucose levels in obese mice [49]. A role for RBP4 in insulin resistance and diabetes is strongly supported by studies that show correlations between RBP4 levels and the magnitude of insulin resistance [50]. RBP4 levels are also indirectly correlated with a number of cardiovascular risk indexes including BMI, waist-to-hip ratio, serum triglycerides, systolic blood pressure and inversely with plasma HDL [50]. Whether RBP4 contributes to the pathogenesis of CVD in obesity is yet to be elucidated. RBP4 binds to LRP2/megalin with low affinity and a high-affinity cell-surface receptor for RBP4 has been recently identified as the multitransmembrane domain protein STRA6, which functions as a major physiological mediator of retinol uptake by cells [51].

Visfatin is a novel adipokine that corresponds to the previously identified pre-B colony-enhancing factor [52,53]. It consist of 491 amino acids and full-length visfatin has a molecular mass of 52 kD. It is highly expressed in adipose tissue, lymphocytes, liver and muscle, with higher expression in visceral than subcutaneous WAT [53,54] and circulates in plasma in a concentration of ~2 ng/mL. Visfatin is thought to bind to an allosteric site on the insulin receptor and induces insulin-like effects [53]. This suggests that it has a role in glucose homeostasis. The exact effects of visfatin remain largely unknown and much controversy exists regarding the relationship of visfatin to obesity, insulin resistance, and type 2 diabetes [55]. Serum levels of the

protein were increased in obese and diabetic mice [53]. In morbidly obese humans, who are defined as subjects with a BMI of 40 to 49.9 kg/m^2, elevated plasma levels were found which were subsequently lowered with weight loss [56]. However, in addition there are studies that show contradictory results [57,58].

Tumor Necrosis Factor-α (TNF-α) is a cytokine and an important inflammatory mediator, which is secreted both by inflammatory cells such as macrophages, NK cells, and T-cells, and by adipocytes. TNF-α is primarily produced as a 212-amino acid-long transmembrane protein, which can be cleaved off, resulting in soluble TNF-α with a molecular mass of 21 kD. TNF-α plasma levels are ~5 pg/mL. It is thought to play a role in the development of insulin resistance [59]. In adipose tissue from *ob/ob* mice, TNF-α mRNA levels were increased compared to wild-type controls [60]. In obese individuals, mRNA and protein levels were increased in adipose tissue compared with lean subjects [59]. Conflicting studies report either increases [61,62] in TNF-α in human subjects with insulin resistance or decreases [63,64]. TNF-α binds to 2 receptors, TNF receptor type 1 and TNF receptor type 2, which start 3 different pathways, namely activation of NF-κB, activation of mitogen-activated protein kinase (MAPK) pathways or induction of death signaling.

Interleukin-6 (IL-6) is a well-known pro-inflammatory cytokine and among the first to be implicated as mediator of insulin resistance and CVD. It consists of 212 amino acids and is a phosphoglycoprotein with a molecular weight ranging from 21 to 29 kDa, depending on the degree of glycosylation and phosphorylation. It is produced in large quantities by abdominal adipose tissue, and circulates in plasma in a concentration of ~1.2 ng/mL. IL-6 production in abdominal adipose tissue is 3-fold higher than in subcutaneous adipose tissue, indicating that IL-6 might be one of the factors that make an increase in abdominal adipose tissue a high risk factor for the development of insulin resistance [65]. Elevated IL-6 plasma levels are associated with insulin resistance and with increased risk of diabetes, independently of body weight [66]. Indeed, in type 2 diabetes patients circulating levels of IL-6 are 3.1-fold increased compared to control subjects [63,67]. A high dose of IL-6 given peripherally results in decreased insulin sensitivity [68]. It is therefore rather unexpected that IL-6 deficient mice are obese, insulin resistant and diabetic. Depletion of IL-6 in mice causes adverse rather than beneficial effects on fat and glucose metabolism, possibly secondary to the increased obesity [69]. Binding of IL-6 to its receptor, a cell-surface type I cytokine receptor complex consisting of the ligand-binding IL-6Rα chain and the signal-transducing component gp130,

also called CD130, results in activation of the JAK/STAT pathway [70].

Apart from leptin, adiponectin, PAI-1, resistin, RBP4, visfatin, TNF-α and IL-6 (summarized in Table 1), currently up to 50 other adipokines have been identified, such as angiotensinogen, acylation-stimulating protein (ASP), adrenomedullin, serum amyloid A and MCP-1.

Table 1. Adipokines (Source: Hajer *et al*, Eur Heart J, 2008)

Adipokine	Full name	Effects on
Leptin	Leptin	Food intake, fat mass
Adiponectin	Adiponectin	Insulin resistance, inflammation
Resistin	Resistin	Insulin resistance, inflammation
Visfatin	Visfatin	Insulin resistance
RBP4	Retinol binding protein 4	Lipid metabolism
TNF-α	Tumor Necosis Factor-α	Inflammation
IL-6	Interleukin-6	Inflammation
PAI-1	Plasminogen Activator Inhibitor-1	Fibrinolysis

Metabolic syndrome

The prevalence of the metabolic syndrome, also known as metabolic syndrome X, syndrome X, insulin resistance syndrome and Reaven's syndrome, is increasing to epidemic proportions throughout the world [71]. In particular, in obesity the prevalence of the components of the metabolic syndrome is increased [72].

Already in the 1920s associations of risk factors for diabetes have been described by Joslin EP, but the term metabolic syndrome was not used until late 1950s and came into common usage in the late 1970s. The metabolic syndrome is a clustering of components that reflect overnutrition and an inactive lifestyle including abdominal fat, insulin resistance, dyslipidemia (high triglycerides and low high-density lipoprotein (HDL)), and hypertension. Because the metabolic syndrome is not a single disease, multiple definitions have been developed over the years. In 2001, the National Cholesterol Education Program's Adult Treatment Panel III (NCEP-ATPIII) definition of the metabolic syndrome was set [73], where subjects suffering from the metabolic syndrome meet in the following criteria; the presence of three or more of the following components: waist circumference ≥ 102 cm for men and ≥ 88 cm for women, triglycerides ≥ 150 mg/dl, HDL-c < 40 mg/dl for men

or < 50 mg/dl for women, blood pressure ≥ 130/85 mm Hg, fasting plasma glucose ≥ 110 mg/dl.

Two additional components have been defined by the American Heart Association on metabolic syndrome definition. These are a proinflammatory and a prothrombotic state [74]. Studies show that the metabolic syndrome is associated with a 2-fold increase in CVD risk [75] and an up to 5-fold increased risk for type 2 diabetes [76,77]. It is accompanied by important changes in the haemostatic system that may change the balance towards a prothrombotic phenotype; hyperactive platelets, hypercoagulability, hypofibrinolysis and endothelial dysfunction. In addition, it is associated with nonalcoholic fatty liver disease, reproductive disorders and obstructive sleep apnea.

The underlying cause of this multifactorial disease has been sought in abdominal obesity and insulin resistance. Lifestyle modification and weight loss should, therefore, be the focus of treating or preventing the metabolic syndrome and its components.

Diabetes mellitus

Diabetes has reached epidemic proportions and currently affects 246 million people worldwide. It is expected to affect 380 million by 2025 and each year a further 7 million people develop diabetes (www.idf.org, Figure 1). In the Netherlands in 2007, 740.000 people were diagnosed with diabetes and an estimated 250.000 people undiagnosed. Currently one million people might be diabetic in the Netherlands (www.diabetesfonds.nl, www.rivm.nl). Diabetes is a chronic metabolic disease, characterized by day-time or fasting hyperglycaemia. There are a number of subtypes of diabetes, the most well known of which comprise of type 1 diabetes mellitus which is an autoimmune disease with destruction of β-cells, and type 2 diabetes mellitus which is a heterogeneous disorder characterized by a combination of defects in both insulin secretion and insulin sensitivity. Other subtypes are gestational diabetes, and specific diabetes disorders mostly due to hereditary syndromes such as Maturity Onset Diabetes of the Young (MODY) and many others. Some 80 to 90% of all diabetic individuals have type 2 diabetes.

Diabetes mellitus is characterized by hyperglycemia and is diagnosed by demonstrating one of the following: fasting plasma glucose level ≥ 126 mg/dL (7.0 mmol/L), plasma glucose ≥ 200 mg/dL (11.1 mmol/L) two hours after a 75 g oral

COUNTRY/TERRITORY	2010 MILLIONS	COUNTRY/TERRITORY	2030 MILLIONS
1 India	50.8	1 India	87.0
2 China	43.2	2 China	62.6
3 United States of America	26.8	3 United States of America	36.0
4 Russian Federation	9.6	4 Pakistan	13.8
5 Brazil	7.6	5 Brazil	12.7
6 Germany	7.5	6 Indonesia	12.0
7 Pakistan	7.1	7 Mexico	11.9
8 Japan	7.1	8 Bangladesh	10.4
9 Indonesia	7.0	9 Russian Federation	10.3
10 Mexico	6.8	10 Egypt	8.6

Figure 1. Number of people with diabetes (20-79 years), 2010 and 2030

Source: International Diabetes Federation (www.eatlas.idf.org)

glucose load, or symptoms of hyperglycemia and plasma glucose ≥ 200 mg/dL (11.1 mmol/L). A second test on a different day has to confirm whether a subject has diabetes.

Insulin plays a central role in this disease. It is the key hormone for regulation of blood glucose level. In general, normoglycaemia, a glucose level within 4.0 and 6.0 mmol/L, is maintained by the balanced interplay between insulin sensitivity in the peripheral tissues and insulin secretion by the pancreatic β-cells. Importantly, the normal pancreatic β-cell adapts to changes in insulin signaling; a decrease in insulin sensitivity is accompanied by upregulation of insulin secretion and vice versa. Dysfunction of the β-cells is a critical component in the pathogenesis of type 2 diabetes. When insulin sensitivity decreases the system usually compensates by increasing β-cell function. However, at the same time, the concentration of blood glucose at fasting and several hours after glucose load will increase mildly. Although this increase may be small, it is thought that in type 2 diabetes this leads to a compensatory increase in insulin secretion by the pancreatic β-cells and, ultimately, β-cell failure. Insulin resistance is characterized by a reduction in sensitivity to the action of insulin in tissues involved in glucose metabolism, including the liver, skeletal muscle and adipocytes. Additionally, insulin resistance occurs in other cell types, such as platelets, macrophages and cardiac myocytes.

Atherothrombosis

Diabetes is a chronic disease with severe complications, such as CVD, visual impairment, nephropathy, neuropathy and amputations. Each year 3.8 million

deaths are attributable to diabetes. And this number is even higher when death caused by secondary effects, such as diabetes-related lipid disorders and hypertension, are added. On average, people with type 2 diabetes will die 5-10 years earlier than people without diabetes. CVD is the major cause of death in diabetes patients. Diabetes is considered to be a prothrombotic state; eighty percent of type 2 diabetes patients will die a thrombotic death. The risk of cardiovascular mortality and morbidity in type 2 diabetes patients is 2 to 8 fold increased compared with healthy controls. Atherothrombotic disorders affect the coronary and cerebral circulation and peripheral arteries in the lower limbs. The combination of hyperactive platelets, hypercoagulability, hypofibrinolysis and endothelial dysfunction creates a thrombotic clustering which explains the increased risk of CVD in type 2 diabetes patients (Diabetes Atlas, 3rd edition, IDF 2007, Diabetes and CVD: Time to Act, IDF 2001, WHO Diabetes Unit Epidemiology).

Increased platelet reactivity

Activated platelets adhere to the vascular subendothelium which is exposed following endothelial cell damage; platelets will then release storage granules and aggregate to form thrombi. Activation of platelets is accompanied by the increased expression of activated GPIIb/IIIa receptors which bind fibrinogen and facilitate platelet aggregation. Platelets are activated by a number of different factors including components of the subendothelium such as collagen, and the coagulation factor thrombin, and endogenous factors released from storage granules; ADP, platelet activating factor and thromboxane A2 (TxA_2). The adhesion/aggregation of platelets is regulated by the balance between pro-aggregants and anti-aggregants within the circulation.

Platelet function is also regulated by insulin acting via insulin receptors on the platelet surface [78,79]. Under *in vitro* conditions, insulin has a direct inhibitory effect on platelets from healthy individuals. Inhibition is caused by interference with the suppression of cAMP, a potent intracellular inhibitor of aggregation and secretion. In the presence of insulin, the insulin receptor on the surface becomes Tyr phosphorylated as well as a downstream target of the insulin receptor, IRS-1. Subsequently, IRS-1 binds to the α-subunit of the inhibitory GTP-binding protein of adenylyl cyclase $Gi\alpha-2$, thereby inactivating the subunit through Tyr phosphorylation. Gi is an intermediate in the signaling pathway of the purinergic P2Y12 receptor for ADP. The inhibition of $Gi\alpha-2$ by insulin interferes with the suppression of cAMP levels and attenuates platelet aggregation [78].

Platelet hyperactivity and hyperaggregability play a crucial role in the development

of thrombotic complications associated with type 2 diabetes patients. Diabetic platelets are more sensitive to agonists, likely because they are less sensitive to the inhibitory action of insulin. This insulin resistance can therefore lead more easily to activation. Indeed, platelets from type 2 diabetic individuals adhere to the vascular endothelium and aggregate more easily than from healthy individuals. Under experimental conditions type 2 diabetes platelets show better adhesion, aggregation and TxA_2 production than controls. They circulate while exposing P-selectin caused by fusion of the α-granules with the plasma membrane. Intensive insulin treatment partly normalizes the hyperaggregability, further suggesting that the hyperactivity seen *in vivo* might be caused by the absence of insulin inhibition [80-85].

Hyperactivity of platelets may be caused by abnormal synthesis by their progenitor, the megakaryocytes. Megakaryocytes develop from haematopoietic stem cells in the bone marrow in a two weeks process of differentiation, which involves DNA endoreduplication, cytoplasmic maturation and expansion, and finally release of cytoplasmic fragments into the circulation [86,87]. Megakaryocytopoiesis and platelet production are under control of cytokines, as well as adhesive interactions within the microenvironment of the bone marrow [88]. Thrombopoietin (TPO), also known as c-Mpl ligand, is the most potent cytokine for stimulating the proliferation and maturation of megakaryocytes progenitor cells, stimulating megakaryocytes to increase in cell size and ploidy and to form proplatelet processes that fragment into single platelets [89]. In *in vitro* cultures, maturation is under control of TPO, stem cell factor, interleukin-1β and other cytokines. Interestingly, changes in relative concentrations of growth factors trigger alterations in protein expression, illustrating the high responsiveness of megakaryocytes to cytokines.

Hypercoagulability

In type 2 diabetes, elevated circulating levels of Tissue Factor (TF), plasma fibrinogen, factors VII, VIII, XI and XII, kallikrein and possibly von Willebrand factor (VWF) are detected, explaining the hypercoagulability observed in these patients [90]. Indeed, increased levels of these factors might lead to a more activated state of the coagulation cascade, as reflected by increased levels of prothrombin fragment 1 + 2 and thrombin-anti-thrombin complexes. Elevated levels of blood-borne TF are associated with the metabolic syndrome [91]. And also, TF antigen and protein levels were elevated in diabetic patients with microvascular disease or overt CVD [92,93].

TF, formerly known as thromboplastin, is a 47-kDa protein and the initiator of the

coagulation cascade and thereby a key regulator of coagulation. When it comes into contact with circulating activated factor VII (VIIa), a TF-FVIIa complex is formed which activates factor IX, which in turn activates factor X. Alternatively, factor X is directly converted to factor Xa by TF-FVIIa. In complex with factor Va and Ca^{2+}, factor Xa catalyzes the conversion of prothrombin to thrombin, resulting in fibrin formation, platelet activation and generation of a thrombus. TF is inhibited by tissue factor pathway inhibitor (TFPI), which is mainly synthesized and secreted by endothelial cells. TFPI binds to FXa and thereby inhibits TF/FVIIa activity.
TF is constitutively expressed in subendothelial cells such as vascular smooth muscle cells, resulting in rapid initiation of coagulation in case of vascular damage [94]. In contrast, monocytes express little TF under physiological conditions. However, they are a major source of TF upon contact with LPS, C-reactive protein, CD40 ligand, oxidized LDL, TGFβ, angiotensin II, hyperglycemia and adipokines [95-103]. The most extensively studied agonist LPS binds and activates the Toll-like receptor 4 (TLR4). TLR4 signals to the IκB-kinase pathway and the three MAPK pathways, p38, ERK and JNK [99]. This leads to activation of transcription factors Egr-1, AP-1 and NF-κB and an increase in TF mRNA transcription [100].
TF expression increases in the cytosolic compartment and TF translocates to the plasma membrane, where it becomes a source for TF-rich microparticles. These particles are vehicles for so-called circulating or blood-born TF [104]. This form of TF is detected in the bloodstream and originates from endothelial cells, vascular smooth muscle cells, leukocytes and platelets [105,106]. In addition, TF containing microparticles are released from atherosclerotic plaques [107]. Atheroslerotic plaques from type 2 diabetes patients contain high levels of apoptotic microparticles [108], which are mainly of monocytic origin. They retain almost all TF activity, suggesting a direct link between shed microparticles and plaque thrombogenicity. Indeed, active TF has been identified within thrombi formed in coronary arteries [109]. In animal models for thrombosis blood-born TF from leukocyte-derived microparticles initiated fibrin formation at the luminal site of the growing thrombus [110,111]. However, the relative importance of blood-born TF and vessel wall-associated TF in arterial thrombosis is under debate [111,112].
Recently, a novel source for TF expression was discovered [113] and confirmed [114] (Figure 2). The anucleate platelet has long been considered incapable of synthesizing proteins. Intron removal was thought to be confined to the nucleated megakaryocyte and platelet proteins to be the exclusive result of transcription and translation in this progenitor cell. Schwertz *et al* reported that platelets from healthy human subjects contain TF pre-mRNA and upon platelet activation this is

processed to a mature transcript, resulting in the synthesis of TF protein, procoagulant activity and accelerated plasma clot formation. Pre-mRNA splicing in the platelet is controlled by the dual specificity Cdc2-like kinase (Clk)1, which contains an N-terminal region enriched in serine/arginine dipeptide (SR) that interacts which the splicing factor 2 /alternative splicing factor (SF2/ASF), a regulator of constitutive and alternative splicing [113].

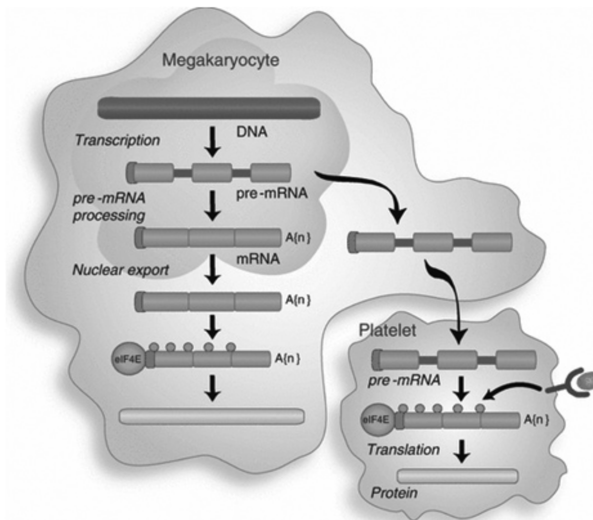


Figure 2. Signal-dependent pre-mRNA splicing in platelets.
During the final stages of differentation, splicing factors accumulate in the cytoplasm of megakaryocytes. The splicing components, along with specific pre-mRNAs, are transferred to ancucleate platelets. Stimulated platelets activate their splicing machinery and generate mature mRNAs that are subsequently translated into protein. This novel pathway of control provides platelets with a mechanism to alter their transcriptome and proteome proflie in response to cellular activation (Source: Weyrich *et al*, J Thromb Haemost, 2008)

Hypofibrinolysis

Studies have demonstrated that type 2 diabetes is associated with decreased fibrinolysis, the process wherein a fibrin clot, the product of coagulation, is broken down. The main cause is the strongly elevated level of the fibrinolytic inhibitor PAI-1 [41], the protein previously discussed. In addition, elevated levels of tPA and changes in clot structure may play a role.
The serine protease tPA, which is synthesized and secreted by endothelial cells, catalyzes the conversion of plasminogen to plasmin, the main protein responsible

for clot breakdown. In clinical studies tPA showed strong association with the metabolic syndrome [115,116] and tPA levels were a strong predictor of future cardiac events [117]. This appears counterintuitive, since elevations of tPA levels would seem to be beneficial for the breakdown of a clot by the generation of plasmin. However, high tPA levels might indicate vascular damage and PAI-1 levels exceed tPA concentration (26 ng/mL versus 8.5 ng/mL respectively [118], thereby favouring inhibition of fibrinolysis [119].
Hyperglycaemia observed in diabetes patients changes fibrin structure and function by glycation, generating a clot which has a denser structure and is more resistant to fibrinolysis leading to prolonged clot lysis times. Indeed, fibrinogen purified from type 2 diabetes patients with poor glycaemic control formed fibrin clots with decreased pore size and smaller fibre thickness with reduced susceptibility to clot lysis [120]. Type 2 diabetes patients had decreased plasminogen and tPA binding to fibrin with less plasmin generated on the fibrin surface compared to controls [121]. In addition, studies showed that clot density increased with increasing number of metabolic syndrome characteristics [122,123].

Endothelial dysfunction

The vascular endothelium plays a crucial role in the maintenance of vascular homeostasis. It provides a physical barrier between the vessel wall and the fluid in the lumen and secretes mediators that regulate vascular tone, coagulation and fibrinolysis, and adherence of leukocytes to the surface and inhibit platelet adhesion [124]. The development of endothelial dysfunction promotes thrombosis and inflammation [124-127]. In healthy subjects with insulin resistance and in type 2 diabetes patients impairment of endothelial-dependent vasodilatation is observed [128-131]. Inflammatory cytokines including TNF-α and IL-6, and the acute phase protein CRP influence endothelial cell function, as do some other adipokines [132-134]. This leads to a proinflammatory phenotype in endothelial cells, demonstrated by the increased release of VCAM-1, ICAM-1, E-selectin, microparticles and VWF.

Outline of the thesis

The aim of the studies presented in this thesis is to elucidate the cause of the prothrombotic condition in type 2 diabetes patients. The studies were focused on platelets and monocytes, which are two important cell types involved in thrombo-

sis.

We addressed the following questions:

1. Platelets from type 2 diabetes patients are hyperactive and could cause thrombosis. Do adipokines that circulate in elevated levels in obese subjects induce changes in the synthesis of platelets by megakaryocytes thereby explaining the hyperactivity? And if so, what are the mechanisms? (Chapter 2)
2. Recent findings suggest that platelets are a new source for TF expression. What is the signaling pathway that leads to TF synthesis in platelets? Does insulin interfere with platelet TF synthesis? And if so, is this disturbed in type 2 diabetes patients? (Chapter 3)
3. Does insulin inhibit TF expression in monocytes and monocyte-derived microparticles and if so, what is the signaling pathway involved (Chapter 4) and is this disturbed in type 2 diabetes patients? (Chapter 5)
4. Why do some investigators not find similar results compared to our studies, concerning the direct effects of insulin on platelets? (Chapter 6)

Finally, in Chapter 7 our findings are discussed in relation to published literature in order to obtain a general view of the cause of the prothrombotic condition in type 2 diabetes patients.

References

1. Poirier P, Giles TD, Bray GA, Hong Y, Stern JS, Pi-Sunyer FX, Eckel RH. Obesity and cardiovascular disease: pathophysiology, evaluation, and effect of weight loss: an update of the 1997 American Heart Association Scientific Statement on Obesity and Heart Disease from the Obesity Committee of the Council on Nutrition, Physical Activity, and Metabolism. Circulation 2006; 113:898-918.
2. Klein S, Burke LE, Bray GA, Blair S, Allison DB, Pi-Sunyer X, Hong Y, Eckel RH. Clinical implications of obesity with specific focus on cardiovascular disease: a statement for professionals from the American Heart Association Council on Nutrition, Physical Activity, and Metabolism: endorsed by the American College of Cardiology Foundation. Circulation 2004; 110:2952-2967.
3. Flegal KM, Carroll MD, Ogden CL, Johnson CL. Prevalence and trends in obesity among US adults, 1999-2000. JAMA 2002; 288:1723-1727.
4. Manson JE, Bassuk SS. Obesity in the United States: a fresh look at its high

toll. JAMA 2003; 289:229-230.
5. Ogden CL, Yanovski SZ, Carroll MD, Flegal KM. The epidemiology of obesity. Gastroenterology 2007; 132:2087-2102.
6. Sturm R, Wells KB. Does obesity contribute as much to morbidity as poverty or smoking? Public Health 2001; 115:229-235.
7. Allison DB, Fontaine KR, Manson JE, Stevens J, VanItallie TB. Annual deaths attributable to obesity in the United States. JAMA 1999; 282:1530-1538.
8. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest 2003; 112:1796-1808.
9. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest 2003; 112:1821-1830.
10. Berg AH, Combs TP, Du X, Brownlee M, Scherer PE. The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. Nat Med 2001; 7:947-953.
11. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 1993; 259:87-91.
12. Kern PA, Ranganathan S, Li C, Wood L, Ranganathan G. Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. Am J Physiol Endocrinol Metab 2001; 280:E745-51.
13. Hajer GR, van Haeften TW, Visseren FL. Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. Eur Heart J 2008; 29:2959-71.
14. Hotta K, Funahashi T, Arita Y, Takahashi M, Matsuda M, Okamoto Y, Iwahashi H, Kuriyama H, Ouchi N, Maeda K, Nishida M, Kihara S, Sakai N, Nakajima T, Hasegawa K, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Hanafusa T, Matsuzawa Y. Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. Arterioscler Thromb Vasc Biol 2000; 20:1595-1599.
15. Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL, Caro JF. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med 1996; 334:292-295.
16. Hu E, Liang P, Spiegelman BM. AdipoQ is a novel adipose-specific gene dysregulated in obesity. J Biol Chem 1996; 271:10697-10703.
17. Savage DB, Sewter CP, Klenk ES, Segal DG, Vidal-Puig A, Considine RV,

O'Rahilly S. Resistin / Fizz3 expression in relation to obesity and peroxisome proliferator-activated receptor-gamma action in humans. Diabetes 2001; 50:2199-202.

18. Shoelson SE, Lee J, Goldfine AB. Inflammation and insulin resistance. J Clin Invest 2006; 116:1793-801.
19. Shoelson SE, Herrero L, Naaz A. Obesity, inflammation, and insulin resistance. Gastroenterology 2007; 132:2169-80.
20. Campfield LA, Smith FJ, Guisez Y, Devos R, Burn P. Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. Science 1995; 269:546-549.
21. Myers MG Jr. Leptin receptor signaling and the regulation of mammalian physiology. Recent Prog Horm Res 2004; 59:287-304.
22. Frederich RC, Hamann A, Anderson S, Lollmann B, Lowell BB, Flier JS. Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. Nat Med 1995; 1:1311-1314.
23. Correia ML, Rahmouni K. Role of leptin in the cardiovascular and endocrine complications of metabolic syndrome. Diabetes Obes Metab 2006; 8:603-610.
24. Wallace AM, McMahon AD, Packard CJ, Kelly A, Shepherd J, Gaw A, Sattar N. Plasma leptin and the risk of cardiovascular disease in the west of Scotland coronary prevention study (WOSCOPS). Circulation 2001; 104:3052-3056.
25. Konstantinides S, Schafer K, Neels JG, Dellas C, Loskutoff DJ. Inhibition of endogenous leptin protects mice from arterial and venous thrombosis. Arterioscler Thromb Vasc Biol 2004; 24: 2196-2201.
26. Nakata M, Yada T, Soejima N, Maruyama I. Leptin promotes aggregation of human platelets via the long form of its receptor. Diabetes 1999; 48:426-429.
27. Dellas C, Schafer K, Rohm IK, Lankeit M, Leifheit M, Loskutoff DJ, Hasenfuss G, Konstantinides SV. Leptin signalling and leptin-mediated activation of human platelets: importance of JAK2 and the phospholipases Cgamma2 and A2. ThrombHaemost 2007; 98:1063-1071.
28. Maeda K, Okubo K, Shimomura I, Funahashi T, Matsuzawa Y, Matsubara K. cDNA cloning and expression of a novel adipose specific collagen-like factor, apM1 (AdiPose Most abundant Gene transcript 1). Biochem Biophys Res Commun 1996; 221:286-289.
29. Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF. A novel serum protein similar to C1q, produced exclusively in adipocytes. J Biol Chem 1995; 270:26746-26749.
30. Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T,

Murakami K, Tsuboyama-Kasaoka N, Ezaki O, Akanuma Y, Gavrilova O, Vinson C, Reitman ML, Kagechika H, Shudo K, Yoda M, Nakano Y, Tobe K, Nagai R, Kimura S, Tomita M, Froguel P, Kadowaki T. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. Nat Med 2001; 7:941-946.

31. Okamoto Y, Kihara S, Ouchi N, Nishida M, Arita Y, Kumada M, Ohashi K, Sakai N, Shimomura I, Kobayashi H, Terasaka N, Inaba T, Funahashi T, Matsuzawa Y. Adiponectin reduces atherosclerosis in apolipoprotein E-deficient mice. Circulation 2002; 106:2767-2770.
32. Kato H, Kashiwagi H, Shiraga M, Tadokoro S, Kamae T, Ujiie H, Honda S, Miyata S, Ijiri Y, Yamamoto J, Maeda N, Funahashi T, Kurata Y, Shimomura I, Tomiyama Y, Kanakura Y. Adiponectin acts as an endogenous antithrombotic factor. Arterioscler Thromb Vasc Biol 2006; 26:224-230.
33. Simpson AJ, Booth NA, Moore NR, Bennett B. Distribution of plasminogen activator inhibitor (PAI-1) in tissues. J Clin Pathol 1991; 44:139-143.
34. Samad F, Yamamoto K, Loskutoff DJ. Distribution and regulation of plasminogen activator inhibitor-1 in murine adipose tissue in vivo. Induction by tumor necrosis factor-alpha and lipopolysaccharide. J Clin Invest 1996; 97:37-46.
35. Konkle BA, Schick PK, He X, Liu RJ, Mazur EM. Plasminogen activator inhibitor-1 mRNA is expressed in platelets and megakaryocytes and the megakaryoblastic cell line CHRF-288. Arterioscler Thromb Vasc Biol 1993; 13:669-674.
36. Paramo JA, Colucci M, Collen D, van de Werf F. Plasminogen activator inhibitor in the blood of patients with coronary artery disease. Br Med J (Clin Res Ed) 1985; 291:573-574.
37. Aznar J, Estelles A, Tormo G, Sapena P, Tormo V, Blanch S, Espana F. Plasminogen activator inhibitor activity and other fibrinolytic variables in patients with coronary artery disease. Br Heart J 1988; 59:535-541.
38. Juhan-Vague I, Alessi MC, Joly P, Thirion X, Vague P, Declerck PJ, Serradimigni A, Collen D. Plasma plasminogen activator inhibitor-1 in angina pectoris. Influence of plasma insulin and acute-phase response. Arteriosclerosis 1989; 9:362-367.
39. Eriksson P, Reynisdottir S, Lonnqvist F, Stemme V, Hamsten A, Arner P. Adipose tissue secretion of plasminogen activator inhibitor-1 in non-obese and obese individuals. Diabetologia 1998; 41:65-71.
40. Morange PE, Alessi MC, Verdier M, Casanova D, Magalon G, Juhan-Vague I. PAI-1 produced ex vivo by human adipose tissue is relevant to PAI-1 blood level. Arterioscler Thromb Vasc Biol 1999; 19:1361-1365.

41. Juhan-Vague I, Roul C, Alessi MC, Ardissone JP, Heim M, Vague P. Increased plasminogen activator inhibitor activity in non insulin dependent diabetic patients--relationship with plasma insulin. Thromb Haemost 1989; 61:370-373.
42. Mansfield,M.W., Stickland,M.H., & Grant,P.J. (1997) PAI-1 concentrations in first-degree relatives of patients with non-insulin-dependent diabetes: metabolic and genetic associations. Thromb Haemost, 77:357-361.
43. Festa A, D'Agostino R Jr, Tracy RP, Haffner SM. Elevated levels of acute-phase proteins and plasminogen activator inhibitor-1 predict the development of type 2 diabetes: the insulin resistance atherosclerosis study. Diabetes 2002; 51:1131-1137.
44. Degryse B, Neels JG, Czekay RP, Aertgeerts K, Kamikubo Y, Loskutoff DJ. The low density lipoprotein receptor-related protein is a motogenic receptor for plasminogen activator inhibitor-1. J Biol Chem 2004; 279:22595-604.
45. Bajaj M, Suraamornkul S, Hardies LJ, Pratipanawatr T, DeFronzo RA. Plasma resistin concentration, hepatic fat content, and hepatic and peripheral insulin resistance in pioglitazone-treated type II diabetic patients. Int J Obes Relat Metab Disord 2004; 28:783-789.
46. Kaser S, Kaser A, Sandhofer A, Ebenbichler CF, Tilg H, Patsch JR. Resistin messenger-RNA expression is increased by proinflammatory cytokines in vitro. Biochem Biophys Res Commun 2003; 309:286-290.
47. Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS, Lazar MA. The hormone resistin links obesity to diabetes. Nature 2001; 409:307-312.
48. Minokoshi Y, Kahn CR, Kahn BB. Tissue-specific ablation of the GLUT4 glucose transporter or the insulin receptor challenges assumptions about insulin action and glucose homeostasis. J Biol Chem 2003; 278:33609-33612.
49. Yang Q, Graham TE, Mody N, Preitner F, Peroni OD, Zabolotny JM, Kotani K, Quadro L, Kahn BB. Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. Nature 2005; 436:356-362.
50. Graham TE, Yang Q, Bluher M, Hammarstedt A, Ciaraldi TP, Henry RR, Wason CJ, Oberbach A, Jansson PA, Smith U, Kahn BB. Retinol-binding protein 4 and insulin resistance in lean, obese, and diabetic subjects. N Engl J Med 2006; 354:2552-2563.
51. Kawaguchi R, Yu J, Honda J, Hu J, Whitelegge J, Ping P, Wiita P, Bok D, Sun H. A membrane receptor for retinol binding protein mediates cellular uptake of vitamin A. Science 2007; 315:820-5.
52. Samal B, Sun Y, Stearns G, Xie C, Suggs S, McNiece I. Cloning and charac-

terization of the cDNA encoding a novel human pre-B-cell colony-enhancing factor. Mol Cell Biol 1994; 14:1431-1437.

53. Fukuhara A, Matsuda M, Nishizawa M, Segawa K, Tanaka M, Kishimoto K, Matsuki Y, Murakami M, Ichisaka T, Murakami H, Watanabe E, Takagi T, Akiyoshi M, Ohtsubo T, Kihara S, Yamashita S, Makishima M, Funahashi T, Yamanaka S, Hiramatsu R, Matsuzawa Y, Shimomura I. Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. Science 2005; 307:426-430.
54. Hammarstedt A, Pihlajamaki J, Rotter Sopasakis V, Gogg S, Jansson PA, Laakso M, Smith U. Visfatin is an adipokine, but it is not regulated by thiazolidinediones. J Clin Endocrinol Metab 2006; 91:1181-1184.
55. Arner P. Visfatin--a true or false trail to type 2 diabetes mellitus. J Clin Endocrinol Metab 2006; 91:28-30.
56. Haider DG, Schindler K, Schaller G, Prager G, Wolzt M, Ludvik B. Increased plasma visfatin concentrations in morbidly obese subjects are reduced after gastric banding. J Clin Endocrinol Metab 2006; 91:1578-1581.
57. Berndt J, Kloting N, Kralisch S, Kovacs P, Fasshauer M, Schon MR, Stumvoll M, Bluher M. Plasma visfatin concentrations and fat depot-specific mRNA expression in humans. Diabetes 2005; 54:2911-2916.
58. Pagano C, Pilon C, Olivieri M, Mason P, Fabris R, Serra R, Milan G, Rossato M, Federspil G, Vettor R. Reduced plasma visfatin/pre-B cell colony-enhancing factor in obesity is not related to insulin resistance in humans. J Clin Endocrinol Metab 2006; 91:3165-3170.
59. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. J Clin Invest 1995; 95:2409-2415.
60. Schafer K, Fujisawa K, Konstantinides S, Loskutoff DJ. Disruption of the plasminogen activator inhibitor 1 gene reduces the adiposity and improves the metabolic profile of genetically obese and diabetic ob/ob mice. FASEB J 2001; 15:1840-1842.
61. Katsuki A, Sumida Y, Murashima S, Murata K, Takarada Y, Ito K, Fujii M, Tsuchihashi K, Goto H, Nakatani K, Yano Y. Serum levels of tumor necrosis factor-alpha are increased in obese patients with noninsulin-dependent diabetes mellitus. J Clin Endocrinol Metab 1998; 83:859-862.
62. Dandona P, Weinstock R, Thusu K, Abdel-Rahman E, Aljada A, Wadden T. Tumor necrosis factor-alpha in sera of obese patients: fall with weight loss. J Clin Endocrinol Metab 1998; 83:2907-2910.
63. Muller S, Martin S, Koenig W, Hanifi-Moghaddam P, Rathmann W, Haastert

B, Giani G, Illig T, Thorand B, Kolb H. Impaired glucose tolerance is associated with increased serum concentrations of interleukin 6 and co-regulated acute-phase proteins but not TNF-alpha or its receptors. Diabetologia 2002; 45:805-812.

64. Bruun JM, Verdich C, Toubro S, Astrup A, Richelsen B. Association between measures of insulin sensitivity and circulating levels of interleukin-8, interleukin-6 and tumor necrosis factor-alpha. Effect of weight loss in obese men. Eur J Endocrinol 2003; 148:535-542.
65. Fried SK, Bunkin DA, Greenberg AS. Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. J Clin Endocrinol Metab 1998; 83:847-850.
66. Wannamethee SG, Lowe GD, Rumley A, Cherry L, Whincup PH, Sattar N. Adipokines and risk of type 2 diabetes in older men. Diabetes Care 2007; 30:1200-1205.
67. Pickup JC, Mattock MB, Chusney GD, Burt D. NIDDM as a disease of the innate immune system: association of acute-phase reactants and interleukin-6 with metabolic syndrome X. Diabetologia 1997; 40:1286-1292.
68. Tsigos C, Papanicolaou DA, Kyrou I, Defensor R, Mitsiadis CS, Chrousos GP. Dose-dependent effects of recombinant human interleukin-6 on glucose regulation. J Clin Endocrinol Metab 1997; 82:4167-4170.
69. Wallenius V, Wallenius K, Ahren B, Rudling M, Carlsten H, Dickson SL, Ohlsson C, Jansson JO. Interleukin-6-deficient mice develop mature-onset obesity. Nat Med 2002; 8:75-79.
70. Heinrich PC, Behrmann I, Müller-Newen G, Schaper F, Graeve L. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. Biochem J 1998; 334 (Pt 2):297-314.
71. Grundy SM. Metabolic syndrome pandemic. Arterioscler Thromb Vasc Biol 2008; 28:629-636.
72. Abbasi F, Brown BW Jr, Lamendola C, McLaughlin T, Reaven GM. Relationship between obesity, insulin resistance, and coronary heart disease risk. J Am Coll Cardiol 2002; 40:937-943.
73. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). JAMA 2001; 285:2486-2497.
74. Grundy SM, Brewer HB Jr, Cleeman JI, Smith SC Jr, Lenfant C. Definition of metabolic syndrome: Report of the National Heart, Lung, and Blood Institute/

American Heart Association conference on scientific issues related to definition. Circulation 2004; 109:433-438.

75. Jeppesen J, Hansen TW, Rasmussen S, Ibsen H, Torp-Pedersen C, Madsbad S. Insulin resistance, the metabolic syndrome, and risk of incident cardiovascular disease: a population-based study. J Am Coll Cardiol 2007; 49:2112-2119.
76. Grundy SM. Metabolic syndrome: connecting and reconciling cardiovascular and diabetes worlds. J Am Coll Cardiol 2006; 47: 1093-1100.
77. Ford ES, Li C, Sattar N. Metabolic syndrome and incident diabetes: current state of the evidence. Diabetes Care 2008; 31:1898-1904.
78. Ferreira IA, Eybrechts KL, Mocking AI, Kroner C, Akkerman JW. IRS-1 mediates inhibition of Ca2+ mobilization by insulin via the inhibitory G-protein Gi. J Biol Chem 2004; 279:3254-3264.
79. Hajek AS, Joist JH, Baker RK, Jarett L, Daughaday WH. Demonstration and partial characterization of insulin receptors in human platelets. J Clin Invest 1979; 63:1060-1065.
80. Trovati M, Anfossi G. Insulin, insulin resistance and platelet function: similarities with insulin effects on cultured vascular smooth muscle cells. Diabetologia. 1998; 41:609-22.
81. Anfossi G, Trovati M. Pathophysiology of platelet resistance to anti-aggregating agents in insulin resistance and type 2 diabetes: implications for anti-aggregating therapy. Cardiovasc Hematol Agents Med Chem 2006; 4:111-28.
82. Davì G, Guagnano MT, Ciabattoni G, Basili S, Falco A, Marinopiccoli M, Nutini M, Sensi S, Patrono C. Platelet activation in obese women: role of inflammation and oxidant stress. JAMA 2002; 288:2008-14.
83. Trovati M, Anfossi G. Influence of insulin and of insulin resistance on platelet and vascular smooth muscle cell function. J Diabetes Complications 2002; 16:35-40.
84. Arteaga RB, Chirinos JA, Soriano AO, Jy W, Horstman L, Jimenez JJ, Mendez A, Ferreira A, de Marchena E, Ahn YS. Endothelial microparticles and platelet and leukocyte activation in patients with the metabolic syndrome. Am J Cardiol 2006; 98:70-4.
85. Basili S, Pacini G, Guagnano MT, Manigrasso MR, Santilli F, Pettinella C, Ciabattoni G, Patrono C, Davì G. Insulin resistance as a determinant of platelet activation in obese women. J Am Coll Cardiol 2006; 48:2531-8.
86. Patel SR, Hartwig JH, Italiano JE Jr. The biogenesis of platelets from megakaryocyte proplatelets. J Clin Invest 2005; 115:3348-3354.
87. Richardson JL, Shivdasani RA, Boers C, Hartwig JH, Italiano JE Jr. Mecha-

nisms of organelle transport and capture along proplatelets during platelet production. Blood 2005; 106:4066-4075.

88. Avecilla ST, Hattori K, Heissig B, Tejada R, Liao F, Shido K, Jin DK, Dias S, Zhang F, Hartman TE, Hackett NR, Crystal RG, Witte L, Hicklin DJ, Bohlen P, Eaton D, Lyden D, de Sauvage SF, Rafii S. Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. Nat Med 2004; 10:64-71.
89. Kaushansky K. The molecular mechanisms that control thrombopoiesis. J Clin Invest 2005; 115:3339-3347.
90. Carr ME. Diabetes mellitus: a hypercoagulable state. J Diabetes Complications 2001; 15:44-54.
91. Diamant M, Nieuwland R, Pablo RF, Sturk A, Smit JW, Radder JK. Elevated numbers of tissue-factor exposing microparticles correlate with components of the metabolic syndrome in uncomplicated type 2 diabetes mellitus. Circulation 2002; 106:2442-2447.
92. Zumbach M, Hofmann M, Borcea V, Luther T, Kotzsch M, Muller M, Hergesell O, Andrassy K, Ritz E, Ziegler R, Wahl P, Nawroth PP. Tissue factor antigen is elevated in patients with microvascular complications of diabetes mellitus. Exp Clin Endocrinol Diabetes 1997;105: 206-212.
93. Lim HS, Blann AD, Lip GY. Soluble CD40 ligand, soluble P-selectin, interleukin-6, and tissue factor in diabetes mellitus: relationships to cardiovascular disease and risk factor intervention. Circulation 2004; 109:2524-2528.
94. Wilcox JN, Smith KM, Schwartz SM, Gordon D. Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. Proc Natl Acad Sci USA 1998; 86:2839-2843.
95. Cermak J, Key NS, Bach RR, Balla J, Jacob HS, Vercellotti GM. C-reactive protein induces human peripheral blood monocytes to synthesize tissue factor. Blood 1993; 82:513-520.
96. Mach F, Schonbeck U, Bonnefoy JY, Pober JS, Libby P. Activation of monocyte/macrophage functions related to acute atheroma complication by ligation of CD40: induction of collagenase, stromelysin, and tissue factor. Circulation 1997; 96:396-399.
97. He M, He X, Xie Q, Chen F, He S. Angiotensin II induces the expression of tissue factor and its mechanism in human monocytes. Thromb Res 2006; 117:579-590.
98. Wada H, Kaneko T, Wakita Y, Minamikawa K, Nagaya S, Tamaki S, Deguchi K, Shirakawa S. Effect of lipoproteins on tissue factor activity and PAI-II antigen in

human monocytes and macrophages. Int J Cardiol 1994; 47:S21-S25.

99. Guha M, Mackman N. The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. J Biol Chem 2002; 277:32124-32132.
100. Mackman N. Regulation of the tissue factor gene. Thromb Haemost 1997; 78:747-754.
101. Bierhaus A, Chevion S, Chevion M, Hofmann M, Quehenberger P, Illmer T, Luther T, Berentshtein E, Tritschler H, Muller M, Wahl P, Ziegler R, Nawroth PP. Advanced glycation end product-induced activation of NF-kappaB is suppressed by alpha-lipoic acid in cultured endothelial cells. Diabetes 1997; 46:1481-1490.
102. Bierhaus A, Illmer T, Kasper M, Luther T, Quehenberger P, Tritschler H, Wahl P, Ziegler R, Muller M, Nawroth PP. Advanced glycation end product (AGE)-mediated induction of tissue factor in cultured endothelial cells is dependent on RAGE. Circulation 1997; 96:2262-2271.
103. Napoleone E, DI Santo A, Amore C, Baccante G, di Febbo C, Porreca E, de Gaetano G, Donati MB, Lorenzet R. Leptin induces tissue factor expression in human peripheral blood mononuclear cells: a possible link between obesity and cardiovascular risk? J Thromb Haemost 2007; 5:1462-1468.
104. Giesen PL, Rauch U, Bohrmann B, Kling D, Roque M, Fallon JT, Badimon JJ, Himber J, Riederer MA, Nemerson Y. Blood-borne tissue factor: another view of thrombosis. Proc Natl Acad Sci USA 1999; 96:2311-2315.
105. Llorente-Cortes V, Otero-Vinas M, Camino-Lopez S, Llampayas O, Badimon L. Aggregated low-density lipoprotein uptake induces membrane tissue factor procoagulant activity and microparticle release in human vascular smooth muscle cells. Circulation 2004; 110:452-459.
106. Schecter AD, Spirn B, Rossikhina M, Giesen PL, Bogdanov V, Fallon JT, Fisher EA, Schnapp LM, Nemerson Y, Taubman MB. Release of active tissue factor by human arterial smooth muscle cells. Circ Res 2000; 87:126-132.
107. Mallat Z, Benamer H, Hugel B, Benessiano J, Steg PG, Freyssinet JM, Tedgui A. Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes. Circulation 2000; 101:841-843.
108. Mallat Z, Hugel B, Ohan J, Leseche G, Freyssinet JM, Tedgui A. Shed membrane microparticles with procoagulant potential in human atherosclerotic plaques: a role for apoptosis in plaque thrombogenicity. Circulation 1999;

99:348-353.

109. Marmur JD, Thiruvikraman SV, Fyfe BS, Guha A, Sharma SK, Ambrose JA, Fallon JT, Nemerson Y, Taubman MB. Identification of active tissue factor in human coronary atheroma. Circulation 1996; 94:1226-1232.
110. Furie B, Furie BC. Thrombus formation in vivo. J Clin Invest 2005; 115:3355-3362.
111. Chou J, Mackman N, Merrill-Skoloff G, Pedersen B, Furie BC, Furie B. Hematopoietic cell-derived microparticle tissue factor contributes to fibrin formation during thrombus propagation. Blood 2004; 104:3190-3197.
112. Day SM, Reeve JL, Pedersen B, Farris DM, Myers DD, Im M, Wakefield TW, Mackman N, Fay WP. Macrovascular thrombosis is driven by tissue factor derived primarily from the blood vessel wall. Blood 2005; 105:192-198.
113. Schwertz H, Tolley ND, Foulks JM, Denis MM, Risenmay BW, Buerke M, Tilley RE, Rondina MT, Harris EM, Kraiss LW, Mackman N, Zimmerman GA, Weyrich AS. Signal-dependent splicing of tissue factor pre-mRNA modulates the thrombogenicity of human platelets. J Exp Med 2006; 203:2433-2440.
114. Panes O, Matus V, Saez CG, Quiroga T, Pereira J, Mezzano D. Human platelets synthesize and express functional tissue factor. Blood 2007; 109:5242-5250.
115. Nilsson TK, Boman K, Bjerle P, Hallmans G, Hellsten G. von Willebrand factor and fibrinolytic variables are differently affected in the insulin resistance syndrome. J Intern Med 1994; 235:419-423.
116. Carter AM, Catto AJ, Grant PJ. Determinants of tPA antigen and associations with coronary artery disease and acute cerebrovascular disease. Thromb Haemost 1998; 80:632-636.
117. Sahli D, Eriksson JW, Boman K, Svensson MK. Tissue plasminogen activator (tPA) activity is a novel and early marker of asymptomatic LEAD in type 2 diabetes. Thromb Res 2009; 123:701-706.
118. Flores J, García-Avello A, Flores VM, Navarro JL, Canseco F, Pérez-Rodríguez E. Tissue plasminogen activator plasma levels as a potential diagnostic aid in acute pulmonary embolism. Arch Pathol Lab Med. 2003; 127:310-5.
119. Grant PJ. Diabetes mellitus as a prothrombotic condition. J Intern Med 2007; 262:157-172.
120. Dunn EJ, Ariens RA, Grant PJ. The influence of type 2 diabetes on fibrin structure and function. Diabetologia 2005; 48:1198-1206.
121. Dunn EJ, Philippou H, Ariens RA, Grant PJ. Molecular mechanisms involved in the resistance of fibrin to clot lysis by plasmin in subjects with type 2 diabetes

mellitus. Diabetologia 2006; 49:1071-1080.

122. Carter AM, Cymbalista CM, Spector TD, Grant PJ Heritability of clot formation, morphology, and lysis: the EuroCLOT study. Arterioscler Thromb Vasc Biol 2007; 27:2783-2789.
123. Collet JP, Allali Y, Lesty C, Tanguy ML, Silvain J, Ankri A, Blanchet B, Dumaine R., Gianetti J, Payot L, Weisel JW, Montalescot G. (2006) Altered fibrin architecture is associated with hypofibrinolysis and premature coronary atherothrombosis. Arterioscler Thromb Vasc Biol 2006; 26:2567-2573.
124. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med 1999; 340:115-126.
125. Suwaidi JA, Hamasaki S, Higano ST, Nishimura RA, Holmes DR Jr, Lerman A. Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction. Circulation 2000; 101:948-954.
126. Pasqualini L, Marchesi S, Vaudo G, Siepi D, Angeli F, Paris L, Schillaci G, Mannarino E. Association between endothelial dysfunction and major cardiovascular events in peripheral arterial disease. Vasa 2003; 32:139-143.
127. Halcox JP, Schenke WH, Zalos G, Mincemoyer R, Prasad A, Waclawiw MA, Nour KR, Quyyumi AA. Prognostic value of coronary vascular endothelial dysfunction. Circulation 2002; 106:653-658.
128. Laine H, Yki-Jarvinen H, Kirvela O, Tolvanen T, Raitakari M, Solin O, Haaparanta M, Knuuti J, Nuutila P. Insulin resistance of glucose uptake in skeletal muscle cannot be ameliorated by enhancing endothelium-dependent blood flow in obesity. J Clin Invest 1998, 101:1156-1162.
129. Steinberg HO, Chaker H, Leaming R, Johnson A, Brechtel G, Baron AD. Obesity/insulin resistance is associated with endothelial dysfunction. Implications for the syndrome of insulin resistance. J Clin Invest 1996; 97:2601-2610.
130. Perticone F, Ceravolo R, Candigliota M, Ventura G, Iacopino S, Sinopoli F, Mattioli PL. Obesity and body fat distribution induce endothelial dysfunction by oxidative stress: protective effect of vitamin C. Diabetes 2001; 50:159-165
131. Taylor AA. Pathophysiology of hypertension and endothelial dysfunction in patients with diabetes mellitus. Endocrinol Metab Clin North Am 2001; 30:983-997.
132. Yudkin JS, Stehouwer CD, Emeis JJ, Coppack SW. C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for cytokines originating from adipose tissue? Arterioscler Thromb Vasc Biol 1999; 19:972-978.
133. Mark AL, Correia ML, Rahmouni K, Haynes WG. Selective leptin resistance: a

new concept in leptin physiology with cardiovascular implications. J Hypertens 2002; 20:1245-1250.

134. Ouchi N, Kihara S, Arita Y, Maeda K, Kuriyama H, Okamoto Y, Hotta K, Nishida M, Takahashi M, Nakamura T, Yamashita S, Funahashi T, Matsuzawa Y. Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. Circulation 1999; 100:2473-2476.

Chapter 2

The adipokines resistin, leptin, PAI-1 and RBP4 induce degradation of IRS-1 in megakaryocytes and production of insulin-resistant platelets

AJ Gerrits[1]
E Gitz[1]
CA Koekman[1]
FL Visseren[2]
TW van Haeften[3]
JW Akkerman[1]

[1]Thrombosis and Haemostasis Laboratory, Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, the Netherlands
[2]Department of Vascular Medicine, University Medical Center Utrecht, Utrecht, the Netherlands
[3]Department of Internal Medicine, University Medical Center Utrecht, Utrecht, the Netherlands

Submitted for publication

Abstract

Obesity is a major risk factor of insulin resistance, type 2 diabetes and CVD. Blood from obese individuals contains elevated levels of adipokines, which control adipocyte metabolism and functions ranging from inflammation to feeding behaviour. We identified four adipokines that induce insulin resistance in megakaryocytes, the cells that produce platelets. Short contact with resistin, leptin, PAI-1 and RBP4 but not with visfatin, IL-6 and TNF-α aborts insulin signaling through IRS-1 and is reversible; prolonged incubation causes IRS-1 degradation and is irreversible. Plasma samples from obese men mimick the effect of megakaryocyte-disturbing adipokines. Platelets from type 2 diabetic patients are insulin-resistant and have reduced expression of IRS-1. Insulin resistance in megakaryocytes and platelets increases their responsiveness to agents that induce atherothrombosis. We conclude that increased release of leptin, resistin, PAI-1 and RBP4 by adipocytes causes synthesis of insulin-resistant platelets and increases CVD risk.

Introduction

Obesity increases risk of insulin resistance and type 2 diabetes [1]. Weight gain and appearance of insulin resistance go hand in hand [2] and are thought to be caused by abnormal adipokine release by visceral fat [3]. Products from adipocytes are resistin, leptin, plasminogen activator inhibitor-1 (PAI-1), retinol binding protein 4 (RBP4) and visfatin with elevated plasma levels in individuals with abdominal obesity, and adiponectin, with a plasma level that correlates inversely with body mass [4]. Also interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) released by macrophages present in adipose tissue might induce insulin resistance [1].
Type 2 diabetes patients have a 2-8 fold increased cardiovascular disease (CVD)-risk compared to matched controls [5]. Their platelets circulate in an activated state [6] and in laboratory tests better adhere to thrombogenic components of the vessel wall, form bigger aggregates at lower agonist concentration and produce more platelet-activating and vasoconstricting thromboxane A_2 (TxA_2) than platelets from healthy individuals [7]. The hyperactivity correlates with loss of insulin sensitivity and intensive insulin treatment partly normalizes aggregation [8]. Normally, insulin inhibits platelets by interfering with the suppression of cAMP, a potent intracellular inhibitor of aggregation and secretion. In type 2 diabetes, this interference is much

reduced and platelets show faster aggregation, secretion of granule content, increased synthesis of TxA_2 and generation of a procoagulant surface than controls [9-14].

Type 2 diabetes patients are often obese and their platelets circulate in an environment enriched in specific adipokines [15]. This might be a cause for platelet hyperactivity. Platelets have the leptin receptor, ObRb [16]. Although they do not respond to physiological leptin concentrations, levels found in obesity (30-100 ng/mL) stimulate the rise in cytosolic Ca^{2+}, formation of TxA_2, ADP-induced aggregation and the cytoskeletal re-arrangements required for adhesion to a prothrombotic surface [16-21]. *Ob/ob* mice, that are genetically deficient in the gene for leptin, show a delayed occlusion time in a model for arterial thrombosis [16]. Opposite to findings that leptin increases platelet reactivity is the failure of a four-days leptin infusion in healthy volunteers to change aggregation [19]. In addition, the observation that leptin deficient subjects show increased aggregation by platelets suggests that leptin does not stimulate platelet functions through direct interaction [20]. Findings with adiponectin are equally contradictory. Studies in knockout mice suggest that adiponectin is an antithrombotic factor in wild-type mice [20], but adhesion and aggregation of human platelets incubated with a supra-physiological adiponectin concentration do not change [22].

Although platelets are capable of protein synthesis while adhering to a thrombogenic surface, the process is slow and quantities produced are small. It is therefore unlikely that adipokines change platelet properties by directly changing protein composition. Platelets are synthesized by megakaryocytes and these cells do have a high capacity to synthesize proteins [23]. Megakaryocytes develop from haematopoietic stem cells in the bone marrow in a two weeks process of differentiation. Maturation is under control of thrombopoietin, stem cell factor, interleukin-1β and other cytokines, which determine cell size, ploidity and protein expression. Interestingly, variations in growth factor contribution induce variations in protein expression, illustrating the high responsiveness of megakaryocytes to cytokines [23].

Given the sensitivity of megakaryocytes for different growth factors, we speculated that these cells might be responsive to adipokines. The megakaryocytic CHRF-288-11 cell line has many properties in common with matured megakaryocytes [23-25]. Upon stimulation with thrombin, they mobilize Ca^{2+} ions from intracellular storage sites similar as do mature megakaryocytes [23] and platelets [9,10]. Ca^{2+} mobilization in platelets is enhanced by simultaneous release of ADP which binds to the purinergic P2Y12 receptor thereby activating the GTP-protein Gi and sup-

pressing formation of cAMP, an intracellular inhibitor of Ca^{2+} rises and many other platelet responses. Interference with ADP-P2Y12 interaction aborts suppression of cAMP, retards platelet aggregation and is the basis of anti-thrombotic therapy with clopidogrel in patients with increased CVD risk [26].

Methods

Materials - Human α-thrombin was obtained from Enzyme Research Laboratories (South Bend, IN, USA). Recombinant insulin (solubilized according to the recommendations of the manufacturer in 10 mmol/L acetic acid, 100 mmol/L NaCl, and 0.01% BSA to a stock concentration of 100 μmol/L), Fura-2/AM, bovine serum albumin (BSA) and the adenylyl cyclase inhibitor SQ22536 were from Sigma-Aldrich (St. Louis, MO, USA). Resistin, PAI-1 and cantharidin were purchased from Calbiochem (Darmstadt, Germany). Leptin and IL-6 were from R&D Systems (Minneapolis, MN, USA). RBP4 was obtained from Cayman Chemical (Ann Arbor, MI, USA). TNF-α was from Reprotech Inc (Rocky Hill. NJ, USA), visfatin was from Biovision (Mountain View, CA, USA). Cycloheximide (CHX) was obtained from MP Biomedicals (Morgan Irvine, CA, USA). RGDS (Arg-Gly-Asp-Ser) peptide was from Bachem (Bubendorf, Switzerland). The ADP receptor P2Y12 antagonist, N6-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene ATP (AR-C69931MX) was a kind gift from Astra Zeneca (Loughborough, UK). The JAK2 inhibitor AG490 and proteasome inhibitor MG132 were obtained from Calbiochem (Darmstadt, Germany).

The phosphospecific PKB Serine (473) (P-PKB-Ser^{473}) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against the phosphospecific IRS-1 Serine (307) (P-IRS-Ser^{307}), IRS-2 and ubiquitin were from Upstate Biotechnology (Bucks, UK) and mouse anti-PKB from R&D Systems (Minneapolis, MN, USA). Antibodies against the INS-R, P-INS-R-$Tyr^{1150/1151}$ and horseradish peroxidase-labeled anti-rabbit antibody were from Cell Signaling Technology (Danvers, MA, USA) and antibodies against Giα-2 and SOCS3 from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against F-actin was from Abcam (Cambridge, UK). The monoclonal mouse anti-IGF-1 Receptor was obtained from Calbiochem (Darmstadt, Germany). All other chemicals were of analytical grade.

Cell culture - Megakaryocytic CHRF-288-11 cells were cultured as described earlier [52].

Subjects – These studies have been approved by the Medical Ethical Review Board of the University Medical Center Utrecht, Utrecht, The Netherlands. Plasma samples from metabolic syndrome subjects were obtained as described previously [41]. Their characteristics are given in Table 1.
Type 2 diabetes subjects were recruited from the out-patient clinic of the University Medical Center Utrecht (Utrecht, the Netherlands). Their characteristics are given in Table 2. Patients and matched control subjects gave written informed consent prior to participating in the study.

Platelet isolation - Freshly drawn venous blood from healthy, medication-free volunteers and type 2 diabetes patients was collected into 0.1 volume of 130 mmol/L Na_3 citrate. Platelets were isolated as described [9].

Measurement of Ca^{2+} mobilization - CHRF-288-11 cells (2×10^5 cells per sample) or platelet-rich plasma (PRP) were incubated in the dark with 3 mol/L Fura 2-AM (1 hr, 37°C). PRP was acidified with ACD to pH 6.5 and centrifuged (330xg, 15 min, 20°C), while CHRF-288-11 cells were directly centrifuged (150×g, 5 min, 20°C). Both were resuspended in Ca^{2+}-free HEPES–Tyrode (HT) buffer (145 mmol/L NaCl, 5 mmol/L KCL, 0.5 mmol/L Na_2HPO_4, 1 mmol/L $MgSO_4$, 10 mmol/L HEPES) supplemented with 5 mmol/L D-glucose, pH 7.25. The final platelet concentration was adjusted to 2.0×10^{11} cells/L. Five minutes before the start of the analysis, suspensions were prewarmed at 37°C. Measurements and calibrations were performed as described [9].

Immunoprecipitation and blotting - To study phosphorylation or ubiquitination of IRS-1, CHRF-288-11 cells were incubated with insulin for 15 min and adipokines at indicated concentrations at 37°C and collected in lysis buffer (20 mM Tris, 5 mM EGTA, 1% TX-100, pH 7.2) supplemented with 10% protease inhibitor cocktail, 1 mmol/L $NaVO_3$ and 1 μmol/L cantharidin. Samples were incubated overnight (4°C) with protein G-sepharose and anti-IRS-1 phospho-Ser^{307} or anti-IRS-1 antibodies (1 μg/mL). Precipitates were washed 3 times with lysis buffer and taken up in reducing Laemmli sample buffer. The phosphorylation of INS-R, PKBα, upregulation of SOCS3 and total Giα-2 protein, was measured in lysates in reducing Laemmli sample buffer. Samples were separated by SDS-PAGE and proteins

transferred to polyvinyldine difluoride membranes. After blocking with 4% BSA in TBS or Odyssey blockbuffer (1 hr, 22 °C), membranes were incubated with appropriate primary antibodies (16 hrs, 4°C). Immunoblots for PKBα, phospho-Ser^{473}, F-actin and Giα-2 were visualized by Odyssey infrared imaging (LI-COR Biosciences) using Alexa-labeled antibodies according to the manufacturer's instructions. Detection of INS-R phospho-Tyr$^{1150/1151}$, IRS-1 phospho-Ser^{307}, ubiquitinated IRS-1 and INS-R-, IRS-1-, PKBα and SOCS3 protein, was performed with HRP-labelled secondary antibodies and enhanced chemiluminescence. Quantification of the bands was performed using ImageJ.

Aggregation studies - CHRF-288-11 cells (3×10^6 per sample) were incubated in 12 wells plates with adipokines for 24 hours. Cells were taken up in 0.5 mL HT buffer and stimulated with 0.5 mM arachidonic acid. Platelets of type 2 diabetes patients or controls were stimulated with 1 μg/mL collagen. Optical aggregation was monitored in a Chrono-Log lumiaggregometer at 37°C at a stirring speed of 900 rpm. Changes in light transmission in the suspension were recorded.

Leptin and resistin measurements – Leptin and resistin levels in plasma from metabolic syndrome men and controls were determined by ELISA (R&D systems, Minneapolis, MN, USA).

Statistics - Data is expressed as mean ± S.E.M. with n observations and was analyzed with Student's test for unpaired observations or Mann Whitney U test, as indicated. Differences were considered significant at $p < 0.05$.

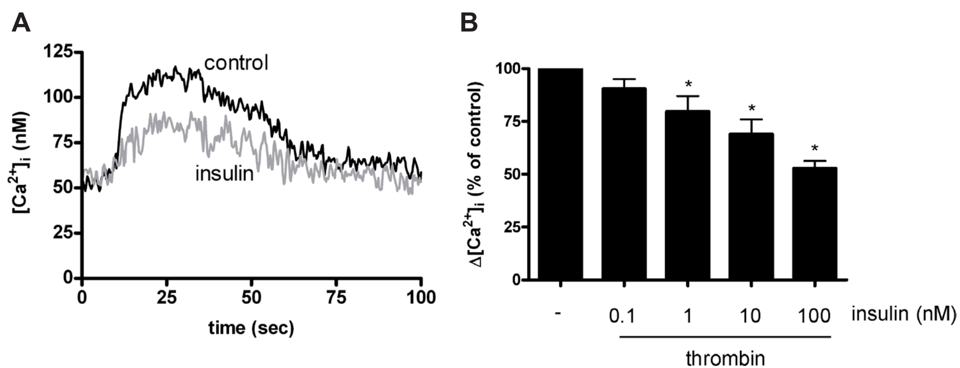
Results

Resistin, leptin, PAI-1 and RBP4 induce insulin resistance in megakaryocytes

Stimulation of megakaryocytes by the platelet activator thrombin initiates a rapid rise in cytosolic Ca^{2+} content caused by release from intracellular storage sites. Insulin induces a dose-dependent fall in Ca^{2+} mobilization (Fig. 1a,b). This inhibition is also seen in platelets and is the result of insulin binding to its receptor and Tyr-phosphorylation of IRS-1 which subsequently associates and thereby inactivates the inhibitory G-protein of adenylyl cyclase, Giα-2 [9]. As in platelets, cangrelor

(AR-C69931MX), which mimicks the action of the active metabolite of clopidogrel, inhibits thrombin-induced Ca^{2+} rises confirming the presence of P2Y12 signaling in megakaryocytes (Supplement Fig. 1a). Inhibition of Ca^{2+} rises by insulin is prevented by the adenylyl cyclase inhibitor SQ22536, confirming that insulin acts by interfering with cAMP production, and independent of the insulin-like growth factor-1 receptor (IGF-1 R), a second receptor for insulin signaling (Supplement Fig. 1b,c).

We investigated whether adipokines interfere with inhibition of Ca^{2+} rises by insulin. Maximal inhibition by insulin was set at 100% and converted in an insulin-sensitivity index (ISI), reflecting the degree by which combinations of insulin and adipokines reduce the Ca^{2+} response (insert Fig. 1c). Megakaryocytes were incubated with different adipokines at concentrations 10 fold the physiological range for 1 to 7 days to allow interference with protein synthesis. Resistin, leptin, PAI-1 and RBP4 induced an 80-100% fall in insulin sensitivity but visfatin, IL-6 and TNF-α did not (Fig. 1c). Adipokines alone failed to change basal [Ca^{2+}] (data not shown). Thus, megakaryocytes become insulin-resistant upon contact with resistin, leptin, PAI-1 and RBP4. In platelets, suppression of Ca^{2+} signaling by insulin is not affected by adipokines, indicating that when platelets are shed from the megakaryocyte, the capacity of resistin, leptin, PAI-1 and RBP4 to interfere with insulin signaling is lost (Fig. 1d). For reasons not yet clarified, loss of platelet sensitivity to insulin is accompanied by a 1.4-fold upregulation of P2Y12 signaling [10] and an increase in responsiveness to activating agents. The same is seen in megakaryocytes which upon contact with leptin lose their sensitivity to insulin (Fig. 1c) and show hyperaggregation (Fig. 1e).



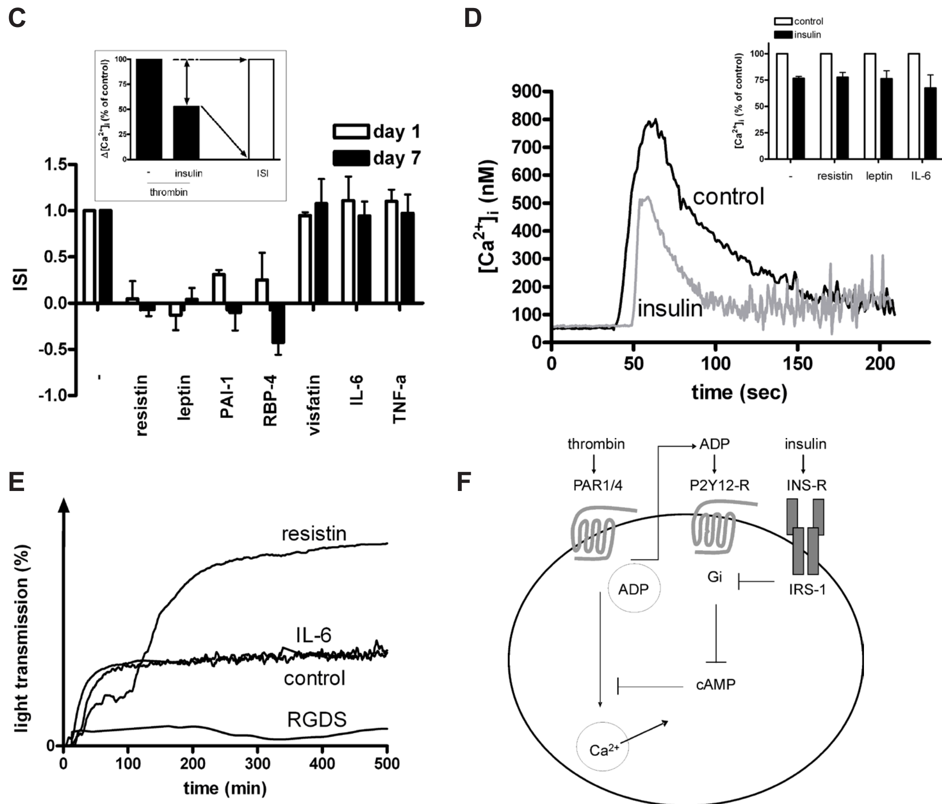


Figure 1. Resistin, leptin, PAI-1 and RBP4 induce insulin resistance and hyperactivity in megakaryocytes

(a,b) Insulin inhibits thrombin-induced Ca^{2+} mobilization in megakaryocytes. Thrombin (1 U/mL)-induced Ca^{2+} mobilization in Fura 2-AM loaded megakaryocytic CHRF-288-11 cells pre-incubated without and with insulin (100 nM, 5 min, 37°C) and different concentrations of insulin. (c) Resistin, leptin, PAI-1 and RBP4 induce insulin resistance in megakaryocytes. Inhibition of Ca^{2+} mobilization by insulin (100 nM) in megakaryocytes following 1 and 7 days culture in the presence of the resistin (150 ng/mL), leptin (300 ng/mL), PAI-1 (260 ng/mL), RBP4 (500 ng/mL), visfatin (20 ng/mL), IL-6 (12 ng/mL) and TNF-α (50 pg/mL). These concentrations were used in all experiments. Inhibition by insulin (100 nM) was set at 100%. The extent by which adipokines interfered was expressed as insulin sensitivity index (ISI, insert). Resistin, leptin, PAI-1 and RBP4 induce insulin resistance but visfatin, IL-6 and TNF-α do not. (d) Resistin, leptin and Il-6 do not induce insulin resistance in platelets. Thrombin (0.25 U/mL)-induced Ca^{2+} mobilization in resistin treated platelets pre-incubated without and with insulin (100 nM, 5 min, 37°C). Platelets were incubated with resistin, leptin and IL-6 (insert, 2 hrs, 37°C). (e) Resistin induces hyperactivity in megakaryocytes. Arachidonic acid (0.5 mM, 37°C) induced aggregation without and with pre-incubation (24 hrs) with resistin and IL-6. The change in light transmission was inhibited by a blocker of fibrinogen-integrin $\alpha_{IIb}\beta_3$ interaction (100 μM RGDS), confirming the true nature of the aggregation responses. (f) Schematic representation of signaling pathways initiated by thrombin and ADP and the interference by insulin in megakaryocytes and platelets. (for b-e: means ± SEM, n=3, data was analyzed with Student's test, * denotes a significant difference, p<0.05)

Short adipokine contact induces reversible insulin resistance

In order to investigate how long adipokines had to make contact with megakaryocytes before interference with insulin signaling could be detected, we performed incubations for 2 to 72 hrs. Interestingly, only 2 hrs is sufficient to induce insulin resistance by resistin and leptin (Fig. 2a,b). The short duration of the incubation period together with the lack of interference by an inhibitor of protein synthesis (100 μg/mL cycloheximide, data not shown) suggest that induction of insulin resistance by these adipokines is independent of transcription/translation. We therefore applied pharmacological inhibitors that interfere with rapid cell responses and found that the Ser phosphatase inhibitor cantharidin almost completely restores insulin sensitivity in megakaryocytes treated with resistin and leptin. These findings are best explained by assuming that one or more proteins involved in insulin signaling depend on a phosphorylated Ser residue for full activation and that adipokines interfere with this Ser phosphorylation.

A key element in insulin signaling is IRS-1, which is upstream of pathways that activate phosphoinositide-3 kinase (PI3-K)/protein kinase B (PKB), the GTP-ase Ras and mitogen-activated protein kinases (MAPK), in addition to inhibition of Giα–2 [9,27]. IRS-1 is activated through Tyr-phosphorylation, which forms binding sites for downstream signaling proteins. This process is under control of Ser^{307} but whether its phosphorylation facilitates or prevents IRS-1 signaling differs between cell types. In Chinese hamster ovary cells, Ser^{307} phosphorylation reduces IRS-1 interaction with the insulin receptor (INS-R) and impairs insulin signaling [28,29]. In Rat hepatoma Fao cells and CHO cells overexpressing the INS-R, the insulin-induced Ser^{307} phosphorylation protects active IRS-1 against Tyr phosphatases [30]. In human adipocytes, phosphorylation of Ser^{307} appears part of a positive feed back loop [31,32]. We find in megakaryocytes that leptin inhibits the phosphorylation and that cantharidin rescues the phosphorylation induced by insulin in the presence of leptin (Fig. 2c). These findings suggest that leptin inactivates IRS-1 through a Ser^{307} phosphatase. This conclusion was confirmed by measuring the insulin-induced activation of PKBα, a downstream element of IRS-1. Phosphorylation of PKBα Ser^{473} by insulin is suppressed by resistin and leptin, but not by IL-6, such in parallel with phosphorylation of IRS-1 Ser^{307} (Fig. 2d). A second candidate for interference by adipokines is the INS-R. However, leptin fails to change insulin-induced β-subunit $Tyr^{1150\text{-}1151}$ phosphorylation, which contributes to receptor kinase activity and recruitment of downstream proteins (Fig. 2e).

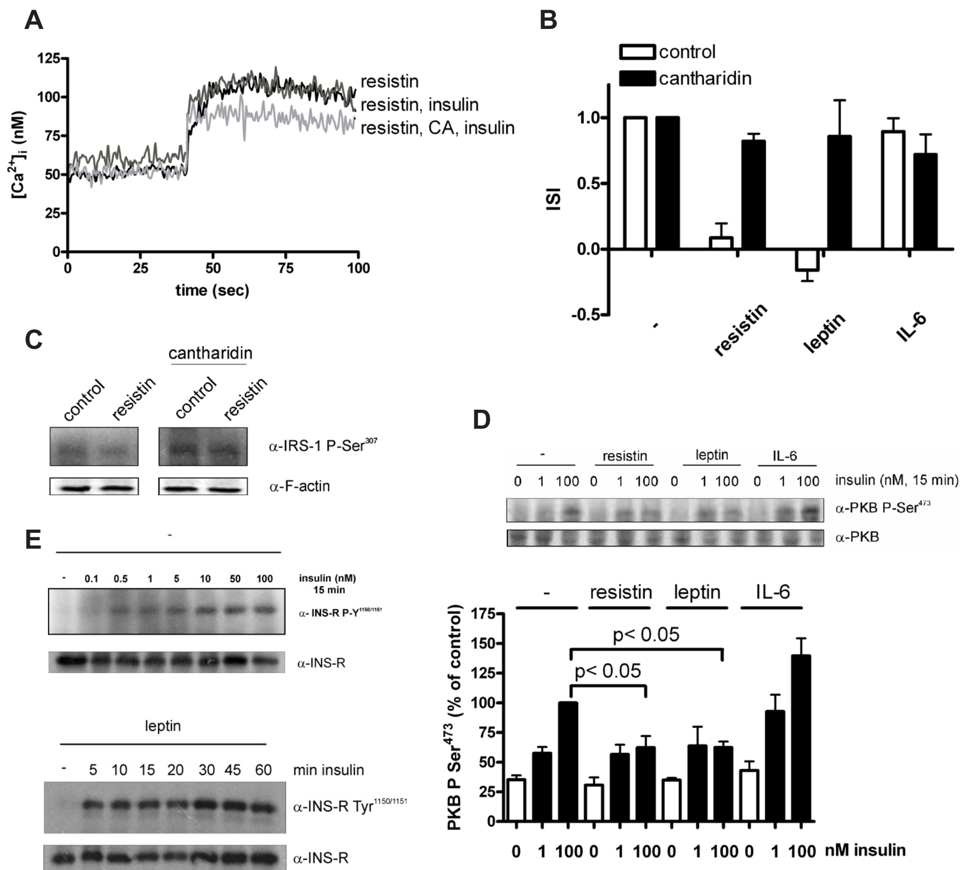


Figure 2. Short adipokine-megakaryocyte contact induces transient insulin resistance by interfering with IRS-1 regulation

(a,b) Megakaryocytes lose insulin sensitivity upon short contact with resistin. Cells were pre-incubated with resistin, leptin and IL-6 for 2 hrs and insulin inhibition of thrombin-induced Ca^{2+} mobilization was measured in the absence and presence of the Ser phosphatase inhibitor cantharidin (CA, 1 μmol/L, 15 min, 37°C). Resistin and leptin but not IL-6 induce induce insulin resistance; cantharidin rescues insulin sensitivity. (c) Loss of insulin sensitivity correlates with loss of IRS-1 Ser^{307} phosphorylation. Megakaryocytes were incubated with resistin for 2 hrs and stimulated with insulin. Detection of phospho IRS-1 Ser^{307} shows decreased levels of insulin-induced phosphorylation in the presence of resistin and restoration by cantharidin (1 μmol/L, 15 min, 37°C). (d) Loss of insulin sensitivity correlates with loss of Protein Kinase Bα (AKT1) activation. Megakaryocytes were incubated with resistin, leptin and IL-6 for 2 hrs and stimulated with 1 and 100 nM insulin (15 min, 37°C). Analysis of phosphor-PKBα Ser^{473} shows interference by resistin and leptin and not by IL-6. (e) Activation of the insulin receptor (INS-R) is not affected by leptin. Megakaryocytes were left untreated or incubated with leptin for 2 hrs and stimulated with different concentrations of insulin or for different time periods with 100 nM insulin. Levels of phospho-INS-R $Tyr^{1150/1151}$ and protein were not affected by leptin. Further details as in Fig.1.

Prolonged adipokine contact triggers persistent insulin resistance

When megakaryocytes are incubated with resistin and leptin for 72 hrs, loss of insulin sensitivity can not be restored by cantharidin, indicating that adipokine-induced IRS-1 Ser^{307} dephoshorylation is no longer involved (Fig. 3a,b). In hepatocytes, hematopoietic cells, pancreatic β-cells and enterocytes, cytokines activate the Janus Kinase and Signaling Transducers and Activators of Transcription (JAK/STAT) pathway. One of the downstream effects is the up-regulation of Suppressor Of Cytokine Signaling (SOCS) proteins [33-36]. The SOCS family negatively regulates their own synthesis by feed back inactivation of receptors and effector molecules. A second property of SOCS is their capacity to induce destruction of these adipokine signaling elements [36,37]. Megakaryocytes incubated with the JAK inhibitor AG-490 preserved insulin sensitivity in the presence of resistin and leptin, confirming that these adipokines signal through JAK (Fig. 3c). Analysis of leptin-stimulated megakaryocytes reveals a strong increase in SOCS3 expression after 2 hrs followed by a return to the pre-stimulation range 32 hrs later (Fig. 3d). This fall reflects involvement of kinase inhibitory domains, which abort their own expression [38]. SOCS are also known to interfere with insulin signaling, either through blocking receptor Tyr kinase activity or by degrading downstream signaling elements [39,40]. In megakaryocytes, resistin and leptin neither change receptor Tyr kinase activity (Fig. 2e) nor protein content (Fig. 3e). Also Giα-2 expression remains stable as does expression of IRS-2, a second member of the IRS family present in megakaryocytes. In contrast, resistin and leptin but not IL-6 induce a reduction in IRS-1 protein, suggesting that these adipokines initiate degradation of IRS-1 by ubiquitination and degradation in the proteosome (Fig. 3e). Indeed, immunoprecipitation of IRS-1 followed by blotting with an antibody against ubiquitin shows that leptin alone but especially in the presence of the proteosome inhibitor MG-132 triggers a shift in molecular mass from 180 to 250 kD (Fig. 3f). Thus, upon prolonged contact with resistin and leptin (and probably also with PAI-1 and RBP4), megakaryocytes lose insulin sensitivity by the ubiquitin-mediated degradation of IRS-1.

Plasma from men with metabolic syndrome induces insulin resistance and type 2 diabetes platelets have a reduced IRS-1 content

Having established that resistin and leptin induce insulin-resistance in cultures of megakaryocytes, we searched for evidence that a similar mechanism is operational *in vivo*. To this end, blood was collected from obese males without clinical evidence of diabetes who meet the criteria of the metabolic syndrome (MetS)

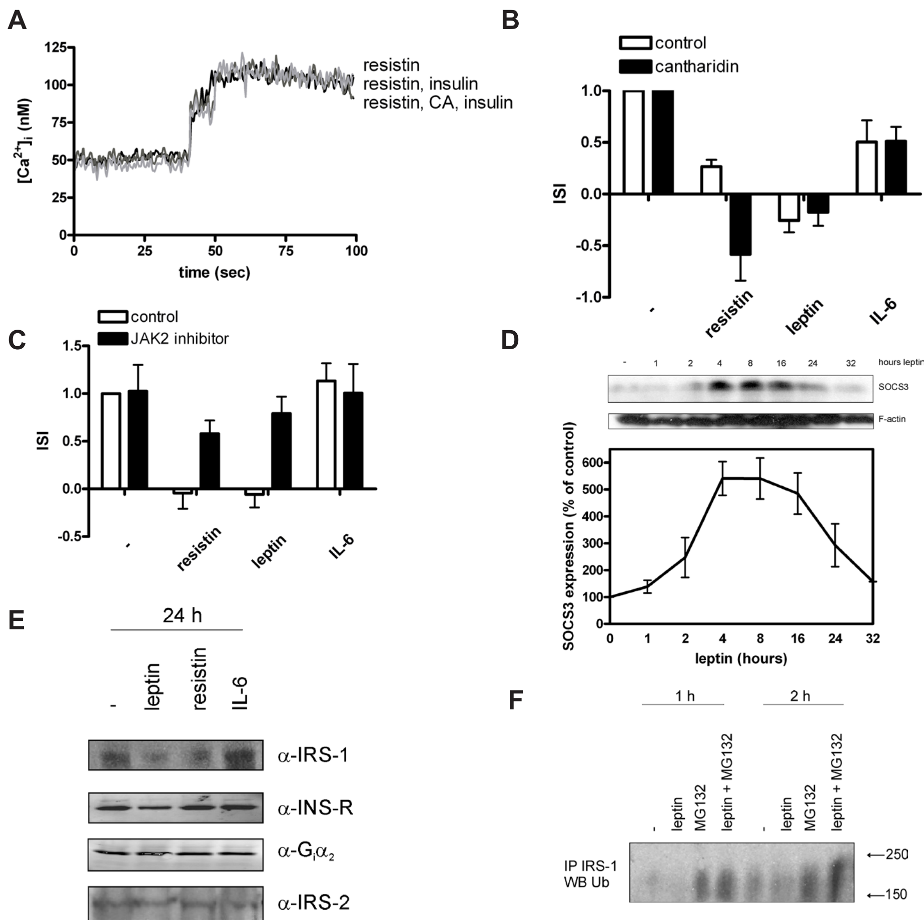
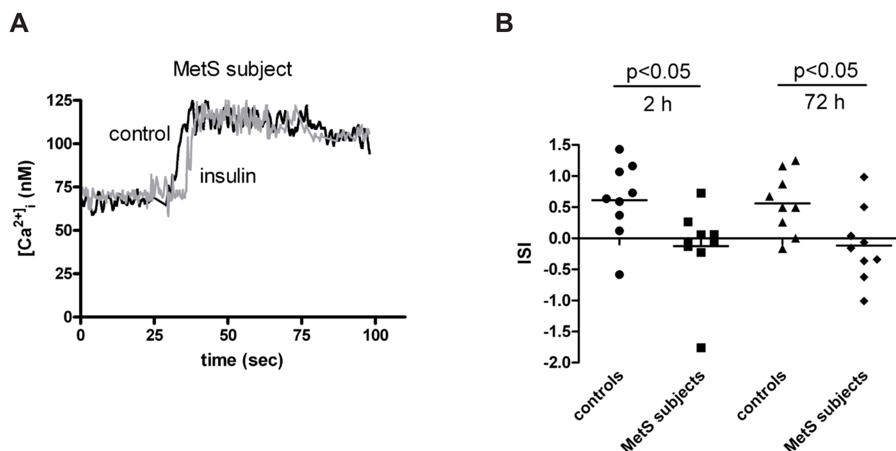


Figure 3. Prolonged adipokine-megakaryocyte contact induces persistant insulin resistance by interfering with IRS-1 expression

(a,b) Megakaryocytes lose insulin sensitivity upon prolonged contact with resistin. Cells were pre-incubated with resistin, leptin and IL-6 for 72 hrs and insulin inhibition of thrombin-induced Ca^{2+} mobilization was measured in the absence and presence of the Ser phosphatase inhibitor cantharidin (CA, 1 μmol/L, 15 min, 37°C). Resistin and leptin but not IL-6 induce induce insulin resistance; cantharidin fails to rescue insulin sensitivity. (c) A JAK2 inhibitor rescues insulin sensitivity. Megakaryocytes incubated with resistin, leptin and IL-6 for 72 hrs were pre-incubated with the JAK2 inhibitor AG490 (100 nM, 15 min, 37°C) and the ISI was determined. Induction of insulin resistance by resistin and leptin was prevented by JAK2 blockade (d) Leptin initiates upregulation of SOCS3. Megakaryocytes were incubated with leptin for 0 – 32 hrs and expression of SOCS3 was measured. (e). Leptin and resistin but not Il-6 induce degradation of IRS-1. Under the same conditions (d), expression of IRS-1 was detected showing the fall induced by resistin and leptin but not by IL-6. Expression of INS-R, Giα-2 and IRS-2 remains unchanged. (f). Leptin induces ubiquitination of IRS-1. Megakaryocytes were incubated with leptin, the proteasome inhibitor MG132 (10 μM) or both (1 or 2 hrs, 37°C) and expression of ubiquitinated IRS-1 was measured. Further details as in Fig.1.

defined by the Adult Treatment Panel III of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults [42] (Supplement Table 1). MetS-plasma samples were incubated with megakaryocytes for 2 and 72 hrs. Seven out of 9 samples induced almost complete insulin resistance. Most samples from matched controls had no effect but a few induced a similar resistance as plasma samples from obese men, possibly reflecting the onset of acquiring MetS properties at a later stage (Fig. 4a,b). Obesity is known to alter the levels of many adipokines and induction of insulin resistance by plasma from obese individuals must have a multifactorial origin. Analysis of resistin and leptin in the plasma samples revealed an increased leptin and normal resistin content, suggesting that a high leptin concentration might be at least one of the factors causing insulin resistance in megakaryocytes (Fig. 4c,d).

The abnormalities in plasma adipokine content seen in MetS subjects are also found in patients with type 2 diabetes [43-46]. This implies that type 2 diabetes- megakaryocytes mature in an environment that make them insulin-resistant and form insulin-resistent platelets. Indeed, platelets from obese type 2 diabetes patients (Supplement Table 2 for patient characteristics) showed a decreased IRS-1 content most likely caused by increased ubiquitin-mediated degradation of IRS-1 initiated by abnormalities in plasma adipokine content (Fig. 4e). Thus, the loss of insulin sensitivity in platelets is the result of a disturbed biogenesis in the megakaryocyte and accompanied by hyperactivity at the level of aggregation by megakaryocytes (Fig. 1e), platelets ([10] and Fig. 4f) and generation of a procoagulant surface [14].



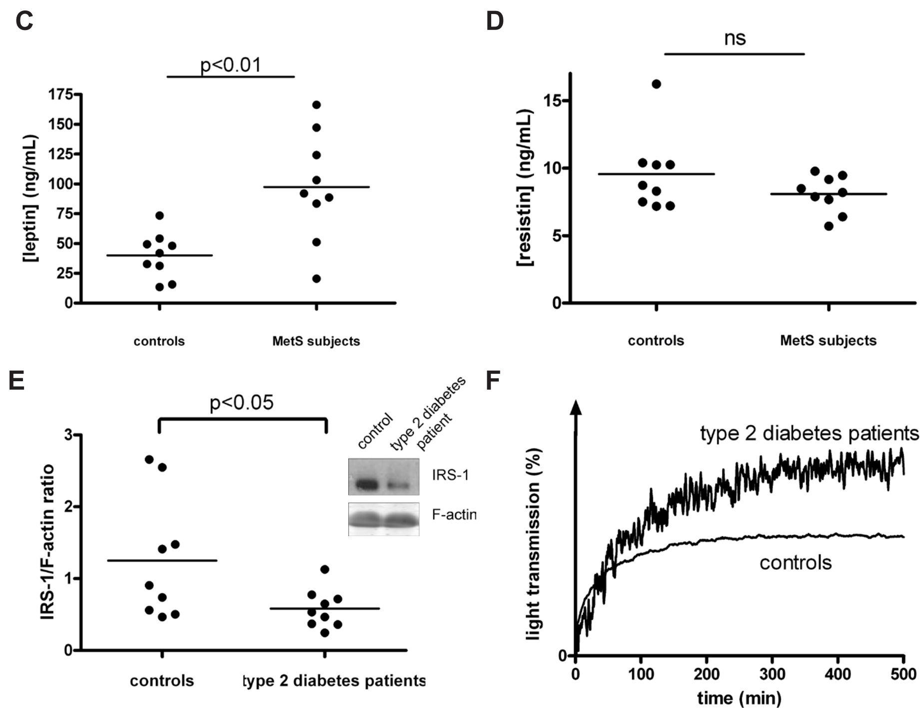


Figure 4. Plasma from men with metabolic syndrome induces insulin resistance and lower the IRS-1/F-actin ratio in type 2 diabetes platelets

(a,b) Megakaryocytes become insulin-resistant upon contact with plasma from men with metabolic syndrome (MetS). Megakaryocytes were incubated in MetS- and control plasma (n=9 for both groups) for 2 and 72 hrs and the thrombin-induced Ca^{2+} mobilization and ISI were determined. Both after 2 and 72 hrs, MetS-plasma but not plasma from controls induces insulin resistance. (c,d) MetS plasma samples contain elevated concentrations of leptin but resistin levels are normal (n=9). (e) Type 2 diabetes platelets contain reduced amounts of IRS-1. Platelets were isolated from type 2 diabetes patients and controls (n=9 for both groups) and expression of IRS-1 and F-actin was meaured. Data was analyzed with Mann Whitney U test. (f) Platelets from type 2 diabetes patients are hyperactive. Aggregation induced by collagen by platelets from a normal subject and a patient with type 2 diabetes (more details in 10). (for b-e: data was analyzed with Mann Whitney U test)

Table 1. Characteristics of male control subjects and male subjects with metabolic syndrome

	Control group (n=9)	Metabolic syndrome patients (n=9)	P
Age (years)	52 ± 8	54 ± 7	0.62
Height (m)	1.80 ± 0.08	1.83 ± 0.04	0.34
Weight (kg)	80.2 ± 11.3	101.5 ± 10.2	<0.001
Body mass index (kg/m^2)	24.6 ± 2.6	30.1 ± 2.7	<0.001
Body fat (%)	23 ± 2	30.3 ± 4.3	<0.001
Laboratory parameters			
Creatinine (μmol/L)	93 ± 18	95 ± 7	0.77
HbA1c (%)	5.5 ± 0.2	5.8 ± 0.4	<0.05
Hs-CRP (mg/l)	0.96 ± 1.2	3.39 ± 2.3	<0.02
Total cholesterol (mmol/l)	6.05 ± 1.2	6.46 ± 1.2	0.47
LDL-cholesterol (mmol/l)	2.98 ± 0.63	4.33 ± 1.15	<0.01
Plasma Insulin (mU/l)	6 ± 4	20 ± 8	<0.001
Homocysteine (μmol/l)	12.6 ± 1.6	9.3 ± 1.7	<0.001
HOMA-IR	1.26 ± 0.27	5.5 ± 2.2	<0.0001
Components of the metabolic syndrome			
Glucose (mmol/l)	5.2 ± 0.6	6.3 ± 0.6	<0.002
Waist (cm)	90 ± 7	111 ± 7	<0.0001
Systolic blood pressure (mmHg)	139 ± 19	131 ± 13	0.30
Diastolic blood pressure (mmHg)	86 ± 10	88 ± 7	0.51
Triglycerides (mmol/l)	1.65 ± 0.83	2.28 ± 0.83	0.13
HDL-c (mmol/l)	1.22 ± 0.20	1.11 ± 0.12	0.19

Table 2. Characteristics of male control subjects and men with type 2 diabetes

	Control group (n= 9)	Type 2 diabetes patients (n=9)	P
Age (years)	55 ± 2	58 ± 3	0.4
BMI (kg/m^2)	25 ± 0.7	28 ± 1	0.08
Systolic blood pressure (mmHg)	136 ± 7	132 ± 2	0.5
Diastolic blood pressure (mmHg)	82 ± 4	80 ± 4	0.7
HbA1c (%)	5.4 ± 0.08	7.3 ± 0.4	<0.0006
Cholesterol (mmol/L)	6.3 ± 0.3	4 ± 0.3	<0.0003
Triglycerides (mmol/L)	1.5 ± 0.3	1.9 ± 0.3	0.4
HDL-cholesterol	1.3 ± 0.07	1.1 ± 0.09	0.05
Creatinine (μmol/L)	100 ± 5.2	100 ± 6	1.0
Management with insulin	-	9	-
Duration of diabetes (years)	-	14 ± 2	-

Discussion

The observation that resistin, leptin, PAI-1 and RBP4 induce insulin resistance in megakaryocytes by interfering with IRS-1 adds to the list of plasma abnormalities that link obesity with development of type 2 diabetes. At levels found in severe obesity, these adipokines reduce insulin signaling by affecting IRS-1 control and degradation. Interference with IRS-1 activity by a Ser Phosphatase is also seen in adipocytes incubated with RBP4 and in adipocytes from type 2 diabetes patients. Danielsson *et al* suggest that Ser^{307} phosphorylation of IRS-1 is not required for the initial Tyr phosphorylation but for the subsequent steady-state level of Tyr phosphorylation [32,47]. Control of IRS-1 is a vital step in the insulin signaling pathway that initiates glucose uptake in muscle cells and adipocytes and it would be of great interest to delineate whether these cells show a similar responsiveness to adipokines as megakaryocytes.

A key finding in the present study is the induction of insulin resistance in megakaryocytes by plasma samples from obese men. These samples have a 2-fold elevated level of leptin. A 10 years follow-up assessment in Aboriginal Canadians who were free of diabetes at baseline showed that high leptin levels at baseline were associated with increased risk of incident type 2 diabetes [43]. Also a study among Japanese men showed that the leptin concentration predicted diabetes [44]. Although the significance of both associations decreased with adjustment for waist circumference, the studies support the concept that abnormalities in content of circulating adipokines link obesity to risk of type 2 diabetes. An adipokine with an even stronger association with diabetes risk is adiponectin, with levels that correlate inversely with diabetes risk. Also restistin, PAI-1 and RBP4 make megakaryocytes insulin resistant and correlation studies with risk for type 2 diabetes have appeared for PAI-1 and RBP4 [45,46], but not for resistin. At present about 50 different adipokines have been identified and their effects alone and in combination should be clarified before their impact on platelet sensitivity for insulin can be understood.

Prolonged contact initiates IRS-1 degradation. The reduced IRS-1 expression found in patients with type 2 diabetes, suggests that these megakaryoctes shed platelets devoid of a normal amount of IRS-1 thereby reducing sensitivity for insulin. This is consistent with previous data, showing SOCS-induced degradation of IRS proteins in hepatic cells in mice [40] and reduced IRS-1 levels in adipocytes from type 2 diabetes patients [48].

Resistin, leptin, PAI-1 and RBP4 induce insulin resistance, whereas visfatin, IL-6

and TNF-α do not. Apparently, interference with megakaryocyte behaviour is not an exclusive property of adipocytes (leptin), but also occurs in macrophages and other immune cells which are present in visceral adipose tissue (resistin, PAI-1, RBP4, visfatin, IL-6 and TNF-α).

Adipokines that make megakaryocytes insulin-resistant do not change insulin signaling in platelets. Platelets have the leptin receptor Ob-Ra and Ob-Rb and short contact enhances platelet activation [16-18]. However, they fail to interfere with insulin suppression of Ca^{2+} increases and probably leave IRS-1 activity unchanged. Also during prolonged contact these adipokines do not change insulin suppression of Ca^{2+} rises. Platelets adhering to a thrombotic surface are capable of synthesizing proteins such as tissue factor but synthesis is slow and even further reduced when cells are in suspension (preliminary data: Enhanced platelet tissue factor synthesis in type 2 diabetes patients, A.J.G., C.A.K., T.W.H., J.W.A., 2009). A high leptin concentration induces production of proinflammatory cytokines by human leukocytes [49] and upregulates tissue factor expression in monocytes inducing hypercoagulability [50].

Platelets from type 2 diabetic patients are hyperactive with increased adhesion, aggregation and TxA_2 production *in vitro* [10] and circulate while exposing P-selectin caused by fusion of the α-granules with the plasma membrane explaining the increased risk for atherothrombosis. The loss of insulin sensitivity in these platelets is accompanied by a 1.4-fold increase in signaling through the P2Y12 pathway which might contribute to the hyperactivity of pathways signaling to rises in Ca^{2+}, aggregation and shear-induced platelet function measured in the PFA-100 system [10,11]. Our observations that resistin makes megakaryocytes insulin-resistant and at the same time increases their aggregability suggests that disturbances in IRS-1 regulation affect the P2Y12 pathway. Since upregulation of P2Y12 signaling reduces the sensitivity for pharmacological inhibition, this might be a cause for the increased resistance against anti-thrombotic therapy with clopidogrel in type 2 diabetic patients [51].

Acknowledgements

The cooperation of patients and control subjects is gratefully acknowledged. This study was supported by the Dutch Diabetes Research Foundation (grant 2004.00.029) and the Netherlands Thrombosis Foundation (2006-1). We wish to thank Prof. Dr. G. Strous and E. Tournoij for scientific advice.

References

1. Kahn SE, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 2006; 444:840-846.
2. Qatanani M, Lazar MA. Mechanisms of obesity-associated insulin resistance: many choices on the menu. Genes Dev 2007; 21:1443-1455.
3. Despres JP, Lemieux I. Abdominal obesity and metabolic syndrome. Nature 2006; 444:881-887.
4. Gualillo O, Gonzalez-Juanatey JR, Lago F. The emerging role of adipokines as mediators of cardiovascular function: physiologic and clinical perspectives. Trends Cardiovasc Med 2007; 17:275-283.
5. Grundy SM, Howard B, Smith S Jr, Eckel R, Redberg R, Bonow RO. Prevention Conference VI: Diabetes and Cardiovascular Disease: executive summary: conference proceeding for healthcare professionals from a special writing group of the American Heart Association. Circulation 2002; 105:2231-2239.
6. Tschoepe D, Roesen P, Esser J, Schwippert B, Nieuwenhuis HK, Kehrel B, Gries FA. Large platelets circulate in an activated state in diabetes mellitus. Semin Thromb Hemost 1991; 17:433-438.
7. Andrade FI, Akkerman JW. IRS-1 and vascular complications in diabetes mellitus. Vitam Horm 2005; 70:25-67.
8. Turk Z, Flego I, Kerum G. Platelet aggregation in type 1 diabetes without microvascular disease during continuous subcutaneous insulin infusion. Horm Metab Res 1996; 28:95-100.
9. Ferreira IA, Eybrechts KL, Mocking AI, Kroner C, Akkerman JW. IRS-1 mediates inhibition of Ca2+ mobilization by insulin via the inhibitory G-protein Gi. J Biol Chem 2004; 279:3254-3264.
10. Ferreira IA, Mocking AI, Feijge MA, Gorter G, van Haeften TW, Heemskerk JW, Akkerman JW. Platelet inhibition by insulin is absent in type 2 diabetes mellitus. Arterioscler Thromb Vasc Biol 2006; 26:417-422.
11. Angiolillo DJ, Bernardo E, Ramírez C, Costa MA, Sabaté M, Jimenez-Quevedo P, Hernández R, Moreno R. Insulin therapy is associated with platelet dysfunction in patients with type 2 diabetes mellitus on dual oral antiplatelet treatment. J Am Coll Cardiol 2006; 48:298-304.
12. Halushka PV, Mayfield R, Wohltmann HJ, Rogers RC, Goldberg AK, McCoy SA, Loadholt CB, Colwell JA. Increased platelet arachidonic acid metabolism in diabetes mellitus. Diabetes 1981; 30:44-48.
13. Trovati M, Mularoni EM, Burzacca S, Ponziani MC, Massucco P, Mattiello L,

Piretto V, Cavalot F, Anfossi G. Impaired insulin-induced platelet antiaggregating effect in obesity and in obese NIDDM patients. Diabetes 1995; 44:1318-22.
14. Colwell JA, Nair RM, Halushka PV, Rogers C, Whetsell A, Sagel J. Platelet adhesion and aggregation in diabetes mellitus. Metabolism 1979; 28:394-400.
15. Anfossi G, Russo I, Trovati M. Platelet dysfunction in central obesity. Nutr Metab Cardiovasc Dis 2009; 19:440-9.
16. Konstantinides S, Schafer K, Koschnick S, Loskutoff DJ. Leptin-dependent platelet aggregation and arterial thrombosis suggests a mechanism for atherothrombotic disease in obesity. J Clin Invest 2001; 108:1533-1540.
17. Nakata M, Yada T, Soejima N, Maruyama I. Leptin promotes aggregation of human platelets via the long form of its receptor. Diabetes 1999; 48:426-429.
18. Corsonello A, Perticone F, Malara A, De Domenico D, Loddo S, Buemi M, Ientile R, Corica F. Leptin-dependent platelet aggregation in healthy, overweight and obese subjects. Int J Obes Relat Metab Disord 2003; 27:566-573.
19. Canavan B, Salem RO, Schurgin S, Koutkia P, Lipinska I, Laposata M, Grinspoon S. Effects of physiological leptin administration on markers of inflammation, platelet activation, and platelet aggregation during caloric deprivation. J Clin Endocrinol Metab 2005; 90:5779-5785.
20. Ozata M, Avcu F, Durmus O, Yilmaz I, Ozdemir IC, Yalcin A. Leptin does not play a major role in platelet aggregation in obesity and leptin deficiency. Obes Res 2001; 9:627-630.
21. Kato H, Kashiwagi H, Shiraga M, Tadokoro S, Kamae T, Ujiie H, Honda S, Miyata S, Ijiri Y, Yamamoto J, Maeda N, Funahashi T, Kurata Y, Shimomura I, Tomiyama Y, Kanakura Y. Adiponectin acts as an endogenous antithrombotic factor. Arterioscler Thromb Vasc Biol 2006; 26:224-230.
22. Elbatarny HS, Netherton SJ, Ovens JD, Ferguson AV, Maurice DH. Adiponectin, ghrelin, and leptin differentially influence human platelet and human vascular endothelial cell functions: implication in obesity-associated cardiovascular diseases. Eur J Pharmacol 2007; 558:7-13.
23. den Dekker DE, Gorter G, Heemskerk JW, Akkerman JW. Development of platelet inhibition by cAMP during megakaryocytopoiesis. J Biol Chem 2002; 277:29321-29329.
24. Fugman DA, Witte DP, Jones CL, Aronow BJ, Lieberman MA. In vitro establishment and characterization of a human megakaryoblastic cell line. Blood 1990; 75:1252-1261.
25. Jones CL, Witte DP, Feller MJ, Fugman DA, Dorn GW 2nd, Lieberman MA. Re-

sponse of a human megakaryocytic cell line to thrombin: increase in intracellular free calcium and mitogen release. Biochim Biophys Acta 1992; 1136:272-282.

26. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). CAPRIE Steering Committee. Lancet 1996; 348: 1329-1339.
27. Ogawa W, Matozaki T, Kasuga M. Role of binding proteins to IRS-1 in insulin signalling. Mol Cell Biochem 1998; 182:13-22.
28. Paz K, Hemi R, LeRoith D, Karasik A, Elhanany E, Kanety H, Zick Y. A molecular basis for insulin resistance. Elevated serine/threonine phosphorylation of IRS-1 and IRS-2 inhibits their binding to the juxtamembrane region of the insulin receptor and impairs their ability to undergo insulin-induced tyrosine phosphorylation. J Biol Chem 1997; 272:29911-8.
29. Aguirre V, Uchida T, Yenush L, Davis R, White MF. The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). J Biol Chem 2000; 275:9047-9054.
30. Paz K, Liu YF, Shorer H, Hemi R, LeRoith D, Quan M, Kanety H, Seger R, Zick Y. Phosphorylation of insulin receptor substrate-1 (IRS-1) by protein kinase B positively regulates IRS-1 function. J Biol Chem 1999; 274:28816-28822.
31. Danielsson A, Nystrom FH, Stralfors P. Phosphorylation of IRS1 at serine 307 and serine 312 in response to insulin in human adipocytes. Biochem Biophys Res Commun 2006; 342:1183-1187.
32. Danielsson A, Ost A, Nystrom FH, Stralfors P. Attenuation of insulin-stimulated insulin receptor substrate-1 serine 307 phosphorylation in insulin resistance of type 2 diabetes. J Biol Chem 2005; 280:34389-34392.
33. Paz K, Liu YF, Shorer H, Hemi R, LeRoith D, Quan M, Kanety H, Seger R, Zick Y. A family of cytokine-inducible inhibitors of signalling. Nature 1997; 387:917-921.
34. Naka T, Narazaki M, Hirata M, Matsumoto T, Minamoto S, Aono A, Nishimoto N, Kajita T, Taga T, Yoshizaki K, Akira S, Kishimoto T. Structure and function of a new STAT-induced STAT inhibitor. Nature 1997; 387:924-929.
35. Endo TA, Masuhara M, Yokouchi M, Suzuki R, Sakamoto H, Mitsui K, Matsumoto A, Tanimura S, Ohtsubo M, Misawa H, Miyazaki T, Leonor N, Taniguchi T, Fujita T, Kanakura Y, Komiya S, Yoshimura A. A new protein containing an SH2 domain that inhibits JAK kinases. Nature 1997; 387:921-924.
36. Krebs DL, Hilton DJ. SOCS: physiological suppressors of cytokine signaling. J. Cell Sci 2000; 113 (Pt 16):2813-2819.

37. Zhang JG, Farley A, Nicholson SE, Willson TA, Zugaro LM, Simpson RJ, Moritz RL, Cary D, Richardson R, Hausmann G, Kile BJ, Kent SB, Alexander WS, Metcalf D, Hilton DJ, Nicola NA, Baca M. The conserved SOCS box motif in suppressors of cytokine signaling binds to elongins B and C and may couple bound proteins to proteasomal degradation. Proc Natl Acad Sci USA 1999; 96:2071-2076.
38. Wang Q, Miyakawa Y, Fox N, Kaushansky K. Interferon-alpha directly represses megakaryopoiesis by inhibiting thrombopoietin-induced signaling through induction of SOCS-1. Blood 2000; 96:2093-2099.
39. Krebs DL, Hilton DJ. A new role for SOCS in insulin action. Suppressor of cytokine signaling. Sci STKE 2003; 2003:PE6.
40. Rui L, Yuan M, Frantz D, Shoelson S, White MF. SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2. J Biol Chem. 2002; 277: 42394-42398.
41. Hajer GR, Dallinga-Thie GM, van Vark-van der Zee LC, Olijhoek JK, Visseren FL. Lipid-lowering therapy does not affect the postprandial drop in high density lipoprotein-cholesterol (HDL-c) plasma levels in obese men with metabolic syndrome: a randomized double blind crossover trial. Clin Endocrinol (Oxf) 2008; 69:870-877.
42. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). JAMA 2001; 285:2486-2497.
43. Ley SH, Harris SB, Connelly PW, Mamakeesick M, Gittelsohn J, Hegele RA, Retnakaran R, Zinman B, Hanley AJ. Adipokines and incident type 2 diabetes in an Aboriginal Canadian [corrected] population: the Sandy Lake Health and Diabetes Project. Diabetes Care 2008; 31:1410-1415.
44. McNeely MJ, Boyko EJ, Weigle DS, Shofer JB, Chessler SD, Leonnetti DL, Fujimoto WY. Association between baseline plasma leptin levels and subsequent development of diabetes in Japanese Americans. Diabetes Care 1999; 22:65-70.
45. Festa A, D'Agostino R Jr, Tracy RP, Haffner SM. Elevated levels of acute-phase proteins and plasminogen activator inhibitor-1 predict the development of type 2 diabetes: the insulin resistance atherosclerosis study. Diabetes 2002; 51:1131-1137.
46. Graham TE, Yang Q, Blüher M, Hammarstedt A, Ciaraldi TP, Henry RR, Wason CJ, Oberbach A, Jansson PA, Smith U, Kahn BB. Retinol-binding protein 4 and

insulin resistance in lean, obese, and diabetic subjects. N Engl J Med 2006; 354:2552-2563.

47. Ost A, Danielsson A, Lidén M, Eriksson U, Nystrom FH, Strålfors P. Retinol-binding protein-4 attenuates insulin-induced phosphorylation of IRS1 and ERK1/2 in primary human adipocytes. FASEB J 2007; 21:3696-3704.
48. Rondinone CM, Wang LM, Lonnroth P, Wesslau C, Pierce JH, Smith U. Insulin receptor substrate (IRS) 1 is reduced and IRS-2 is the main docking protein for phosphatidylinositol 3-kinase in adipocytes from subjects with non-insulin-dependent diabetes mellitus. Proc Natl Acad Sci USA 1997; 94:4171-4175.
49. Zarkesh-Esfahani H, Pockley G, Metcalfe RA, Bidlingmaier M, Wu Z, Ajami A, Weetman AP, Strasburger CJ, Ross RJ. High-dose leptin activates human leukocytes via receptor expression on monocytes. J Immunol 2001; 167:4593-4599.
50. Napoleone E, DI Santo A, Amore C, Baccante G, di Febbo C, Porreca E, de Gaetano G, Donati MB, Lorenzet R. Leptin induces tissue factor expression in human peripheral blood mononuclear cells: a possible link between obesity and cardiovascular risk? J Thromb Haemost 2007; 5:1462-1468.
51. Angiolillo DJ, Shoemaker SB, Desai B, Yuan H, Charlton RK, Bernardo E, Zenni MM, Guzman LA, Bass TA, Costa MA. Randomized comparison of a high clopidogrel maintenance dose in patients with diabetes mellitus and coronary artery disease: results of the Optimizing Antiplatelet Therapy in Diabetes Mellitus (OPTIMUS) study. Circulation 2007; 115:708-716.
52. van der Vuurst H, van Willigen G, van Spronsen A, Hendriks M, Donath J, Akkerman JW. Signal transduction through trimeric G proteins in megakaryoblastic cell lines. Arterioscler Thromb Vasc Biol 1997; 17:1830-1836.

SUPPLEMENTARY DATA

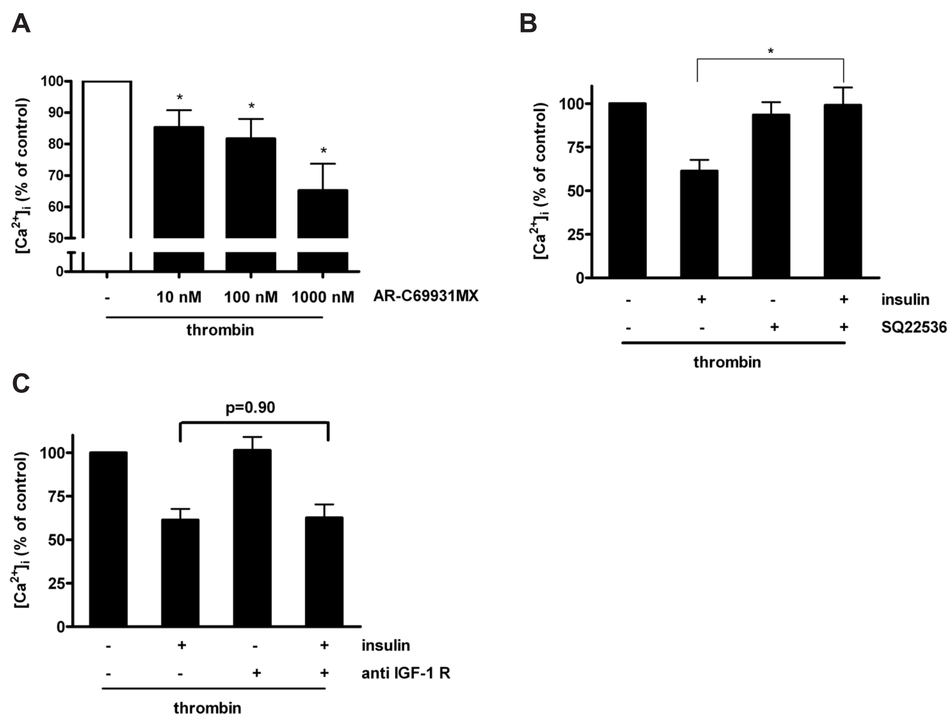


Figure S1. Insulin signaling suppresses thrombin-induced Ca^{2+} mobilization in megakaryocytes

(a) Functional P2Y12 signaling in megakaryocytes. Megakaryocytes were pre-incubated with different concentration AR-C69931MX (5 minutes, 37°C) and thrombin-induced Ca^{2+} mobilization was measured. (b) Insulin inhibition is mediated by adenylyl cyclase. Megakaryocytes were pre-incubated with the adenylyl cyclase SQ22536 (50 μM, 15 min, 37°C) and insulin inhibition of thrombin-induced Ca^{2+} mobilization was measured. (c) No role for the IGF-1 receptor in the inhibition by insulin. Megakaryocytes were pre-incubated with a blocking antibody against the IGF-1 receptor (1 μg/mL, 20 minutes, 37°C) and insulin inhibition of thrombin-induced Ca^{2+} mobilization was measured. (for a-c, means ± SEM, n=3, data was analyzed with Student's test, * denotes a significant difference, $p<0.05$)

Chapter 3

Enhanced platelet tissue factor synthesis in type 2 diabetes patients

AJ Gerrits[1]
CA Koekman[1]
TW van Haeften[2]
JW Akkerman[1]

[1]Thrombosis and Haemostasis Laboratory, Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, the Netherlands
[2]Department of Internal Medicine, University Medical Center Utrecht, Utrecht, the Netherlands

Diabetes, accepted pending modifications

Abstract

Objective Patients with type 2 diabetes have an increased risk of cardiovascular disease and show abnormalities in the coagulation cascade. We investigated whether increased synthesis of Tissue Factor (TF) by platelets could contribute to the hypercoagulant state.

Research Design and Methods Platelets from type 2 diabetes patients and matched controls were adhered to different surface-coated proteins and TF-pre-mRNA splicing, -protein and -procoagulant activity were measured.

Results Different adhesive proteins induced different levels of TF-synthesis. A mimetic of active clopidogrel metabolite (AR-C699331 MX) reduced TF synthesis by 56 ± 10%, an aspirin-like inhibitor (indomethacin) by 82 ± 9% and the combination by 96 ± 2%, indicating that ADP release and thromboxane A_2 production followed by activation of P2Y12- and thromboxane receptors mediate surface-induced TF-synthesis. Interference with intracellular pathways revealed inhibition by agents that raise cAMP and interfere with PI3-K/PKB. Insulin is known to raise cAMP in platelets and inhibited collagen-III induced TF-pre-mRNA splicing and reduced TF activity by 35±5 and 47±5% at 1 and 100 nM. Inhibition by insulin was reduced in type 2 diabetes platelets resulting in a ~1.6-fold higher TF synthesis than in matched controls.

Discussion We characterized the extra- and intracellular mechanisms that couple surface activation to TF-synthesis in adhering platelets. In healthy individuals, TF-synthesis is inhibited by insulin but in patients with type 2 diabetes inhibition is impaired. This leads to the novel finding that platelets from type 2 diabetes patients produce more TF than platelets from matched controls.

Introduction

Type 2 diabetes patients have a 2–8-fold higher risk of cardiovascular disease than healthy individuals and eighty percent will die of arterial thrombosis-related disorders [1]. Type 2 diabetes induces a prothrombotic state caused by imbalance of the hemostatic mechanism with evidence of hypercoagulation, decreased fibrinolysis, platelet hyperaggregability and endothelial dysfunction [2,3]. Circulating markers for activated coagulation such as prothrombin fragment 1 + 2 and thrombin-anti-thrombin complexes are increased. Also the elevated levels of fibrinogen,

factors (F) VII, VIII, XI and von Willebrand factor (VWF) might contribute to the prothrombotic tendency (4). In particular, circulating tissue factor (TF) is increased [5], which, as the main initiator of the coagulation cascade, might contribute to hypercoagulability. Interestingly, improvement of glycaemic control lowers plasma TF [6].

The hyperaggregability of type 2 diabetes platelets might be caused by loss of sensitivity to insulin. Under experimental conditions, platelets of type 2 diabetes patients show better adhesion to a prothrombotic surface, make larger aggregates, have an increased pro-coagulant surface and an elevated cytosolic Ca^{2+} concentration compared to platelets from healthy controls [7]. Intensive insulin treatment partly normalizes platelet aggregation [8]. Indeed, insulin inhibits platelets from healthy individuals but not from type 2 diabetes subjects. Normally, platelet activators start signaling pathways that initiate aggregation and secretion while concurrently reducing the level of the intracellular inhibitor cAMP through the G-protein subunit Giα–2. Insulin inactivates Giα–2 through binding of insulin receptor substrate-1 (IRS-1) to Giα–2 [7-9]. In type 2 diabetes platelets, this property is lost presumably due to a defect in IRS-1. In addition to signaling through Giα–2, insulin releases Gi $\beta\gamma$ activating the phosphatidylinositol 3-kinase (PI3-K)/ protein kinase B (PKB) pathway and increasing glucose uptake through GLUT3 transporters [10,11].

A key step in the initiation of a haemostatic plug is TF exposure. TF is an integral transmembrane protein (43 kD) located on the plasma membrane [12]. TF binds activated FVII and the complex activates FVII, FIX and FX, generating thrombin and inducing clotting. TF is constitutively expressed by vascular smooth muscle cells and vessel damage rapidly starts coagulation. Also monocytes and endothelial cells produce TF following a shift to a prothrombotic phenotype induced by inflammatory factors such as lipopolysaccaride (LPS). Studies in mice with a general and a bone marrow-specific TF suppression show that fibrin propagation depends exclusively on blood-born TF. The source of blood-born TF is sought in microparticles. They deliver TF in an encrypted state which upon contact with the thrombus changes to an active conformation that initiates clotting [13]. We showed recently that TF production by LPS-stimulated monocytes is suppressed by insulin via a mechanism similar but not identical to insulin's inhibition of platelet functions [14]. Preliminary data indicate that in type 2 diabetes monocytes have lost sensitivity to insulin and produce more TF than their normal counterparts.

Recent findings suggest a new source for TF expression [15]. The anucleate platelet has long been considered incapable of synthesizing proteins, but the presence of

a splicing machinery together with pre-mRNAs for a number of proteins including TF make them a source for TF synthesis.

Here we show for the first time that different adhesive proteins initiate different levels of TF synthesis in platelets. We identify extra- and intracellular mechanisms that couple surface activation to TF synthesis and evaluate the effect of platelet inhibiting medication. We show that in normal platelets, insulin interferes with surface-induced TF synthesis. In platelets from type 2 diabetes patients, inhibition by insulin is much reduced resulting in a 1.6-fold increase in TF synthesis compared with platelets from healthy controls.

Methods

Materials - We obtained human recombinant insulin (solubilized according to the recommendations of the manufacturer in 10 mmol/L acetic acid, 100 mmol/L NaCl, and 0.01% BSA to a stock concentration of 100 µmol/L), wortmannin, 1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine (ML-9), lipopolysaccharide (LPS, E. coli 0111:B4), indomethacin, puromycin, collagen type I and III, ADP from Sigma (St. Louis, MO, USA), Akt inhibitor 1701-1 from Biovision (Mountain View, CA, USA), iloprost from Schering AG (Berlin, Germany), forskolin, BAPTA(AM), Tg003 and cantharidin from Calbiochem (La Jolla, CA, USA), LY294002 from Biomol (Plymouth Meeting, PA, USA), cycloheximide from MP Biochemicals (Santa Ana, CA, USA), collagen reagent Horm from Nycomed Pharma (Munich, Germany), thrombin and fibrinogen from Enzyme Research Laboratories (South Bend, IN, USA), U-44619 from Cayman Chemicals (Ann Arbor, MI, USA). Plasma-derived VWF was purified from VWF/FVIII concentrate (Haemate P, Behring, Marburg, Germany) [16]. Factor X (FX) was purified from fresh-frozen plasma [17]. Recombinant Factor VIIa (rFVIIa) was from Novo Nordisk (Bagsværd, Denmark) and recombinant TF (Innovin) from Dade Behring, Liederbach, Germany. Antibodies against the horseradish peroxidase-labeled anti-rabbit antibody were from Cell Signaling Technology (Danvers, MA, USA), anti-phospho-Ser polyclonal antibody from Upstate Biotechnology (Bucks, UK), peroxidase-linked goat anti-mouse antibody from DAKO (Glostrup, Denmark), anti-SF2/ASF antibody from Abcam (Cambridge, UK), anti-TF antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA) for immunoprecipitation and Affinity Biologicals (Ancaster, ON, Canada) for Western blotting and anti-TF pathway inhibitor (TFPI) anti-

body from American Diagnostica (Stamford, UK) An inhibitory antibody against TF was a generous gift from Dr M Kjalke (Hemostasis Biology, Novo Nordisk, Malov, Denmark). The ADP receptor P2Y12 antagonist, N6-(2-methylthioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene ATP (AR-C69931MX) was a kind gift from Astra Zeneca (Loughborough, UK). All other chemicals used were of analytical grade.

Subjects - The study had been approved by the Medical Ethical Review Board. Type 2 diabetes subjects were recruited from the out patient clinics of the Utrecht Medical University Hospital (Utrecht, Netherlands). Their characteristics are given in Table 1. Patients and matched control subjects gave their informed consent prior to participation in the study.

Platelet isolation - Freshly drawn venous blood from healthy volunteers and type 2 diabetes patients was collected into 0.1 volume of 130 mmol/L Na_3 citrate. Platelets were isolated as described [9].
Attempts to remove contaminating monocytes included (i) incubation of a platelet suspension (2×10^8 platelets/mL) with either anti-CD14 or CD45 antibody coupled beads (15 min, 22°C, 35 μL anti-CD14 or anti-CD45 beads per 40×10^8 platelets) and subsequent magnetic separation (Miltenyi Biotec, Bergisch Gladbach, Germany) and (ii) a double incubation of a concentrated platelet suspension (~15×10^8 platelets/mL) with a cocktail of anti-CD14 and anti-CD45 antibody coupled beads (15 min, 22°C, 35 μL anti-CD14 and 35 μL anti-CD45 beads per 40×10^8 platelets) and magnetic separation. Contamination with monocytes was measured by RT-PCR of CD14 mRNA. To this end, total RNA was isolated using the kit from RNA-Bee (Tell Tech, Friendswood, TX, USA) and RT-PCR was performed using the forward primer 5'-AAAGCACTTCCAGAGCCTGC-3' and the reverse primer 5'-TCGAGCGTCAGTTCCTTGAGG-3' on a PTC-200 apparatus (MJ Research, Waltham, MA, USA).

TF expression - An aliquot of platelet suspension (2×10^8 platelets) was incubated for static adhesion experiments in 6-wells plates (Corning Ultra-Low Attachment surface, Corning, Acton, MA, USA) or kept in suspension for the indicated times at 37°C. Wells were coated with 50 μg/mL horm collagen, 50 μg/mL collagen type I, 50 μg/mL collagen type III, 1 U/mL thrombin, 100 μg/mL fibrinogen and 10 μg/mL VWF for 2 hrs at 37°C. For analysis of TF procoagulant activity, adhered platelets, collected in 250 μL HEPES/Tyrode buffer, or suspensions were lysed by

3 times freeze/ thawing and centrifuged (16200 × g, 2 min, 22°C). Pellet fractions were dissolved in 50 μL HEPES/Tyrode buffer. Samples were incubated with 2 μg/mL anti-TFPI antibody (15 min, 22°C) and thereafter with 10 μg/mL FX, 5 U/mL rFVIIa and 5 mmol/L $CaCl_2$ (45 min, 37°C). FXa generation was measured in a fluorescence reader at 405 nm after addition of FXa substrate (Pentapharm, Basel, Switzerland). Procoagulant activity was determined based on a standard curve derived from serial dilutions of recombinant TF.

For analysis of splicing of TF pre-mRNA, 12 × 10^8 platelets were adhered to a coated surface or kept in suspension, as indicated. Total RNA was isolated (RNA-Bee, Tell Tech) and RNA levels adjusted for variations per sample (by measuring the optical density OD260 on a NanoDrop spectrophotometer) and RT-PCR was performed on a PCR apparatus (PTC-200, MJ Research, Waltham, MA, USA) using primers that overlapped intron four, with forward primer 5'-CTCGGACAGC-CAACAATTCAG-3' and reverse primer 5'-CGGGCTGTCTGTACTCTTCC-3'.

Alkaline phosphatase assay –The number of adhered platelets was inferred from the content of alkaline phosphatase in collected samples, as published [18]. Alkaline phosphatase activity was related to platelet number using a serial dilution of platelets in suspension.

Immunoprecipitation –To determine levels of TF protein and the Ser phosphorylation of SF2, aliquots of 2 × 10^8 platelets were adhered to a coated surface in 6-wells plates or kept in suspension (1 hr, 37°C). Adhered platelets and suspensions were collected in lysis buffer (1% NP40, 0.5% octylglucoside, 0.1% SDS, 5 mmol/L EDTA in PBS) supplemented with 10% protease inhibitor cocktail, 1 mmol/L $NaVO_3$ and 10 μM cantharidin. Proteins were precipitated with protein G-sepharose in combination with 1 μg/mL anti-TF or anti-SF2 antibody overnight at 4°C. Precipitates were washed 3 times and dissolved in reducing Laemmli sample buffer.

SDS-PAGE and Western blotting - Proteins were analyzed by SDS-PAGE and Western blotting. After blocking with Odyssey buffer (1 hr, 22°C), membranes were incubated (16 hrs, 4°C) with 1 μg/mL anti-TF, anti-phospho-Ser or anti-SF2 antibody. Immunoblots were visualized using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA) and Alexa-labelled antibodies according to the manufacturer's instructions. The intensity of the bands was quantified with ImageJ software.

Statistics - Data is expressed as mean ± S.E.M. with n observations and was analyzed with Student's test for unpaired observations or Mann Whitney U test, as indicated. Differences were considered significant at $p < 0.05$.

RESULTS

Preparation of pure platelet suspensions

The finding that platelets synthesize TF has been refuted in the past by critics who claimed that platelet suspensions are often contaminated with monocytes which have a many fold higher translation capacity [19-21]. To address this issue, we searched for the monocyte-specific marker CD14-mRNA by RT-PCR in platelet preparations prepared by conventional centrifugation/resuspension [7,9] and found them positive (Fig. 1a, top panel). Also, an extra treatment with anti-CD45 magnetic beads was insufficient to remove all monocytes. Pure platelet preparations were obtained by a twice repeated incubation of a concentrated platelet suspension with a cocktail of anti-CD14 and anti-CD45 magnetic beads and monocyte removal by magnetic sorting. After 35 cycli, RT-PCR of CD14 was negative in the platelet preparation and positive in the elution (Fig. 1a, bottom panel). We found that suspensions containing CD14-mRNA that were allowed to adhere to fibrinogen responded to LPS with formation of TF activity detected in the Factor Xa assay (Fig. 1b). CD14-mRNA negative preparations did not. In order to ensure purity of the platelet suspension, a sample of adhered platelets incubated with LPS was therefore included in all experiments and samples showing LPS-induced TF synthesis were discarded. Addition of anti-TF antibody (0.5 mg/mL, 15 min, 22°C) completely blocked TF activity in activated platelets confirming specificity of the FXa assay.

Activated platelets express TF by splicing of pre-mRNA

The number of adhered platelets was determined by measuring alkaline phosphatase content and a standard curve relating enzyme activity to platelet number established in platelet suspensions (Fig. 1c). TF synthesis and TF pre-mRNA splicing were studied on fibrinogen- and collagen-coated surfaces. Platelets adhering to a fibrinogen-coated surface (4 hr, 37°C) synthesized 0.24 ± 0.04 ng TF/ 2×10^8 platelets (n=10). TF synthesis in adhered platelets was inhibited by the translation blockers cycloheximide (58 ± 2%, n=3) and puromycin (76 ± 6%, n=12)

and the splicing blocker Tg003 (51 ± 7%, n=6; Fig. 1d), confirming earlier work [15,22]. To demonstrate splicing of TF pre-mRNA, a RT-PCR with primers overlapping intron four was performed on platelets adhered to collagen type I (2 hr, 37°C). The result showed the expected band for spliced mRNA at 297 bp and for unspliced mRNA at 904 bp in the presence of Tg003 (Fig. 1e). Regulation of TF pre-mRNA splicing was examined by measuring the Ser phosphorylation of SF2, an effector of Cdc2-like kinase (Clk-1; Fig. 1f). The low levels of SF2-Ser phosphorylation in platelets in suspension were unchanged after stimulation with thrombin and increased when platelets had adhered to fibrinogen (4 hrs, 37°C). Tg003 reduced the phosphorylation, confirming its dependence on Clk1. These results confirm that adhering platelets synthesize TF through splicing of pre-mRNA.

Different splicing at different adhesive surfaces

The question was addressed which adhesive surface induced the highest TF pre-mRNA splicing. Platelets were incubated (4 hr, 37°C) on a surface coated with horm collagen, a mixture of 95% type I and 5% type III collagen, collagen I, which has strong platelet adhesive properties [23], immobilized thrombin, fibrinogen which binds to the damaged vessel wall, collagen III, which is present in the vasculature and VWF which also binds to the sub-endothelium. Puromycin was used to separate translation-dependent TF activity from background TF. Compared with fibrinogen (set at 100%), TF synthesis on horm collagen, collagen I and thrombin was 143 ± 42, 132 ± 29 and 126 ± 20%, respectively. A lower TF activity was found on collagen III (71 ± 10%) and VWF (38 ± 14%) (n=4; Fig. 2a). Since different adhesive surfaces induced different extents of adhesion/aggregation, bound platelets were quantified on the basis of alkaline phosphatase content. The highest adhesion/aggregation was found with collagen I and horm collagen (~125 × 106 platelets), whereas adhesion to thrombin, fibrinogen and VWF was about 30% lower (Fig. 2b). Expression per platelet and correction for background TF (defined as ΔTF activity), indicated that horm collagen, thrombin and collagen I induced the highest TF synthesis followed by fibrinogen (set at 100%), collagen III and VWF (Fig. 2c). We examined whether adhered platelets produce more TF than platelets in suspension. Collagen III-adhered platelets (4 hrs, 37°C) produced 600 fold more TF than platelets in suspension stimulated with collagen III (0.17 ± 0.09 and 0.0003 ± 0.0001 ng TF/ 2.0 × 10^8 platelets respectively, n=3), indicating that splicing of TF pre-mRNA is strongly facilitated by adherence to a surface (Fig. 2d), such in contrast with an earlier finding [15,22].

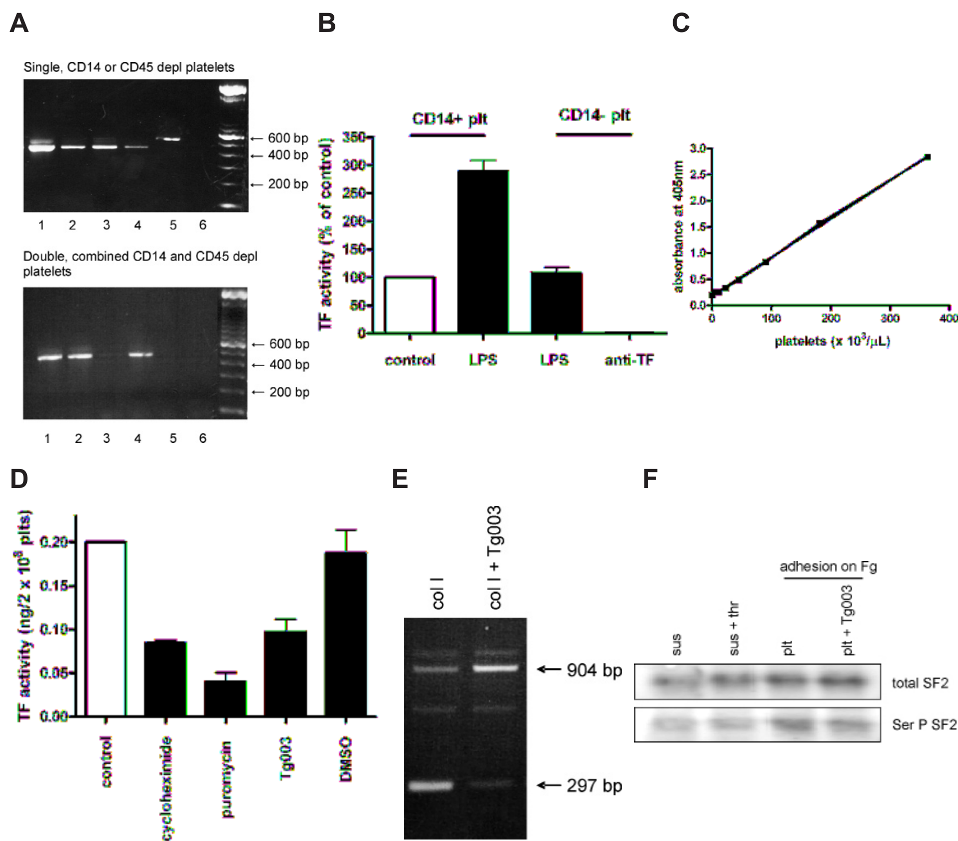


Figure 1. Platelet-specific splicing of TF pre-mRNA and TF synthesis

(a) Detection of leukocyte-specific CD14 RNA (422 bp) in monocytes (lanes 1), platelet suspensions prepared by conventional centrifugation/resuspension (2), leukocyte-depleted platelet suspensions obtained by incubation with either anti-CD14 or anti-CD45 magnetic beads (3, top panel) or double incubation with anti-CD14 and anti-CD45 magnetic beads (3, bottom panel) and elution (4, leukocyte-rich fraction). Controls -RT and –RNA show the presence of genomic DNA and the specificity of the PCR (5,6). (b) Lack of LPS induced TF synthesis in CD14 mRNA-negative suspensions. CD14 mRNA-positive and CD14 mRNA-negative platelet suspensions without (open bar) and with (closed bars) 1 µg/mL LPS. Incubation with a blocking antibody against TF completely inhibited TF activity in CD14 mRNA-negative suspensions. TF activity in fibrinogen-adhered platelets without LPS stimulation was expressed as 100%. (c) Relation between alkaline phosphatase level and platelet number used to quantify the number of adhered platelets. (d) TF activity in fibrinogen-adhered platelets (4 hr, 37°C; open bar) is inhibited by the translation blockers cycloheximide and puromycin and the splicing blocker Tg003 (in DMSO); closed bars. (e) Splicing by platelets adhering to collagen I (4 hr, 37°C) demonstrating a shift from pre-mRNA (904 bp) to mature mRNA (297 bp). (f) Negative SF2 phosphorylation in resting and thrombin-stimulated platelet suspensions and positive SF2 phosphorylation in fibrinogen-adhered platelets which is inhibited by Tg003. Data are means ± SEM; n=3-12 (a), (c), (e).

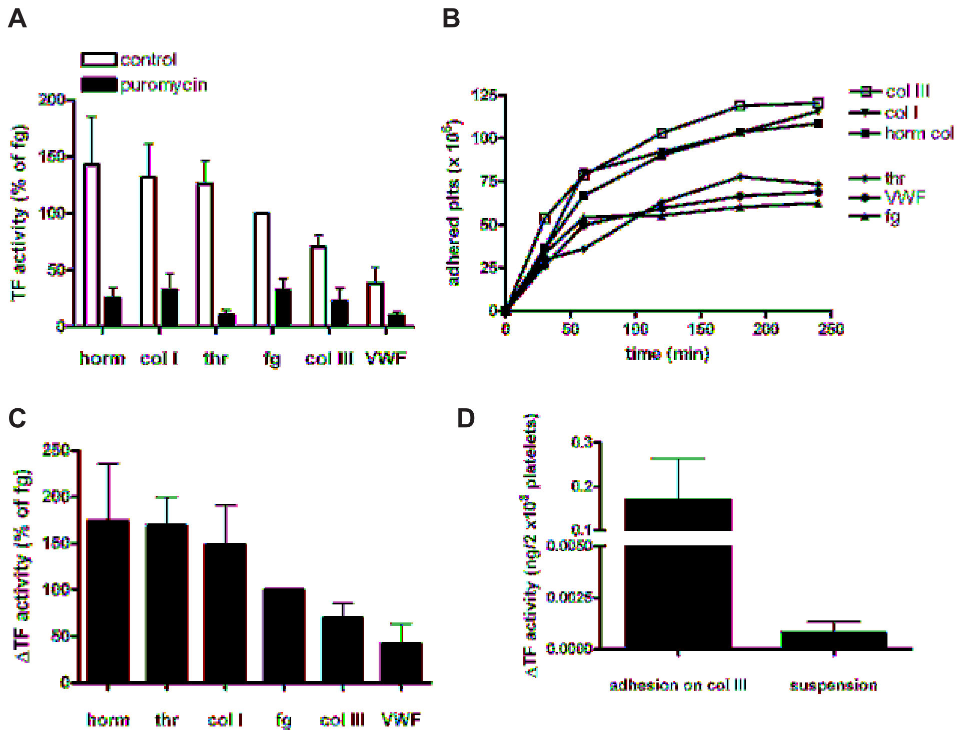


Figure 2. Surface-dependent platelet TF activity

(a) TF synthesis in platelets adhering to surface-coated horm collagen (horm), collagen I (col I), thrombin (thr), fibrinogen (fg), collagen III (col III) and VWF (4 hr, 37°C). TF activity in fibrinogen-adhered platelets was set at 100%. Pre-incubation with puromycin (15 min, 37°C) inhibited TF activity to background. (b) Platelets were adhered to different adhesive proteins and at different times alkaline phosphatase was measured and the number of adhered platelets determined. A representative example of n=4 is shown. (c) For different adhesive proteins surface-induced TF activity was expressed per platelet, corrected for background TF and defined as ΔTF activity. (d) Platelets adhering to collagen III (4 hr, 37°C) produce 600-fold more TF activity than the same platelet number in suspension stimulated with a same concentration of collagen III. (a) and (c) TF activity in fibrinogen-adhered platelets was expressed as 100%. Data are means ± SEM; n=3-4.

Inhibition of platelet TF synthesis

To examine the mechanisms that couple surface activation to TF synthesis, platelets were adhered to collagen III without and with co-incubation with AR-C699331 MX (which mimics blockade of the P2Y12-receptor by the active metabolite of clopidogrel), with indomethacin, an easily soluble COX-1 inhibitor which mimics the action of aspirin, or a combination. The inhibitors did not change platelet adhesion during prolonged contact with collagen III (Fig. 3a). AR-C699331 induced a 56 ± 16% decrease in TF synthesis, indicating that in its absence TF synthesis is

enhanced by ADP secretion and P2Y12 receptor-mediated signaling. Indomethacin induced an even steeper reduction and inhibited TF synthesis by 82 ± 9%. The combination of the two inhibitors reduced TF synthesis by 96 ± 2% (n=4, Fig. 3b). Thus, platelet TF synthesis strongly depends on the extracellular feed-back loops mediated by secretion of granule ADP and release of TxA_2.
Since platelets adhered to different adhesive proteins synthesized different amounts of TF, intracellular pathways linking surface receptors with splicing regulation were investigated. There was little effect of metabolic inhibitors on adhesion (Fig. 3c and not shown). The adenylyl cyclase activator forskolin and the stable prostacyclin mimetic iloprost raise cAMP and inhibited TF synthesis on collagen III by 51 ± 14 and 82 ± 6% respectively. BAPTA/AM, which lowers the cytosolic Ca^{2+} concentration and quenches Ca^{2+} increases in activated platelets, inhibited TF synthesis almost completely. Inhibitors of PI3-K (wortmannin, LY294002) and PKB (Akt inhibitor 1701-1, ML-9) decreased TF synthesis by 60% or more, indicating that in the absence of inhibitors, the PI3-K/PKB pathway signals to TF pre-mRNA splicing (Fig. 3d). To investigate whether the inhibitors interfere with release of ADP/TxA_2 and/or with signaling from ADP/TxA_2 receptors to the spliceosome, experiments were repeated in the presence of excess of ADP (50 μM) and of the stable TxA_2 analog U-46619 (2 μM). Inhibition of TF synthesis by the metabolic inhibitors was preserved, indicating that in addition to the known suppression of ADP/TxA_2 release, these blockers interfere with the control of TF pre-mRNA splicing (Fig. 3e). Western blot analysis confirmed that iloprost inhibited synthesis of TF protein (Fig. 3f). Together, these data reveal that surface-induced TF synthesis is inhibited by a rise in cAMP, strongly depends on cytosolic Ca^{2+} and is induced by signaling through the PI3-K/PKB pathway.

Insulin inhibits collagen-induced TF synthesis in platelets

Earlier studies in platelets showed that insulin induces Tyr-phosphorylation of IRS-1, which is an upstream regulator of the Gi-mediated suppression of adenylyl cyclase and the PI3-K mediated activation of PKB [9]. Since surface-induced TF synthesis was sensitive to agents that raise cAMP or inhibit the PI3-K/PKB pathway, we addressed the question whether insulin interferes with adhesion-induced TF synthesis. Platelets were pre-incubated with 100 nM insulin (5 min, 37°C) and adhered to different adhesive surfaces. The degree of inhibition differed between adhesive proteins and reached 94 ± 6, 64 ± 27, 76 ± 27, 67 ± 14, 41 ± 7% (n=5) for horm collagen, thrombin, collagen I, fibrinogen and collagen III respectively. On VWF no inhibition by insulin was found (Fig. 4a). To elucidate whether insulin

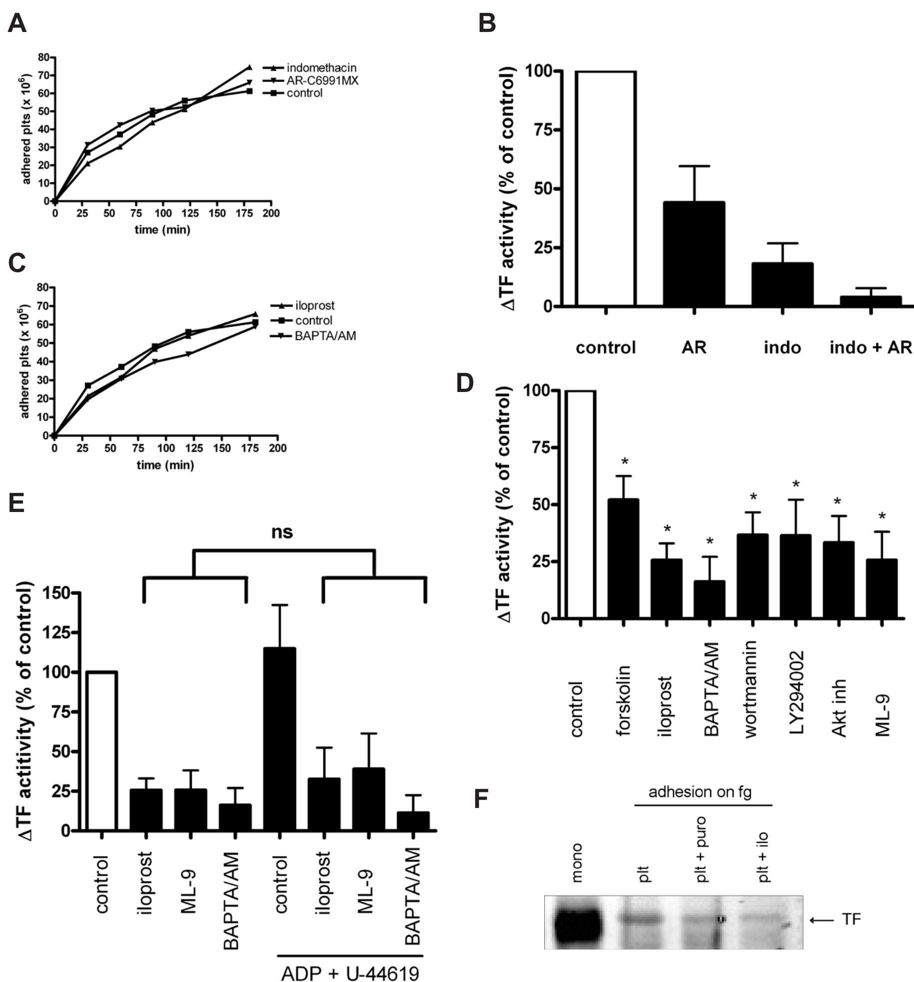


Figure 3. Effect of inhibitors of signaling pathways on TF synthesis in platelets
(a,b) Platelets were pre-incubated with a mimetic of active clopidogrel metabolite (AR-C699331 MX, AR, 250 nM), the aspirin-like inhibitor indomethacin (Indo, 100 μM) or the combination (15 min, 37°C). AR-C699331 MX and indomethacin did not affect platelet adhesion but reduced surface-induced TF synthesis (4 hr, 37°C). (c,d). Platelets were preincubated with the adenylyl cyclase activator forskolin (40 μM), the stable prostacyclin mimetic iloprost (1 μg/mL), the Ca^{2+} quencher BAPTA/AM (25 μM), the PI3-K/PKB inhibitors wortmannin (0.5 μM) and LY294002 (10 μM), the Akt inhibitor 1701-1 (2 μM) and ML-9 (100 μM) (15 min, 37°C). The inhibitors did not change platelet adhesion but reduced the surface-induced TF synthesis (4 hr, 37 °C). (e) Platelets in the presence of excess of ADP and the TxA_2 mimetic U-44619 were pre-incubated with iloprost, BAPTA/AM, wortmannin, LY294002, the Akt inhibitor 1701-1 and ML-9 before platelets were adhered to collagen III (4 hr, 37°C) and TF activity was determined. (f) Platelets were adhered to fibrinogen (4 hr, 37°C). The blot shows the 60 kD band (in presence of DTT) of monocyte TF, surface-induced platelet TF protein, the inhibition by puromycin and by the cAMP raising agent iloprost. (a), (c) and (f) representative examples for n=3. (b), (d) and (e) TF activity in collagen III-adhered platelets was expressed as 100%. Data are means ± SEM; n=3-4.

interferes with splicing before or after release of ADP/TxA_2, experiments were repeated in the presence of excess of ADP and TxA_2 mimetic. Inhibition of collagen III-induced TF synthesis was preserved, indicating that insulin inhibits TF-synthesis by interfering with the control of pre-mRNA splicing induced by ADP and thromboxane A_2 receptors (Fig. 4b). Collagen III-adhered platelets were used to further evaluate the inhibition by insulin. Adhered platelets showed a dose-dependent inhibition between 1 and 100 nM insulin (Fig. 4c). Inhibition of pre-mRNA splicing by insulin was clearly detectable, showing decreased levels of mature mRNA compared to platelets in the absence of insulin (Fig. 4d). In type 2 diabetes patients, inhibition by insulin was lost, resulting in higher levels of collagen III-induced TF activity (96 ± 12%, n=8) compared to matched controls (59 ± 12%, n=8; $p<0.05$ with Mann Whitney U test; Fig 4e).

Discussion

Novel findings in the present study are (i) the identification of the extra- and intracellular mechanisms that couple surface activation to initiation of TF synthesis in platelets, (ii) the inhibition of TF synthesis by insulin in normal platelets and, (iii) the loss of insulin inhibition in platelets from patients with type 2 diabetes resulting in a 1.6-fold higher TF synthesis. The increased TF synthesis might be a cause of the hypercoagulant state observed in type 2 diabetes patients and contribute to the increase in thrombotic risk.

The critique that TF synthesis by platelets is caused by contaminating leukocytes appears unjust. The CD14 mRNA-free platelet suspensions showed no TF pre-mRNA splicing upon stimulation with LPS, which is a potent inducer of TF synthesis in monocytes [14]. Apparently, splicing of TF pre-mRNA is not under control of the platelet LPS receptor which contrasts with other studies that show LPS-induced splicing of IL-1β and COX-2 pre-mRNA in platelets [24].

Platelets adhering to surface-coated adhesive proteins start splicing of TF pre-mRNA, synthesis of TF protein and induce the capacity to initiate coagulation, confirming earlier observations [15,22]. The anucleate platelet has long been considered incapable of synthesizing proteins. Intron removal was thought to be confined to the nucleated megakaryocyte and platelet proteins to be the exclusive result of transcription and translation in this progenitor cell [25]. Unexpectedly, the platelet cytoplasm contains splicing factor 2 /alternative splicing factor (SF2/

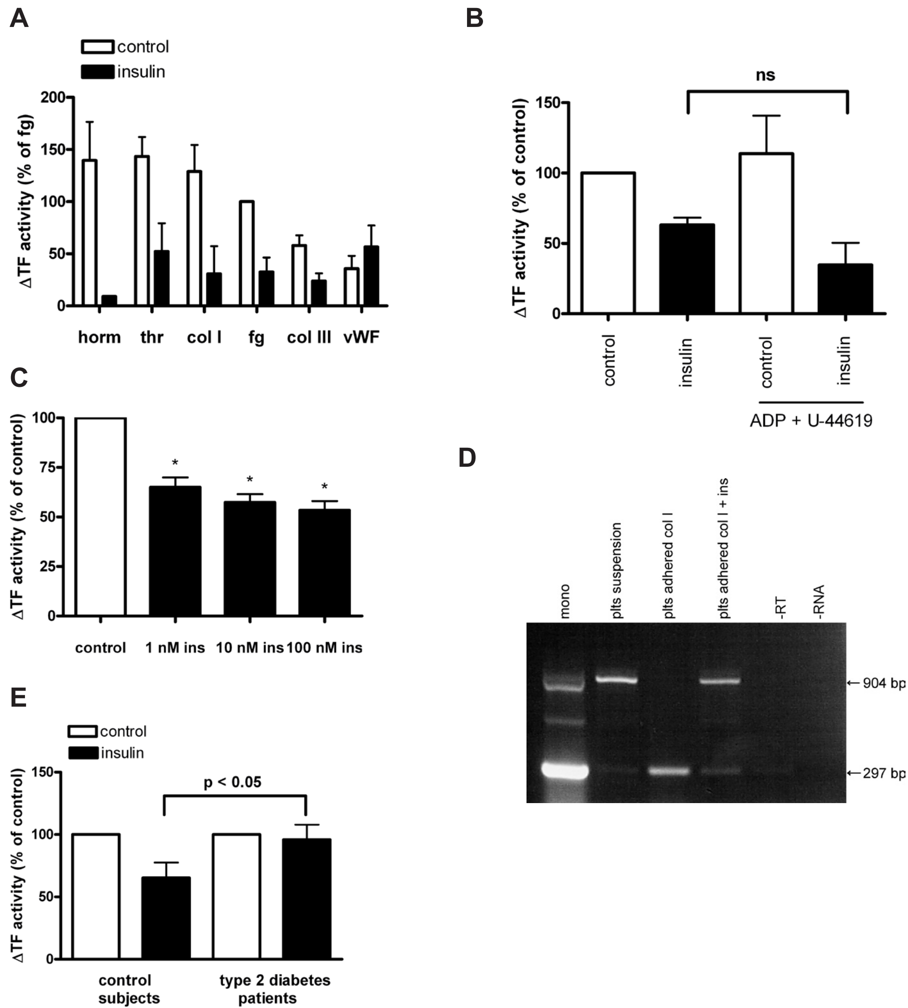


Figure 4. Insulin inhibits synthesis of platelet TF

(a) Platelets were pre-incubated with 100 nM insulin (5 min, 37°C) and adhered to surface- coated horm collagen, thrombin, collagen I, fibrinogen, collagen III and VWF (4 hr, 37°C) and TF activity was measured. (b) To the platelet suspension excess ADP and U-46619 were added and followed by pre-incubation with insulin (5 min, 37°C) before platelets were adhered to collagen III (4 hr, 37°C) and TF activity was determined. (c) Dose-dependent inhibition by insulin (5 min, 37°C) of TF synthesis by collagen III-adhered platelets. (d) Splicing was demonstrated by the presence of either pre-mRNA or mature mRNA in platelets that were untreated or pre-incubated with insulin and allowed to adhere to collagen I (30 min, 37°C). A representative example of n=4 is shown. (e) Platelets from 8 type 2 diabetes patients and 8 matched controls were pre-incubated with insulin, allowed to adhere to collagen III (4 hr, 37 °C) and TF activity was measured. TF activity in adhered platelets without insulin from controls and type 2 diabetes patients were not significant different. (a), (b), (c) and (e) TF activity in fibrinogen or collagen III-adhered platelets was set at 100%. Data are means ± SEM; n=3-8.

Table 1. Characteristics of the study population

	Control group (n= 8)	Type 2 diabetes patients (n= 8)	P
Age (years)	52 ± 3	58 ± 3	0.2
BMI (kg/m^2)	25 ± 0.9	28 ± 2	0.2
Systolic blood pressure (mmHg)	140 ± 7	131 ± 3	0.2
Diastolic blood pressure (mmHg	86 ± 4	80 ± 4	0.3
HbA1c (%)	5.5 ± 0.0	7.3 ± 0.5	0.002
Cholesterol (mmol/l)	6.1 ± 0.	4 ± 0.3	0.001
Triglycerides (mmol/l)	1.7 ± 0.3	1.9 ± 0.4	0.6
HDL-cholesterol	1.3 ± 0.07	1.1 ± 0.11	0.2
Creatinine (mmol/l)	95 ± 6.4	104 ± 6	0.3
Management with insulin	-	8	-
Duration of diabetes (years)	-	16 ± 2	-

Data are expressed as mean ± SEM

ASF), a regulator of constitutive and alternative splicing [26] together with a number of pre-mRNAs with message for IL-1β, B-cell lymphoma 3, plasminogen activator inhibitor-1, cyclo-oxygenase-2 (COX-2) and TF [27-29]. Platelets stimulated with soluble agonists or adhering to fibrinogen, start formation of mature RNAs and translation into functional proteins. Splicing of TF pre-mRNA results in formation of TF protein and the capacity to start coagulation and is under control of dual specificity Cdc2-like kinase (Clk)1, which contains an N-terminal region enriched in serine/arginine dipeptides (SR) that interacts with SF2/ASF [30]. Inhibition of splicing by Tg003 and of protein synthesis by puromycin confirm that platelets are capable of transcription/translation-dependent TF synthesis [15]. However, the inhibition was incomplete, suggesting that platelets contain a small store of encrypted TF that becomes active when the cells adhere to an adhesive surface.

Splicing of TF pre-mRNA was 600-fold more efficient in adhering platelets than in platelet suspensions, suggesting that close contact between platelets accelerates this process. In adhered platelets, the P2Y12-receptor blocker AR-C699331 MX inhibited TF synthesis by 56% and the COX-1 blocker indomethacin by 82%, illustrating that without inhibitors, TF synthesis is strongly enhanced by ADP secretion and TxA_2 formation and subsequent activation of the P2Y12 receptor (ADP) and TPα/β receptors (TxA_2). The combination of the blockers reduced TF synthesis almost by 100%. This observation might indicate that *in vivo* signaling to TF pre-

mRNA splicing is inhibited by the active clopidogrel metabolite as well as aspirin and that the combination abolishes the major part of platelet TF production. Apparently, there is little TF synthesis in the absence of feed back activation by released ADP and TxA_2, indicating that surface receptors that trigger TF pre-mRNA splicing predominantly do so by inducing these positive feed back pathways. Collagen I, horm collagen and thrombin had the strongest capacity to induce splicing of TF pre-mRNA. Fibrinogen and collagen III induced less splicing and VWF was the weakest activator. These differences might reflect the contribution of ADP/ TxA_2 release in platelet activation by these agonists, which is strong in horm collagen [31] and thrombin [32] activation, weak in activation by surface-coated fibrinogen [33] and virtually absent in platelet activation by coated VWF [34].
Iloprost, BAPTA-AM and inhibitors of PI3-K / PKB inhibited surface-induced TF synthesis, indicating that negative control through cAMP-dependent mechanisms and positive control through the PI3-K/PKB route determine the rate of TF synthesis. In addition to a direct effect of these inhibitors on the release of ADP/TxA_2, inhibition of TF activity also takes place after the released products start further signaling through the P2Y12- and TxA_2 receptors. PKAα and PKAβ are activated by an increase in cAMP concentration induced by forskolin or 8-CPT-cAMP and phosphorylate SR proteins *in vitro* and change splice site selection *in vivo* [35]. PKB contributes to phosphorylation of SR proteins, specifically SF2 [36], which potentiate their binding to recently transcribed mRNA and/or directly stimulate SR protein activities. Interestingly, in monocytes TF expression is under negative control by the PKB pathway [14,37]. Here PKB inhibits NF-κB activity [37], such in contrast to anucleated platelets where PKB regulates the splicing mechanism.
The induction of ADP/TxA_2 release followed by signaling from ADP/TxA_2 receptors to the spliceosome is under control of insulin. In normal platelets, insulin interferes with the P2Y12 pathway by inducing the association of IRS-1 with Giα–2, thereby blocking Gi-mediated cAMP suppression, which attenuates platelet functions. In addition, insulin stimulates the PI3-K – PKB pathway, initiating glucose uptake through upregulation of GLUT3 transporters [7,11]. Interference with cAMP suppression by insulin impairs agonist-induced Ca^{2+} rises, aggregation, secretion and the generation of a pro-coagulant surface and the interference with TF synthesis presented here adds to the list of platelet functions under negative control by insulin.
Platelet TF does not seem to play a role in acute injury, since it takes long to express active TF. An animal model of laser induced vessel wall injury [12] shows the first appearance of TF at about 100 sec after induction of vessel wall damage,

which is well before the first sign of platelet TF synthesis. Diabetes increases the risk of developing lower extremity arterial disease, which is one of the first signs of a generalized atherosclerotic disease [38]. A key event in the pathogenesis of diabetes type 2 is loss of atherosclerotic plaque stability. When the fibrous cap is disrupted, a platelet plug forms, which continuously increases in size until the lumen is completely obstructed. In the chronic process of arterial occlusive disease, the slow but consistent production of TF by platelets may well contribute to the propagation and stabilization of a thrombus. Indeed, previous studies have detected TF antigen and activity in the atherosclerotic plaque [39]. The elevated TF synthesis in platelets form type 2 diabetes patients might result in a more stable clot inducing occlusion of an artery at sites where atherosclerotic lesions have developed.

Acknowledgements

This work was supported by the Dutch Diabetes Research Foundation (no. 2004.00.029) and the Netherlands Thrombosis Foundation (2006-1). The authors acknowledge the cooperation of the patients and control subjects.

References

1. Grundy SM, Howard B, Smith S Jr, Eckel R, Redberg R, Bonow RO. Prevention Conference VI: Diabetes and Cardiovascular Disease: executive summary: conference proceeding for healthcare professionals from a special writing group of the American Heart Association. Circulation 2002; 105:2231-2239.
2. Collier A, Rumley A, Rumley AG, Paterson JR, Leach JP, Lowe GD, Small M. Free radical activity and hemostatic factors in NIDDM patients with and without microalbuminuria. Diabetes 1992; 41:909-913.
3. McGill JB, Schneider DJ, Arfken CL, Lucore CL, Sobel BE. Factors responsible for impaired fibrinolysis in obese subjects and NIDDM patients. Diabetes 1994; 43:104-109.
4. Carr ME. Diabetes mellitus: a hypercoagulable state. J Diabetes Complications 2001; 15:44-54.
5. Lim HS, Blann AD, Lip GY. Soluble CD40 ligand, soluble P-selectin, interleukin-6, and tissue factor in diabetes mellitus: relationships to cardiovascular dis-

ease and risk factor intervention. Circulation 2004; 109:2524-2528.
6. Sambola A, Osende J, Hathcock J, Degen M, Nemerson Y, Fuster V, Crandall J, Badimon JJ. Role of risk factors in the modulation of tissue factor activity and blood thrombogenicity. Circulation 2003; 107:973-977.
7. Ferreira IA, Mocking AI, Feijge MA, Gorter G, van Haeften TW, Heemskerk JW, Akkerman JW. Platelet inhibition by insulin is absent in type 2 diabetes mellitus. Arterioscler Thromb Vasc Biol 2006; 26:417-422.
8. Angiolillo DJ, Bernardo E, Ramirez C, Costa MA, Sabate M, Jimenez-Quevedo P, Hernandez R, Moreno R, Escaned J, Alfonso F, Banuelos C, Bass TA, Macaya C, Fernandez-Ortiz A. Insulin therapy is associated with platelet dysfunction in patients with type 2 diabetes mellitus on dual oral antiplatelet treatment. J Am Coll Cardiol 2006; 48:298-304.
9. Ferreira IA, Eybrechts KL, Mocking AI, Kroner C, Akkerman JW. IRS-1 mediates inhibition of Ca2+ mobilization by insulin via the inhibitory G-protein Gi. J Biol Chem 2004; 279:3254-3264.
10. Kim S, Jin J, Kunapuli SP. Akt activation in platelets depends on Gi signaling pathways. J Biol Chem 2004; 279:4186-4195.
11. Ferreira IA, Mocking AI, Urbanus RT, Varlack S, Wnuk M, Akkerman JW. Glucose uptake via glucose transporter 3 by human platelets is regulated by protein kinase B. J Biol Chem 2005; 280:32625-32633.
12. Gross PL, Furie BC, Merrill-Skoloff G, Chou J, Furie B. Leukocyte-versus microparticle-mediated tissue factor transfer during arteriolar thrombus development. J Leukoc Biol 2005; 78:1318-1326.
13. Furie B, Furie BC. Thrombus formation in vivo. J Clin Invest 2005; 115:3355-3362.
14. Gerrits AJ, Koekman CA, Yildirim C, Nieuwland R, Akkerman JW. Insulin inhibits tissue factor expression in monocytes. J Thromb Haemost 2009; 7:198-205.
15. Schwertz H, Tolley ND, Foulks JM, Denis MM, Risenmay BW, Buerke M, Tilley RE, Rondina MT, Harris EM, Kraiss LW, Mackman N, Zimmerman GA, Weyrich AS. Signal-dependent splicing of tissue factor pre-mRNA modulates the thrombogenicity of human platelets. J Exp Med 2006; 203:2433-2440.
16. Sodetz JM, Pizzo SV, McKee PA. Relationship of sialic acid to function and in vivo survival of human factor VIII/von Willebrand factor protein. J Biol Chem 1997; 252:5538-5546.
17. Hackeng TM, Hessing M, van 't Veer C, Meijer-Huizinga F, Meijers JC, de Groot PG, van Mourik JA, Bouma BN. Protein S binding to human endothelial cells is

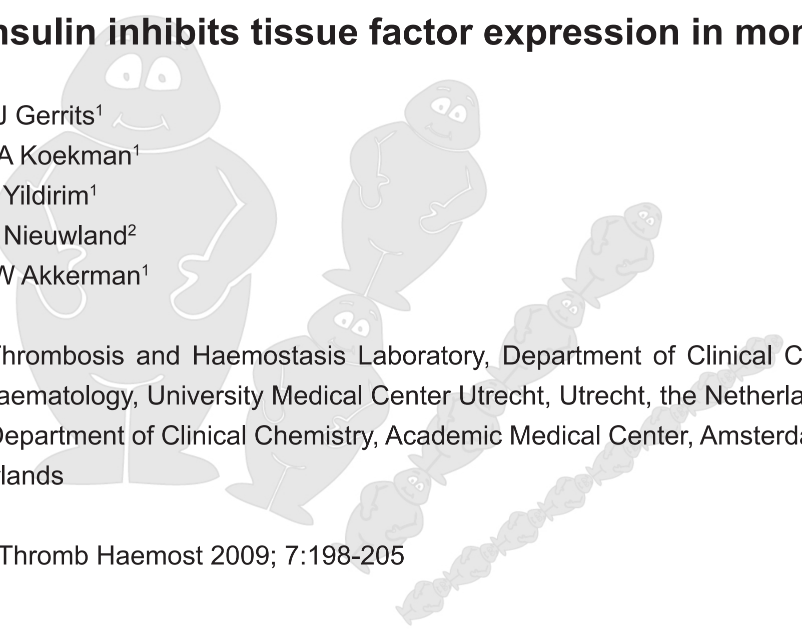
required for expression of cofactor activity for activated protein C. J Biol Chem 1993; 268:3993-4000.

18. Onley DJ, Knight CG, Tuckwell DS, Barnes MJ, Farndale RW. Micromolar Ca2+ concentrations are essential for Mg2+-dependent binding of collagen by the integrin alpha 2beta 1 in human platelets. J Biol Chem 2000; 275:24560-24564.
19. Pillitteri D, Bassus S, Boller K, Mahnel R, Scholz T, Westrup D, Wegert W, Kirchmaier CM. Thrombin-induced interleukin 1beta synthesis in platelet suspensions: impact of contaminating leukocytes. Platelets 2007; 18:119-127.
20. Aye MT, Palmer DS, Giulivi A, Hashemi S. Effect of filtration of platelet concentrates on the accumulation of cytokines and platelet release factors during storage. Transfusion 1995; 35:117-124.
21. Stack G, Snyder EL. Cytokine generation in stored platelet concentrates. Transfusion 1994; 34:20-25.
22. Panes O, Matus V, Saez CG, Quiroga T, Pereira J, Mezzano D. Human platelets synthesize and express functional tissue factor. Blood 2007; 109:5242-5250.
23. Santoro SA, Cunningham LW. Collagen-mediated platelet aggregation. Evidence for multivalent interactions of intermediate specificity between collagen and platelets. J Clin Invest 1977; 60:1054-1060.
24. Shashkin PN, Brown GT, Ghosh A, Marathe GK, McIntyre TM. Lipopolysaccharide is a direct agonist for platelet RNA splicing. J Immunol 2008; 181:3495-3502.
25. Maniatis T, Reed R. An extensive network of coupling among gene expression machines. Nature 2002; 416:499-506.
26. Denis MM, Tolley ND, Bunting M, Schwertz H, Jiang H, Lindemann S, Yost CC, Rubner FJ, Albertine KH, Swoboda KJ, Fratto CM, Tolley E, Kraiss LW, McIntyre TM, Zimmerman GA, Weyrich AS. Escaping the nuclear confines: signal-dependent pre-mRNA splicing in anucleate platelets. Cell 2005; 122:379-391.
27. Lindemann S, Tolley ND, Dixon DA, McIntyre TM, Prescott SM, Zimmerman GA, Weyrich AS. Activated platelets mediate inflammatory signaling by regulated interleukin 1beta synthesis. J Cell Biol 2001; 154:485-490.
28. Weyrich AS, Denis MM, Schwertz H, Tolley ND, Foulks J, Spencer E, Kraiss LW, Albertine KH, McIntyre TM, Zimmerman GA. mTOR-dependent synthesis of Bcl-3 controls the retraction of fibrin clots by activated human platelets. Blood 2007; 109:1975-1983.
29. Brogren H, Karlsson L, Andersson M, Wang L, Erlinge D, Jern S. Platelets syn-

thesize large amounts of active plasminogen activator inhibitor 1. Blood 2004; 104:3943-3948.
30. Colwill K, Pawson T, Andrews B, Prasad J, Manley JL, Bell JC, Duncan PI. The Clk/Sty protein kinase phosphorylates SR splicing factors and regulates their intranuclear distribution. EMBO J 1996; 15:265-275.
31. Cho MJ, Liu J, Pestina TI, Steward SA, Thomas DW, Coffman TM, Wang D, Jackson CW, Gartner TK. The roles of alpha IIb beta 3-mediated outside-in signal transduction, thromboxane A2, and adenosine diphosphate in collagen-induced platelet aggregation. Blood 2003; 101:2646-2651.
32. Cho MJ, Pestina TI, Steward SA, Lowell CA, Jackson CW, Gartner TK. Role of the Src family kinase Lyn in TxA2 production, adenosine diphosphate secretion, Akt phosphorylation, and irreversible aggregation in platelets stimulated with gamma-thrombin. Blood 2002; 99:2442-2447.
33. Soriani A, Moran B, de Virgilio M, Kawakami T, Altman A, Lowell C, Eto K, Shattil SJ. A role for PKCtheta in outside-in alpha(IIb)beta3 signaling. J Thromb Haemost 2006; 4:648-655.
34. van Lier M, Verhoef S, Cauwenberghs S, Heemskerk JW, Akkerman JW, Heijnen HF. Role of membrane cholesterol in platelet calcium signalling in response to VWF and collagen under stasis and flow. Thromb Haemost 2008; 99:1068-1078.
35. Kvissel AK, Orstavik S, Eikvar S, Brede G, Jahnsen T, Collas P, Akusjarvi G, Skalhegg BS. Involvement of the catalytic subunit of protein kinase A and of HA95 in pre-mRNA splicing. Exp Cell Res 2007; 313:2795-2809.
36. Blaustein M, Pelisch F, Tanos T, Munoz MJ, Wengier D, Quadrana L, Sanford JR, Muschietti JP, Kornblihtt AR, Caceres JF, Coso OA, Srebrow A. Concerted regulation of nuclear and cytoplasmic activities of SR proteins by AKT. Nat Struct Mol Biol 2005; 12:1037-1044.
37. Guha M, Mackman N. The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. J Biol Chem 2002; 277:32124-32132.
38. American Diabetes Association. Peripheral arterial disease in people with diabetes. Diabetes Care 2003; 26:3333-3341.
39. Marmur JD, Thiruvikraman SV, Fyfe BS, Guha A, Sharma SK, Ambrose JA, Fallon JT, Nemerson Y, Taubman MB. Identification of active tissue factor in human coronary atheroma. Circulation 1996; 94:1226-1232.

Chapter 4

Insulin inhibits tissue factor expression in monocytes



AJ Gerrits[1]
CA Koekman[1]
C Yildirim[1]
R Nieuwland[2]
JW Akkerman[1]

[1]Thrombosis and Haemostasis Laboratory, Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht, the Netherlands
[2]Department of Clinical Chemistry, Academic Medical Center, Amsterdam, the Netherlands

J Thromb Haemost 2009; 7:198-205

Abstract

Objective Platelets from healthy subjects are inhibited by insulin but type 2 diabetes mellitus (T2DM) platelets have become insulin-resistant which might explain their hyperactivity. In the present study we investigated whether monocytes are responsive to insulin.

Research Design and Methods LPS-induced Tissue Factor (TF) upregulation was measured in human monocytes and monocytic THP-1 cells in a factor Xa generation assay.

Results Insulin (0.1 – 100 nM) induced a dose-dependent inhibition in both cell types and in monocytes 100 nM insulin inhibited cytosolic, membrane-bound and microparticle TF by 32 ± 2, 27 ± 3 and 52 ± 4% (n=3). Insulin induced Tyr phosphorylation of the insulin receptor (INS-R) and formation of an INS-R – Giα–2 complex, suggesting interference with LPS-induced cAMP control. Indeed, insulin interfered with LPS-induced cAMP decrease and TF upregulation in a manner similar to an inhibitor of Gi (pertussis toxin) and agents that raise cAMP (iloprost, forskolin, IBMX) reduced TF upregulation. Although LPS failed to raise cytosolic Ca^{2+}, quenching of Ca^{2+} increases (BAPTA-AM) reduced and induction of Ca^{2+} entry (ionophore, P2X7 activation) enhanced upregulation of TF mRNA and procoagulant activity. Insulin interfered with MCP-1 induced Ca^{2+} mobilization but not with ATP induced Ca^{2+} rises. *Conclusion* Insulin inhibits TF expression in monocytes and monocyte-derived microparticles through interference with Giα–2 mediated cAMP suppression which attenuates Ca^{2+} -mediated TF synthesis.

Introduction

Patients with diabetes mellitus (DM) have a 2-8 fold higher risk of cardiovascular morbidity and mortality compared to matched controls [1]. Patients with type 2 DM (T2DM) show impaired insulin responsiveness or even complete insulin resistance and suffer from atherothrombotic complications and disturbed regulation of haemostasis with endothelial activation, hyperactive platelets, hypercoagulability and hypofibrinolysis [2,3]. The hypercoagulability in T2DM patients is accompanied by increased levels of plasma fibrinogen, factors VII, VIII, XI and XII, kallikrein and possibly von Willebrand Factor [4]. Also levels of prothrombin fragment 1 + 2 and thrombin-anti-thrombin complexes are elevated reflecting activation of the

coagulation cascade. Of specific interest are the elevated levels of Tissue Factor (TF)[5,6] explaining the hypercoagulability and the cardiovascular complications observed in DM.

Under physiological conditions, monocytes express little TF. Upon contact with lipopolysaccharide (LPS), C-reactive protein, CD40 ligand, oxidized LDL and angiotensin II, TF expression increases in the cytosolic compartment and TF translocates to the plasma membrane where it becomes a source for TF-rich microparticles. These particles are vehicles for so called blood-born TF that in animal models for thrombosis initiates fibrin formation at the luminal site of the growing thrombus [7]. LPS binds and activates the Toll-like receptor 4 (TLR4). TLR4 signals to the IκB-kinase pathway and three mitogen-activated protein kinase (MAPK) pathways leading to activation of transcription factors Egr-1, AP-1 and NF-κB [8]. In addition to pathways that promote TF synthesis, LPS initiates pathways that attenuate its production. Activation of the phosphatidyl inositol 3-kinase (PI3-K) – protein kinase B (PKB) pathway interferes with MAPK and transcription factors AP-1 and Egr-1 and the nuclear translocation of the NF-κB [9,10]. A second inhibitory route is formation of cAMP. The cAMP raising agents dibutyryl-cAMP, forskolin and 3-isobutyl-1-methylxanthine (IBMX) inhibit formation of TF mRNA, surface expression of TF protein and procoagulant activity due to interference with NF-κB and gene transcription [11,12].

Platelet functions are strongly enhanced by secreted ADP that binds to the P2Y12 receptor which is coupled to the inhibitory G-protein of adenylyl cyclase, Gi. Activation of Gi initiates PI3-K-mediated activation PKBα,β (Akt1,2) contributing to platelet activation and inhibits adenylyl cyclase through Gi α which attenuates formation of cAMP, which is a potent platelet inhibitor [13-15]. In normal platelets insulin interferes with the P2Y12 pathway by signaling through its receptor (INS-R) and insulin receptor substrate-1 (IRS-1), inducing association of IRS-1 with the Giα–2 subunit. Tyr-phosphorylation of Giα-2 blocks Gi-mediated cAMP suppression and attenuates platelet functions. Platelets from T2DM patients have lost sensitivity to insulin and better respond to inducers of aggregation, release of secretion granules and generation of a pro-coagulant surface [16-18].

In the present study we investigated whether insulin interferes with TF expression in monocytes and monocytic THP-1 cells. We show that insulin concentrations in the physiological range interfere with cAMP suppression and TF formation in cells and monocytes-derived microparticles, making insulin resistance seen in diabetics a possible cause for the hyperactive monocytes seen in this disease.

Methods

Materials - We obtained human recombinant insulin, Fura-2/AM, IBMX, ATP, wortmannin, protease inhibitor cocktail, sodium vanadate ($NaVO_3$) and LPS (E. coli 0111:B4) from Sigma (St. Louis, MO, USA), prostacyclin from Schering AG (Berlin, Germany), H89 from Alexis Biochemicals (Lausen, Switzerland), protein G-Sepharose from Amersham (Uppsala, Sweden), forskolin, pertussis toxin from Bordetella pertussis, ionophore A23187, staurosporin and BAPTA(AM) from Calbiochem (La Jolla, CA, USA), LY294002 from Biomol (Plymouth Meeting, PA, USA) and MCP-1 from R&D Systems (Minneapolis, MN, USA). Factor X (FX) was purified from fresh-frozen plasma as described previously [19]. Recombinant Factor VIIa (rFVIIa) was from Novo Nordisk (Bagsværd, Denmark) and Innovin from Dade Behring (Liederbach, Germany). All other chemicals used were of analytical grade. Antibodies against the INS-R, INS-R phospho-$Tyr^{1150/1151}$, IRS-1 and horseradish peroxidase-labeled anti-rabbit antibody were from Cell Signaling Technology (Danvers, MA, USA), polyclonal antibodies against Giα–2, Gsα, Gqα, Gzα (T-19), PKBα,β total and PKBα phospho-Ser^{473} from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-phospho-Tyr monoclonal antibody 4G10 from Upstate Biotechnology (Bucks, UK), peroxidase-linked goat anti-mouse antibody from DAKO (Glostrup, Denmark), anti-annexin V-APC conjugated antibody from CALTAG laboratories (Burlingame, CA, USA) and anti-CD11b-PE conjugated antibody from Becton, Dickinson and Company (Franklin Lakes, NJ, USA).

Monocytes, monocytic THP-1 cells and microparticles - Freshly drawn venous blood from healthy, medication-free volunteers was collected with informed consent into 0.1 volume of 130 mM trisodium citrate according to procedures approved by the local Medical Ethical Review Board. Peripheral mononuclear cells were isolated by density-gradient centrifugation on Ficoll-Paque columns and monocytes were recovered by magnetic separation with anti-CD14 antibody coupled beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were resuspended in RPMI 1640 culture medium. For analyses that required large cell numbers, monocytic THP-1 (ATCC, Teddington, UK) were grown in RPMI 1640 culture medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5% heat-inactivated fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 50 μM β-mercaptoethanol. For isolation of microparticles, monocytes were stimulated with 1 μg/mL LPS for 5 h (37°C), centrifuged twice (350 g, 5 min, 22°), and the supernatant was collected. After a second centrifugation (17.000 g, 30 min,

4°C), the pellet was collected and dissolved in 100 μL tris-buffered saline (TBS). For quantification of microparticle numbers, 5 μL freshly prepared microparticle suspension was labelled with annexin V-APC or with CD11b-PE (15 min, 22°C, in darkness). Labelled particles were counted in a FACS Calibur (Becton, Dickinson and Company, Franklin Lakes, NY, USA) qualibrated for particles <1.5 μm. Supernatant of monocytes treated with 2 μM staurosporin (5 h, 37°C) served as positive control.

TF expression - For analysis of TF procoagulant activity, monocytes and THP-1 cells (1 × 10^6 cells) were stimulated for 5 h (37°C) with 1 μg/mL LPS, a condition used in all experiments. Suspensions were centrifuged (350 g, 5 min, 22°C) and the pellet dissolved in 100 μL TBS. Lysates were prepared by 4 times freeze/thawing and centrifuged (16100 g, 5 min, 22°C). Supernatant (cytosol fraction) and pellet (membrane fraction) were collected and incubated with 10 μg/mL FX, 5 U/mL rFVIIa and 5 mM $CaCl_2$. FXa generation was measured in a fluorescence reader at 405 nm after addition of FXa substrate (Pentapharm, Basel, Switzerland). Procoagulant activity was expressed as ng/10^7 cells based a standard curve derived from serial dilutions of recombinant TF (Innovin). The cytosol fraction was adjusted for variantions in protein content using the BCA assay.
For analysis of TF mRNA, 1 mL THP-1 suspension (5 × 10^6 cells/mL) was cultured in 6-wells plates. Total RNA was isolated using the kit from Macherey-Nagel (Düren, Germany). cDNA was synthesized by reverse transcription on the PCR machine (PTC-200, MJ Research, Waltham, MA, USA). Quantitative real-time PCR was performed using TF primers 5'-CTC CCC AGA GTT CAC ACC TTA CC-3' and 5'-CCG TTC ATC TTC TAC GGT CAC A-3' and the fluorescent TF probe 5'-AGA CAA ACC TCG GAC AGC CAA CAA TTC A-3'. PBGD mRNA, which is constitutively expressed in cells, was measured for calibration.

IL-1β measurements - Monocytes (1 × 10^6 cells) were stimulated with LPS (5 h, 37 °C) and centrifuged (350g, 5 min, 22°C). Supernatant and pellet were isolated, pellet was dissolved in 250 μL TBS, and Interleukin (IL)-1β was determined by ELISA (PeliKine Compact, Sanquin, Amsterdam, the Netherlands).

Insulin signaling - Aliquots of 1 × 10^6 THP-1 cells or monocytes were incubated with insulin (37°C), collected in lysis buffer and analyzed for Tyr phosphorylation of INS-R, IRS-1, Gα-proteins, associations between these proteins and Ser phosphorylation of PKBα using appropriate antibodies, as previously described [16].

Band intensities were semi-quantified with ImageJ software.

cAMP and cytosolic Ca^{2+} - Monocytes (1×10^6 cells) were incubated with insulin as indicated and stimulated with LPS for 15 min (37°C). Samples were acetylated according to manufacturer instructions and cAMP levels were measured using the cAMP-EIA kit (Cayman Chemical, Ann Arbor, MI, USA). THP-1 cells (10×10^6 cells) were loaded with Fura-2/AM in the dark (1 h, 37°C). Fluorescence was measured and changes in cytosolic Ca^{2+} content, $[Ca^{2+}]_i$, were analyzed as described [16].

Statistics - Data are expressed as means ± S.E.M. with number of observations n and were analyzed with the Student's test for unpaired observations. Differences were considered significant at $p < 0.05$.

Results

Insulin inhibits LPS-induced TF procoagulant activity in monocytes, THP-1 cells and monocyte-derived microparticles

In initial experiments, conditions were sought that optimally revealed interference with TF expression by insulin. Simultaneous addition of insulin and LPS showed no effect but a 15 min pre-incubation time induced maximal inhibition by insulin. In the absence of LPS, insulin failed to change the basal TF procoagulant activity (data not shown). LPS-induced an increase of 84 ± 6% in cytosolic-TF and of 67 ± 2 % in membrane-TF, which is equivalent to 42 ± 10 to 226 ± 39 and from 354 ± 30 to 1075 ± 139 ng/10^7 cells respectively (n=3). Insulin induced a dose-dependent inhibition, resulting in 32 ± 2% and 27 ± 3% (n=3) fall in these fractions at 100 nM insulin (Fig. 1A). THP-1 cells showed a similar sensitivity to insulin with a 40 ± 9% fall in cytosolic TF at 100 nM insulin (Fig.1B). LPS raised TF pro-coagulant activity in the microparticles from from 97 ± 26 to 311 ± 55 ng/10^7 monocytes which was inhibited by 31 ± 8 and 52 ± 4% (n=3) at 0.1 nM and 100 nM insulin respectively (Fig. 1C). Control studies confirmed that insulin treatment left the number of annexin V-positive (Fig. 1D) and CD11b-positive (data not shown) particles unchanged whereas staurosporin increased the number of microparticles with 40 ± 25% compared to untreated cells. Thus insulin decreased the TF pro-coagulant activity of microparticles derived from LPS-treated monocytes. To investigate whether inhibition by insulin was restricted to TF expression, the LPS-induced

surface appearance and secretion of IL-1β were measured. Both responses were inhibited by insulin (Fig.1E). Thus, a physiological relevant concentration of 1 nM insulin or more inhibits the generation of TF-procoagulant activity as well as IL-1β secretion suggesting that insulin triggers a general reduction in the responsiveness of monocytes to stimulation by LPS.

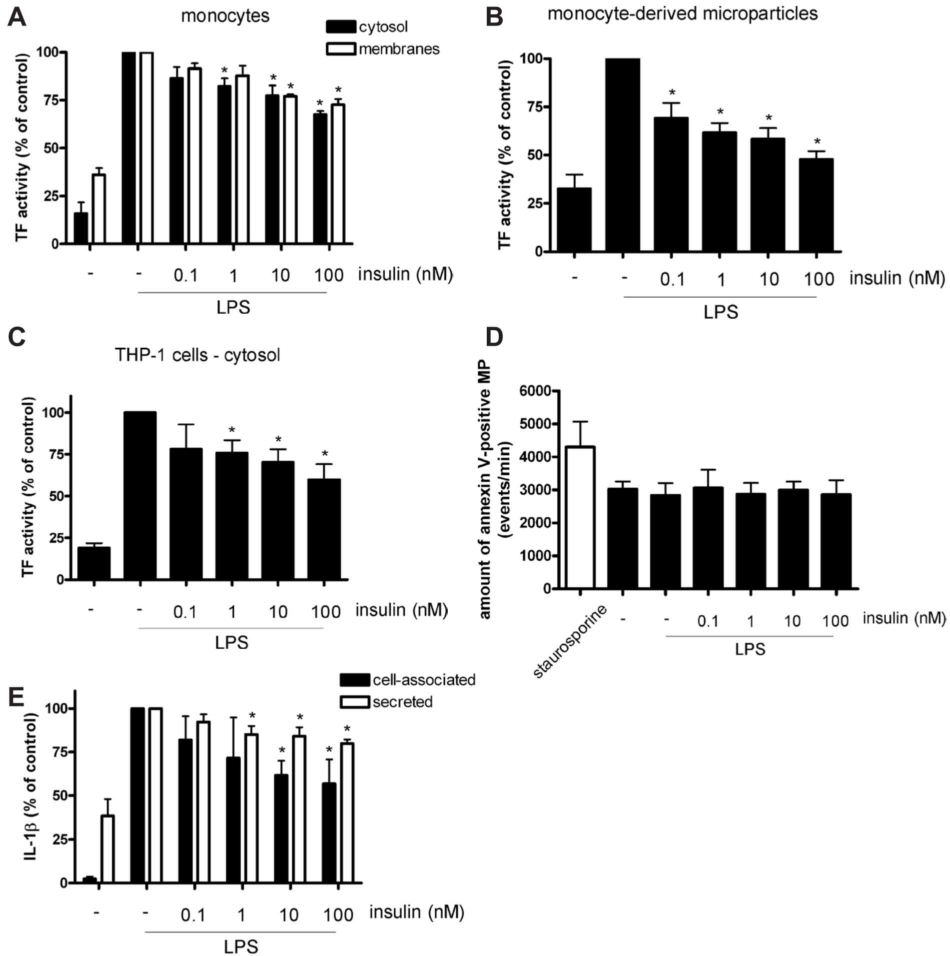
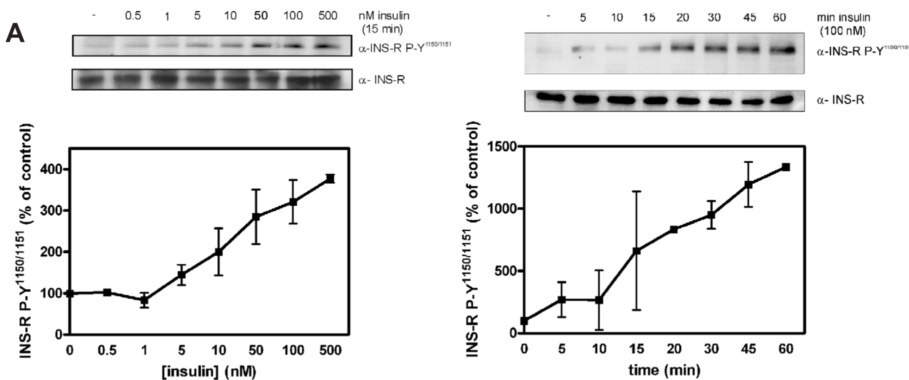


Figure 1. Insulin inhibits LPS-induced upregulation of TF

Human monocytes and THP-1 were treated with 1 μg/mL LPS without and with pre-incubation with insulin (15 min, 37°C). TF-procoagulant activity was measured in monocytes (A), THP-1 cells (B) and monocytes-derived microparticles (C). The number of microparticles was measured as annexin V-positive events using 2 μM staurosporine (5 h, 37°C) treatment as positive control (D). IL-1β surface expression and secretion were measured in monocytes (E). In the absence of LPS, insulin neither changed TF levels in monocytes (354 ± 30 ng/10^7 cells), THP-1 cells (82 ± 24 ng/10^7 cells) and monocytes-derived microparticles (97 ± 26 ng/10^7 monocytes) nor cell-associated and secreted IL-1β levels (583 ± 119 and 7626 ± 562 pg/10^7 cells). Data are percentages of controls (means ± SEM, n=4, * p <0.05 versus controls).

Insulin signaling in THP-1 cells and monocytes

Having established that insulin reduced LPS-induced TF upregulation, we characterized the signaling pathways involved. In platelets, insulin affects INS-R, IRS-1 and a specific isoform of the Gi family, Giα–2, through a series of Tyr phosphorylations and transient formation of an IRS-1 - Giα–2 complex. Similar studies in monocytic THP-1 cells revealed that insulin induced a dose- and time dependent phosphorylation of the INS-R (Fig. 2A) and IRS-1 (Fig. 2B). Phosphorylation of the receptor was detected after 5 min, persisted for one h and was maximal at 500 nM insulin. In contrast, IRS-1 phosphorylation was maximal after 15 min and returned to pre-stimulation values after 30 min. Thus, activation of INS-R and signal generation occurred under conditions where insulin interfered with TF synthesis. In platelets, phosphorylation of IRS-1 induces association with Giα–2 but attempts to demonstrate a similar complex in monocytes had no success. In a search for alternative means for Gi inactivation we found that the INS-R associates directly with Giα–2 in THP-1 cells (Fig. 2C). These findings suggest that insulin activates pathways in monocytes through direct receptor - Giα–2 contact. Immunoprecipitates of the activated INS-R failed to show co-association of Gsα, Gqα and Gzα (data not shown). These findings indicate that activation of INS-R triggers the exclusive association with Giα–2. Complex formation was maximal after 15 min stimulation and thereafter declined (Fig. 2C). Similar results for the transient binding between the INS-R and Giα–2 after insulin stimulation were seen in monocytes (Fig. 2D). This explains the 15 minutes pre-incubation time required for optimal inhibition of TF expression by insulin.



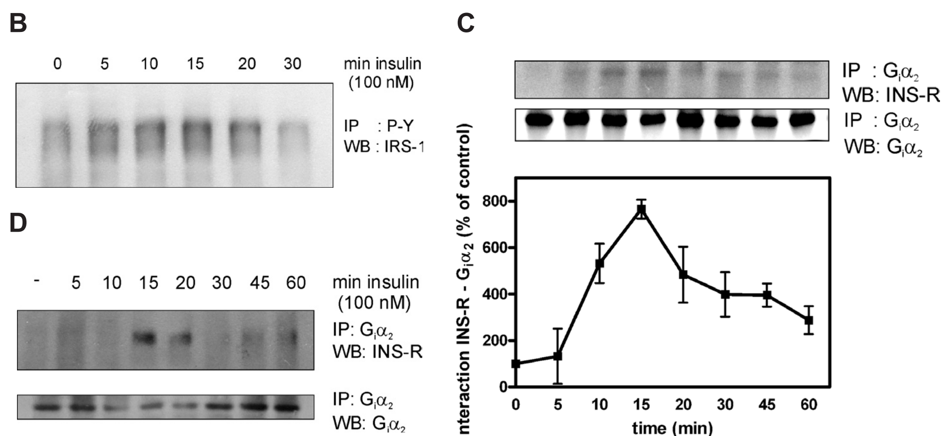


Figure 2. Insulin signaling to Gi-α2

THP-1 cells and monocytes were incubated with insulin at the indicated conditions (37°C). For THP-1 cells phosphorylated and total INS-R was measured on Western blots (A) and immunoprecipitates of Tyr-phosphorylated proteins analyzed on Western blots show Tyr-phosphorylation of IRS-1 (B, representative example of n=3). The insulin-induced association between INS-R and Giα–2 in THP-1 cells (C) and monocytes (D) in immunoprecipitates of Giα–2 are shown on Western blots. Band intensities were semi-quantified with ImageJ software and expressed as percentages of untreated cells (means ± SEM, n=3).

Control of cAMP by insulin

To confirm that interaction between INS-R and Giα–2 leads to inactivation of the G-protein, studies were repeated with pertussis toxin, a known inhibitor of the Gi family [20]. As observed with insulin, pertussis toxin inhibited LPS-induced upregulation of TF procoagulant activity leading to a 49 ± 6% decrease (Fig. 3A). The cAMP level in monocytes is 14 ± 0.7 pmol/10^7 cells and decreased further to 4 ± 0.6 pmol/10^7 cells upon stimulation with LPS ($p<0.01$, n=10). Thus, LPS activates Gi thereby inhibiting adenylyl cyclase and formation of cAMP. Pertussis toxin (Fig. 3A) and insulin (Fig. 3B) both interfered with the fall in cAMP induced by LPS, suggesting that insulin signaling to Gi inactivates the G-protein. To confirm that changes in cAMP directly interfered with TF synthesis, monocytes were treated with a stimulator of Gs (the prostacyclin analog iloprost), a stimulator of adenylyl cyclase (forskolin) and an inhibitor of phosphodiesterases (IBMX). All treatments inhibited LPS-induced TF synthesis and induced the expected increases in cAMP, confirming the role of cAMP as an inhibitor of TF upregulation (Fig. 3C). In platelets, cAMP exerts its inhibition through cAMP dependent protein kinase A. As expected, in the presence of the proten kinase A blocker H89, the effect of insulin

had disappeared (Fig. 3D)
Earlier studies showed that TF expression is under negative control by the PI3-K-PKB pathway [9,10]. Since also in monocytes insulin is an activator of PKB (insert Fig. 3D), it is feasible that inhibition of TF upregulation by insulin is caused by stimulation of the PKB pathway. To evaluate this possibility, monocytes were incubated with wortmannin and LY294002, both potent inhibitors of PI3-K. The inhibitors induced a 1.5 to 2.0 fold increase in TF upregulation, illustrating the strong suppression of TF upregulation through this pathway in untreated monocytes. In the presence of wortmannin and LY294002, insulin reduced TF expression by 27 ± 3% and 37 ± 7% (n=3) respectively, confirming an inhibitory role independent of the PI3-K–PKB pathway (Fig. 4D).

Ca^{2+} rises contribute to TF expression

In platelets, rises in cAMP levels attenuate aggregation and secretion through inhibition of Ca^{2+} mobilization and influx [16]. We investigated whether a similar mechanism controlled TF synthesis in THP-1 cells. Stimulation with LPS alone failed to change $[Ca^{2+}]_i$ in agreement with earlier observations (Fig. 4A, insert and [21]). The Ca^{2+} chelating agent BAPTA-AM abolished the rise in LPS-induced TF procoagulant activity almost completely, both in the cytosol and the membrane fraction (Fig. 4A). Incubation with BAPTA-AM alone did not change basal TF activity. AM-free BAPTA did not change TF expression, reflecting its incapability to cross membranes and demonstrating that these compounds left the Ca^{2+}-dependent factor Xa generating assay undisturbed (data not shown).
When $[Ca^{2+}]_i$ was raised by ionophore A23187 in the presence of extracellular Ca^{2+}, TF expression was 33.3 ± 0.03% of the rise induced by LPS, indicating that a rise in Ca^{2+} alone is a poor activator of TF expression. Together with LPS, this treatments raised TF expression by 240 ± 13%, illustrating strong stimulation by increases in $[Ca^{2+}]_i$ (Fig. 4B). ATP activates the P2X7 receptor and Ca^{2+} influx. In the absence of LPS, ATP had little effect, again illustrating the poor capacity of rises in $[Ca^{2+}]_i$ to induce TF expression. Together with LPS, ATP induced an increase of 68 ± 17% (Fig. 4B).
These results show that Ca^{2+} is required for LPS-induced upregulation of TF procoagulant activity and that increases in $[Ca^{2+}]_i$ strongly enhance this process. To investigate whether Ca^{2+} stimulated TF expression at the level of transcription or the post-transcriptional activation known as de-encryption [22], TF mRNA levels were measured. The slight rise in TF expression by ATP alone, the higher increase by LPS and especially the strong potentation by the combination of LPS

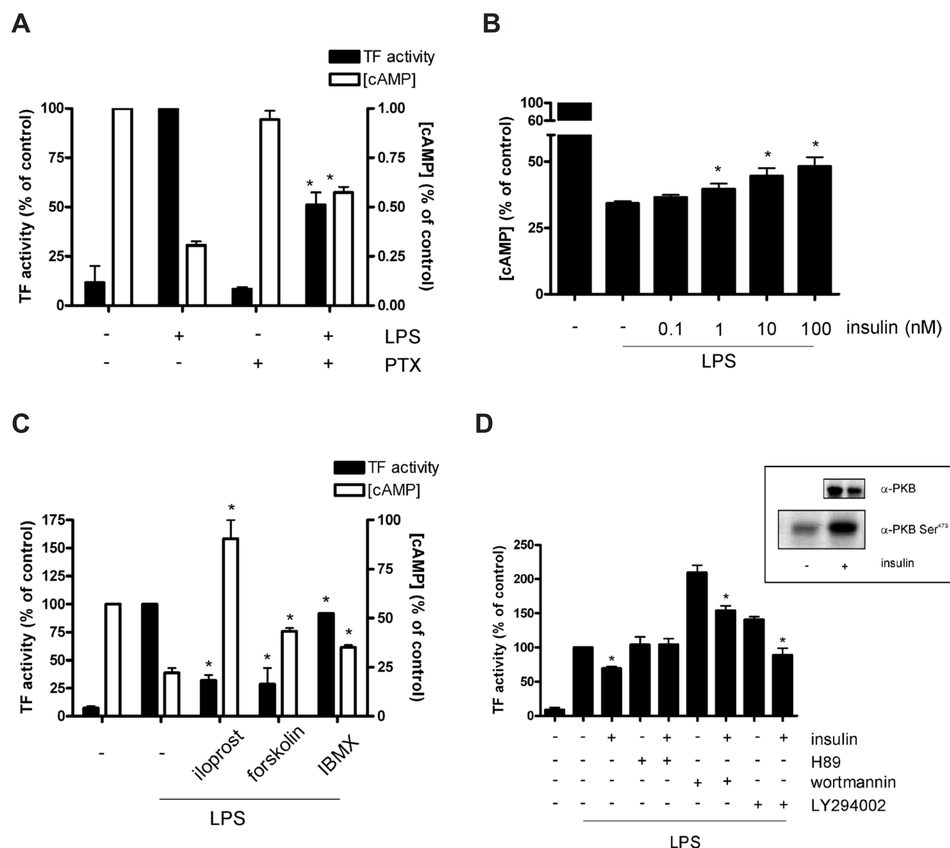


Figure 3. Role of Gi/cAMP pathway in LPS-induced TF expression

Monocytes were pre-incubated at 37°C with PTX (1 μg/mL, 2h), insulin (0-100 nM, 15 min), iloprost (1 μg/mL), forskolin (50 μM) and IBMX (10 μM, 15 min each), wortmannin (50 nM, 30 min), LY294002 (10 μM, 15 min), H89 (10 μM, 15 min) before stimulation with LPS (1 μg/mL; A-D). cAMP levels in untreated cells and TF procoagulant activity induced by LPS were set at 100% (controls). Addition of inhibitors without LPS did not change basal TF levels, nor did cells treated with only vehicle change LPS-induced TF levels (data not shown). Lysates of THP-1 cells show increased Ser^{473} phosphorylation of PKB after stimulation with insulin (D, insert). Data are expressed as percentages of control (means ± SEM, n=3, for C n=5; * $p<0.05$ of controls).

and ATP seen at the level of TF pro-coagulant activity was paralleled by similar changes in TF mRNA expression. Also the inhibition TF pro-coagulant activity by BAPTA/AM was accompanied by inhibition of TF mRNA (Fig. 4C). Together, these data indicate that Ca^{2+} supports the signaling pathways that control transcription of the TF gene, independent from changes in the TF protein.

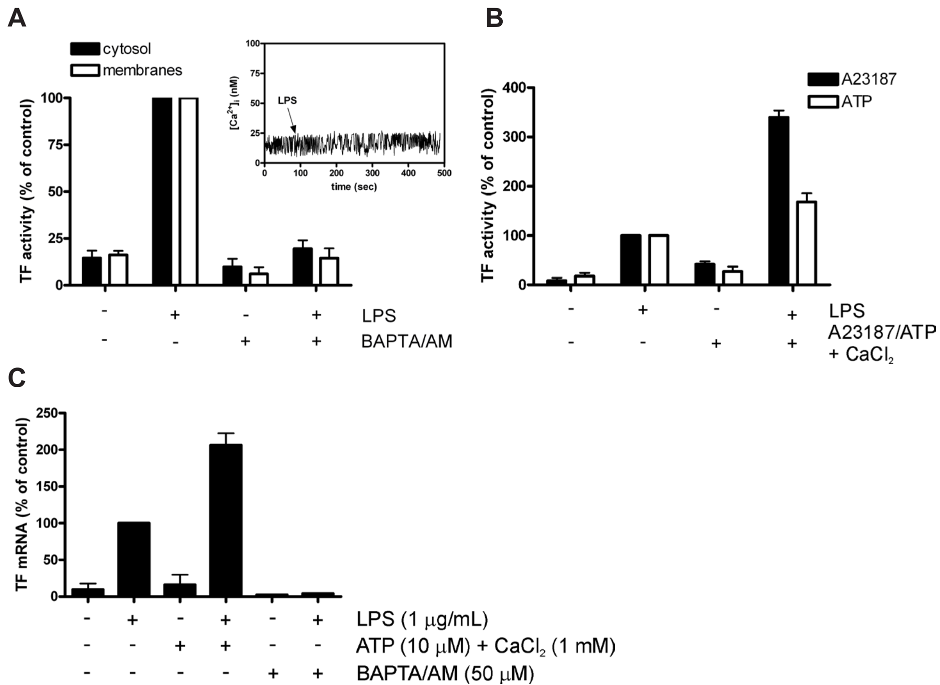


Figure 4. LPS-induced TF upregulation depends on $[Ca^{2+}]_i$

THP-1 cells were incubated with Fura-2/AM at 37° and changes in $[Ca^{2+}]_i$ were measured after stimulation with LPS (1 μg/mL, A, insert). THP-1 cells were pre-incubated at 37°C with BAPTA/AM (50 μM, 15 min, A), Ca^{2+} ionophore A23187 (1 μM, 15 min) with $CaCl_2$ (1 mM, B) or ATP (10 μM, 15 min) with $CaCl_2$ (1 mM, B) and stimulated with LPS (1 μg/mL LPS). TF procoagulant activity was measured after 5 h incubation. TF mRNA was measured by quantitative real-time PCR analysis at the conditions indicated after 2 h stimulation with LPS (C).

Insulin inhibits MCP-1- induced Ca^{2+} mobilization

To investigate whether the inhibition of TF upregulation by insulin was caused by attenuation of Ca^{2+} rises, THP-1 were stimulated with ATP after pre-incubation with increasing concentrations of insulin. Without insulin, this treatment raised $[Ca^{2+}]_i$ to 206 ± 12 nM (n=4). The increase remained unchanged in the presence of insulin, which argues against inhibition by insulin through interference with the P2Y7 receptor (Fig. 5A,B). To assess an effect by insulin on Ca^{2+} mobilization, THP-1 cells were stimulated with 10 ng/mL MCP-1 which induced a rise to 36 ± 10 nM (n=3). Insulin induced a dose-dependent inhibition leading to a fall of 39 ± 7% at 100 nM insulin (Fig. 5C,D). Apparently, pathways that control the mobilization of Ca^{2+} from internal stores are sensitive to inhibition by insulin.

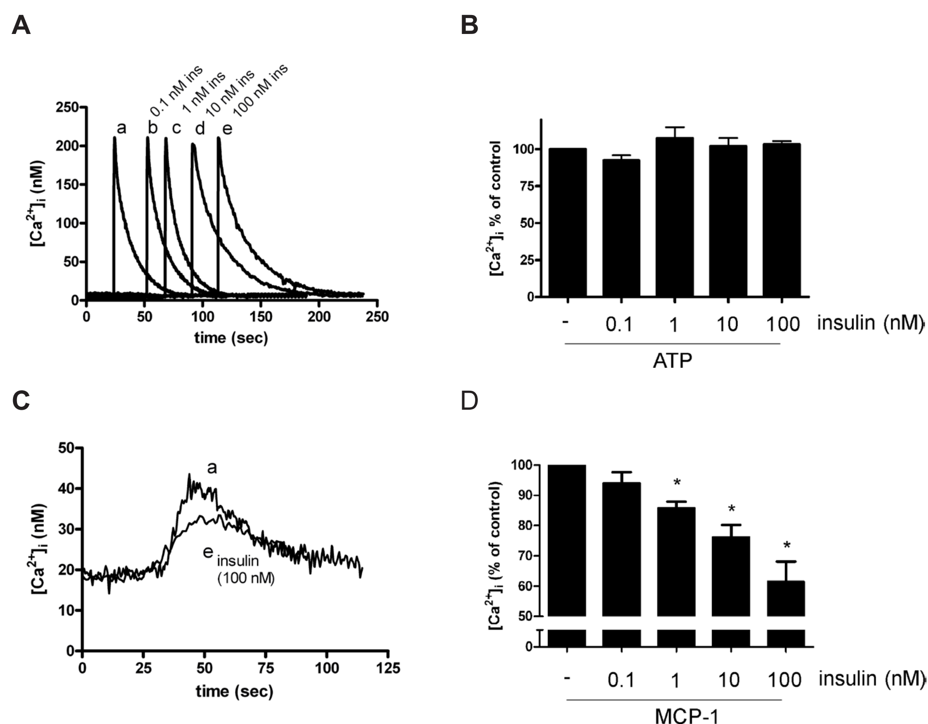


Figure 5. Insulin inhibits MCP-1-induced Ca^{2+} mobilization
In THP-1 cells rises in $[Ca^{2+}]_i$ were measured at 37° upon stimulation with ATP (10 μM, A,B) and MCP-1 (10 ng/mL, C,D). Curve (a) represents Ca^{2+} mobilization in the absence of insulin. Curves (b,c,d,e) represent the Ca^{2+} mobilization after pre-incubation with insulin (15 min, A,C). Agonist-induced increase in $[Ca^{2+}]_i$ was expressed at 100% (control) (B,D). Insulin alone did not change basal $[Ca^{2+}]_i$ (19 ± 2 nM). (Futher details as in Fig.3).

Discussion

Insulin inhibits dose-dependently the upregulation of TF procoagulant activity in monocytes and THP-1 cells stimulated with LPS. The inhibition is caused by a mechanism that interferes with the regulation of cAMP and $[Ca^{2+}]_i$, and has a great impact on the expression of TF. Since this property also affects TF expression on monocyte-derived microparticles, it is likely to interfere with the contribution of blood-born TF to thrombus formation. Insulin also reduces surface expression and secretion of IL-1β consistent with earlier findings [23] and might therefore act as a general suppressor of monocyte activation.

Inhibition by insulin is the result of activation through Tyr phosphorylation of the INS-R (on residues 1150/1151) inducing a transient INS-R – Giα–2 complex. It is

difficult to confirm that insulin inhibits the GTPase activity of a Gα protein [24], but the observation that it aborts the suppression of cAMP synthesis by LPS is proof for interference with a Giα subunit. Since Giα–2, but not Gsα, Gqα, Gzα interacts with the INS-R after stimulation with insulin, blockade of this G-protein subunit is the cause for inhibition of TF expression.

The cause for the inhibition by insulin must be sought in the control of cAMP. Agents that stimulate cAMP production or inhibit cAMP degradation raise cAMP and induce a fall in TF procoagulant activity. The observation that LPS reduces cAMP thereby inducing optimal upregulation of TF expression implies that the LPS receptor signals to a Giα subunit, possibly Giα–2. Previous studies [11,12] and our own data show that elevated cAMP levels inhibit TF expression in monocytes. cAMP induces the expression of several genes mediated by protein kinase A through the phosphorylation of the cAMP response element-binding protein (CREB) and the subsequent recruitment of the co-activator CREB-binding protein (CBP). Besides association with CREB, CBP binds to p65 *in vitro* and *in vivo*. Functional assays suggest that a competition occurs between CREB and p65 for limiting amounts of CBP, which can lead to a decrease in association of CBP with p65, thus inhibiting NF-κB-dependent transcription [25]. In addition to direct interference with TF transcription, cAMP might inhibit TF expression by suppressing rises in $[Ca^{2+}]_i$. In THP-1 cells LPS does not trigger a rise in $[Ca^{2+}]_i$, but the importance of basal $[Ca^{2+}]_i$ and rises in $[Ca^{2+}]_i$ induced by entry and mobilization is evident. Previous studies support a role for $[Ca^{2+}]_i$ in the activation of the transcription factor NF-κB in RAW 264.7 cells [26] and dendritic cells [27]. A similar mechanism might be operational in monocytes.

T2DM patients suffer from atherothrombotic complications. Their atheroslerotic plaques contain high levels of apoptotic microparticles [28]. They are mainly of monocytic and lymphocytic origin and retain almost all TF activity, suggesting a direct link between shed microparticles and plaque thrombogenicity [28]. Indeed, active TF has been identified within thrombi formed in coronaries [29,30]. The observation that insulin suppresses TF expression in monocytes from healthy individuals raises the possibility that insulin-resistance in T2DM patients leads to hyperactivity of monocytes, increased TF expression and microparticles enriched in TF. This would increase the risk for cardiovascular disease.

In healthy individuals, insulin suppresses the activity of platelets through interference with cAMP suppression by the P2Y12 receptor [16]. In T2DM patients, platelets have lost their responsiveness to insulin and show hyperactivity upon stimulation. The clopidogrel-like inhibitor AR-C69931 MX makes T2DM platelets

equally responsive as normal platelets in the presence of insulin, suggesting that clopidogrel is a suitable target to normalize the function of T2DM platelets [17]. Monocytes lack the P2Y12 receptor and alternative approaches must be sought to normalize the function of T2DM monocytes, if these cells have indeed become insulin-resistant. Preliminary experiments with periodate-oxidized ATP show that such a correction is feasible through interference with the P2X7 receptor, thereby suppressing the stimulation by Ca^{2+} without interfering with the basal TF expression.

Acknowledgements

The support of the Dutch Diabetes Research Foundation (no. 2004.00.029) is gratefully acknowledged. JWNA is supported by the Netherlands Thrombosis Foundation.

References

1. Grundy SM, Howard B, Smith S Jr, Eckel R, Redberg R, Bonow RO. Prevention Conference VI: Diabetes and Cardiovascular Disease: executive summary: conference proceeding for healthcare professionals from a special writing group of the American Heart Association. Circulation 2002; 105:2231-9.
2. Collier A, Rumley A, Rumley AG, Paterson JR, Leach JP, Lowe GD, Small M. Free radical activity and hemostatic factors in NIDDM patients with and without microalbuminuria. Diabetes 1992; 41:909-13.
3. McGill JB, Schneider DJ, Arfken CL, Lucore CL, Sobel BE. Factors responsible for impaired fibrinolysis in obese subjects and NIDDM patients. Diabetes 1994; 43:104-9.
4. Carr ME. Diabetes mellitus: a hypercoagulable state. J Diabetes Complications 2001; 15:44-54.
5. Zumbach M, Hofmann M, Borcea V, Luther T, Kotzsch M, Muller M, Hergesell O, Andrassy K, Ritz E, Ziegler R, Wahl P, Nawroth PP. Tissue factor antigen is elevated in patients with microvascular complications of diabetes mellitus. Exp Clin Endocrinol Diabetes 1997; 105:206-12.
6. Lim HS, Blann AD, Lip GY. Soluble CD40 ligand, soluble P-selectin, interleu-

kin-6, and tissue factor in diabetes mellitus: relationships to cardiovascular disease and risk factor intervention. Circulation 2004; 109:2524-8.

7. Furie B, Furie BC. Thrombus formation in vivo. J Clin Invest 2005; 115:3355-62.
8. Guha M, Mackman N. LPS induction of gene expression in human monocytes. Cell Signal 2001; 13:85-94.
9. Guha M, Mackman N. The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. J Biol Chem 2002; 277:32124-32.
10. Schabbauer G, Tencati M, Pedersen B, Pawlinski R, Mackman N. PI3K-Akt pathway suppresses coagulation and inflammation in endotoxemic mice. Arterioscler Thromb Vasc Biol 2004; 24:1963-9.
11. Ollivier V, Houssaye S, Ternisien C, Leon A, de VH, Elbim C, Mackman N, Edgington TS, de Prost D. Endotoxin-induced tissue factor messenger RNA in human monocytes is negatively regulated by a cyclic AMP-dependent mechanism. Blood 1993; 81:973-9.
12. Ollivier V, Parry GC, Cobb RR, de PD, Mackman N. Elevated cyclic AMP inhibits NF-kappaB-mediated transcription in human monocytic cells and endothelial cells. J Biol Chem 1996; 271:20828-35.
13. Kim S, Jin J, Kunapuli SP. Akt activation in platelets depends on Gi signaling pathways. J Biol Chem 2004; 279:4186-95.
14. Kroner C, Eybrechts K, Akkerman JW. Dual regulation of platelet protein kinase B. J Biol Chem 2000; 275:27790-8.
15. Bommakanti RK, Vinayak S, Simonds WF. Dual regulation of Akt/protein kinase B by heterotrimeric G protein subunits. J Biol Chem 2000; 275: 38870-6.
16. Ferreira IA, Eybrechts KL, Mocking AI, Kroner C, Akkerman JW. IRS-1 mediates inhibition of Ca2+ mobilization by insulin via the inhibitory G-protein Gi. J Biol Chem 2004; 279:3254-64.
17. Ferreira IA, Mocking AI, Feijge MA, Gorter G, van Haeften TW, Heemskerk JW, Akkerman JW. Platelet inhibition by insulin is absent in type 2 diabetes mellitus. Arterioscler Thromb Vasc Biol 2006; 26:417-22.
18. Angiolillo DJ, Bernardo E, Ramirez C, Costa MA, Sabate M, Jimenez-Quevedo P, Hernandez R, Moreno R, Escaned J, Alfonso F, Banuelos C, Bass TA, Macaya C, Fernandez-Ortiz A. Insulin therapy is associated with platelet dysfunction in patients with type 2 diabetes mellitus on dual oral antiplatelet treatment. J Am Coll Cardiol 2006; 48:298-304.
19. Hackeng TM, Hessing M, van ‘t Veer C, Meijer-Huizinga F, Meijers JC, de Groot

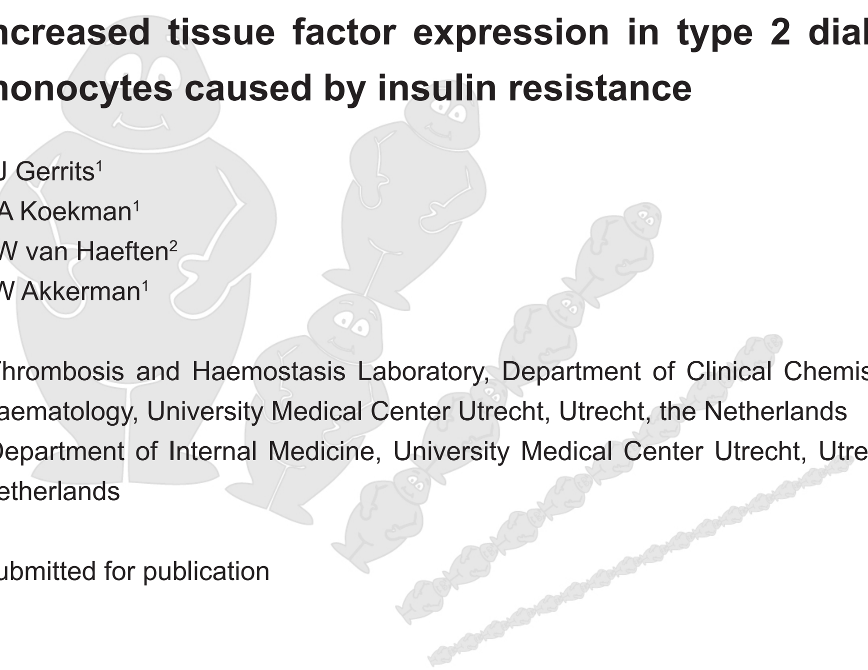
PG, van Mourik JA, Bouma BN. Protein S binding to human endothelial cells is required for expression of cofactor activity for activated protein C. J Biol Chem 1993; 268:3993-4000.

20. Kaslow HR, Burns DL. Pertussis toxin and target eukaryotic cells: binding, entry, and activation. FASEB J 1992; 6:2684-90.
21. Bosshart H, Heinzelmann M. Lipopolysaccharide-mediated cell activation without rapid mobilization of cytosolic free calcium. Mol Immunol 2004;41: 1023-8.
22. Bach RR. Tissue factor encryption. Arterioscler Thromb Vasc Biol 2006; 26:456-61.
23. Leffler M, Hrach T, Stuerzl M, Horch RE, Herndon DN, Jeschke MG. Insulin attenuates apoptosis and exerts anti-inflammatory effects in endotoxemic human macrophages. J Surg Res 2007;143:398-406.
24. Klinker JF, Hageluken A, Grunbaum L, Heilmann I, Nurnberg B, Harhammer R, Offermanns S, Schwaner I, Ervens J, Wenzet-Seifert K, Muller T, Seifert R. Mastoparan may activate GTP hydrolysis by Gi-proteins in HL-60 membranes indirectly through interaction with nucleoside diphosphate kinase. Biochem J 1994; 304:377-83.
25. Parry GC, Mackman N. Role of cyclic AMP response element-binding protein in cyclic AMP inhibition of NF-kappaB-mediated transcription. J Immunol 1997; 159:5450-6.
26. Martin L, Pingle SC, Hallam DM, Rybak LP, Ramkumar V. Activation of the adenosine A3 receptor in RAW 264.7 cells inhibits lipopolysaccharide-stimulated tumor necrosis factor-alpha release by reducing calcium-dependent activation of nuclear factor-kappaB and extracellular signal-regulated kinase 1/2. J Pharmacol Exp Ther 2006; 316:71-8.
27. Lyakh LA, Koski GK, Telford W, Gress RE, Cohen PA, Rice NR. Bacterial lipopolysaccharide, TNF-alpha, and calcium ionophore under serum-free conditions promote rapid dendritic cell-like differentiation in CD14+ monocytes through distinct pathways that activate NK-kappa B. J Immunol 2000; 165:3647-55.
28. Mallat Z, Hugel B, Ohan J, Leseche G, Freyssinet JM, Tedgui A. Shed membrane microparticles with procoagulant potential in human atherosclerotic plaques: a role for apoptosis in plaque thrombogenicity. Circulation 1999; 99:348-53.
29. Marmur JD, Thiruvikraman SV, Fyfe BS, Guha A, Sharma SK, Ambrose JA, Fallon JT, Nemerson Y, Taubman MB. Identification of active tissue factor in human coronary atheroma. Circulation; 94:1226-32.
30. Giesen PL, Rauch U, Bohrmann B, Kling D, Roque M, Fallon JT, Badimon JJ,

Himber J, Riederer MA, Nemerson Y. Blood-borne tissue factor: another view of thrombosis. Proc Natl Acad Sci U S A 1999; 96:2311-5.

Chapter 5

Increased tissue factor expression in type 2 diabetes monocytes caused by insulin resistance



AJ Gerrits[1]
CA Koekman[1]
TW van Haeften[2]
JW Akkerman[1]

[1]Thrombosis and Haemostasis Laboratory, Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht, the Netherlands
[2]Department of Internal Medicine, University Medical Center Utrecht, Utrecht, the Netherlands

Submitted for publication

Abstract

Objective Type 2 diabetes patients are insulin resistant and suffer from thrombotic complications caused by hypercoagulable plasma. Coagulation is initiated by Tissue Factor (TF), which is predominantly produced in activated monocytes. We investigated whether insulin resistance affects TF production in monocytes.

Research Design and Methods LPS-induced TF activity was measured in the absence and presence of insulin in monocytes and monocyte-derived microparticles from type 2 diabetes patients and matched controls. Association of the insulin receptor (INS-R) with Giα–2, which inactivates the G-protein, raises cAMP and inhibits TF production, was investigated by immunoprecipitation/blotting.

Results In controls, insulin inhibited TF production in monocytes to 64 ± 13% and reduced TF in microparticles to 71 ± 12% (means ± SEM, n=9) but not in patients (104 ± 8, 109 ± 10% respectively). Formation of an [INS-R - Giα–2] complex was reduced in patients compared to controls.

Conclusions Contact with insulin suppresses TF production in monocytes in control subjects but not in type 2 diabetes patients. This might explain the increased risk of atherothrombotic complications observed in these patients.

Introduction

Type 2 diabetes is associated with accelerated atherosclerosis [1,2] and a 2-8 fold increased risk of cardiovascular and thrombotic complications [3]. Eighty percent of the patients will die of atherothrombotic-related disorders that affect the coronary and cerebral circulation and peripheral arteries in the lower limbs [4]. Plasma from type 2 diabetes patients shows the characteristics of a hypercoagulable state with elevated levels of coagulation factors and activation of the coagulation cascade [5]. Proper regulation of Tissue Factor (TF) production is important, since TF is the prime initiator of blood coagulation. Type 2 diabetes patients have elevated plasma levels of TF antigen [6,7] and circulating TF-procoagulant activity [8,9]. In addition, there is increased TF expression in circulating monocytes [10,11] and an elevated level of TF-positive microparticles [12]. A positive association between soluble TF and microvascular complications has been found, suggesting that soluble TF is a predictive marker of thrombotic complications in type 2 diabetes patients [13]. Interestingly, improvement of glycemic control induces a 1.5-fold reduction in cir-

culating TF [14].

In healthy individuals, stimulation of monocytes with lipopolysaccharide (LPS) induces TF surface expression and procoagulant activity and insulin interferes with these processes [15]. Insulin induces autophosphorylation of the insulin receptor (INS-R). The activated receptor binds the G-protein Giα-2 forming an INS-R - Giα-2 complex. The association inactivates Giα-2 and interferes with the suppression of cAMP, an inhibitor of TF synthesis. Thus, contact between insulin and monocytes inhibits TF synthesis. Inhibition by insulin also reduces TF expression on monocyte-derived microparticles, without affecting the number of microparticles shed from the cells [15].

A main feature of type 2 diabetes is the loss of the capacity to respond to insulin of many cell types, including platelets. We hypothesized that in type 2 diabetes also monocytes lose responsiveness to insulin and thereby the negative control of TF synthesis. In the presence of insulin, this would result in higher TF production than in normal individuals and might explain the hypercoagulable state in type 2 diabetes patients. To test this hypothesis, levels of LPS-induced TF activity were measured in monocytes and monocyte-derived microparticles in type 2 diabetes patients and matched control subjects.

Methods

Materials - We obtained human recombinant insulin (solubilized according to the recommendations of the manufacturer in 10 mmol/L acetic acid, 100 mmol/L NaCl, and 0.01% BSA to a stock concentration of 100 µmol/L), lipopolysaccharide (LPS, E. coli 0111:B4), protease inhibitor cocktail and sodium vanadate ($NaVO_3$) from Sigma (St Louis, MO, USA) and protein G-Sepharose from Amersham (Uppsala, Sweden). Factor (F) X was purified from fresh-frozen plasma [16]. Recombinant FVIIa (rFVIIa) was from Novo Nordisk (Bagsværd, Denmark) and Innovin from Dade Behring (Liederbach, Germany). Antibodies against the INS-R, and horseradish peroxidase-labeled anti-rabbit antibody were from Cell Signaling Technology (Danvers, MA, USA), polyclonal antibody against Giα-2 from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and antibody against F-actin from Abcam (Cambridge, UK). All other chemicals used were of analytical grade.

Subjects - Type 2 diabetes patients were recruited from the out-patient clinics of

the Utrecht Medical University Hospital, Utrecht, the Netherlands. Their characteristics are given in Table 1. The study was approved by the Medical Ethical Review Board. Patients and matched control subjects gave their informed consent prior to participation in the study.

Monocytes and microparticles - Freshly drawn venous blood was collected into 0.1 volume of 130 mmol/L Na_3citrate. Peripheral mononuclear cells were isolated by density-gradient centrifugation on Ficoll-Paque columns and monocytes recovered by magnetic separation with anti-CD14 antibody coupled beads, as described [15]. For isolation of microparticles, monocytes were stimulated with 1 μg/mL LPS for 5 h (37°C), centrifuged twice (350 g, 5 min, 22°C), and the supernatant was collected. After a second centrifugation (17.000 g, 30 min, 4°C), the pellet was collected and dissolved in 100 μL tris-buffered saline (TBS).

TF procoagulant activity - Monocytes (1×10^6 cells) were pre-incubated for 15 min at 37°C with different concentrations insulin. Cells were stimulated for 5 h (37°C) with 1 μg/mL LPS (E. coli 0111:B4). Suspensions were centrifuged (350 ×g, 5 min, 22°C) and the pellet dissolved in 100 μL TBS. Lysates were prepared by four times freeze/thawing and centrifuged (16100 ×g, 5 min, 22°C). Supernatant (cytosol fraction) was collected and adjusted for variations in protein content using the BCA assay. Cytosol and membrane fraction showed the same interference by insulin (15). Lysate was incubated with 10 μg/mL FX (purified from fresh-frozen plasma [16]), 5 U/mL rFVIIa (from Novo Nordisk, Bagsværd, Denmark) and 5 mmol/L $CaCl_2$. FXa generation was measured in a fluorescence reader at 405 nm after addition of FXa substrate (Pentapharm, Basel, Switzerland). Procoagulant activity was expressed as ng per 10^7 cells, based on a standard curve derived from serial dilutions of recombinant TF (Innovin from Dade Behring, Liederbach, Germany).

Insulin signaling - Aliquots of 1×10^6 monocytes were incubated with insulin (100 nM, 15 min, 37°C), collected in lysis buffer (1% NP40, 0.5% octylglucoside, 0.1% SDS, 5 mmol/L EDTA in PBS) supplemented with 10% protease inhibitor cocktail, 1 mmol/L $NaVO_3$, kept on ice for 15 min and analyzed for association between INS-R and Giα–2 by precipitating proteins with G-sepharose in combination with 1 μg/mL anti-Giα–2 antibody overnight at 4°C. Precipitates were washed 3 times with lysis buffer supplemented with 1 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L $NaVO_3$ and dissolved in reducing Laem-

mli sample buffer. Proteins were analyzed by SDS-PAGE and Western blotting. After blocking with 4% BSA in TBS or Odyssey buffer (1 hr, 22°C), membranes were incubated (16 hrs, 4°C) with 1 μg/mL INS-R or F-actin antibodies. Immunoblots were visualized using secondary antibodies labelled with HRP by enhanced chemiluminescence (for INS-R) or using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA) and Alexa-labelled antibodies according to the manufacturer's instructions (for F-actin). The intensity of the bands was quantified with ImageJ software.

Statistics - Data is expressed as mean ± S.E.M. with n observations and was analyzed with Student's test for unpaired observations or Mann Whitney U test, as indicated. Differences were considered significant at P< 0.05.

Results

LPS-induced TF-procoagulant activity in monocytes from healthy subjects was inhibited by 0.1 nM insulin or more, indicating that insulin is a negative regulator of TF synthesis (Fig. 1).

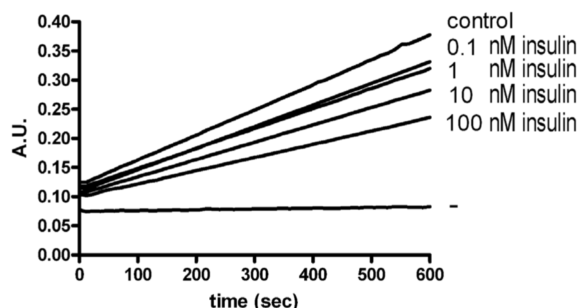


Figure 1. Insulin inhibits TF expression in monocytes

A representative graph of FXa formation in time in monocytes from healthy individuals. Monocytes were unstimulated (-) or stimulated with LPS (control) and pre-incubated without or with different concentrations of insulin (0.1, 1, 10 or 100 nM) for 15 min at 37°C.

In the absence of insulin, LPS increased TF expression from 76 ± 56 to 128 ± 38 ng/10^7 cells (means ± SEM, n=9), an 83 ± 7% increase. In monocytes from type 2 diabetes patients, the increase was from 42 ± 3 to 192 ± 48 ng/10^7 cells, a 98 ± 1% increase. Pre-incubation with insulin reduced TF production in control monocytes to 64 ± 13% (P=0.02, n=9), but in patient monocytes there was no effect (104 ± 8%, P=0.6 by Student's test; Fig. 2a). To investigate whether these

differences also showed up in the microparticles, monocytes were stimulated with LPS and microparticles were isolated. Microparticles shed from 10^7 monocytes showed an increase from 118 ± 42 to 686 ± 94 ng TF in controls, a 70 ± 12% rise, and from 116 ± 22 to 690 ± 134 ng in patients, a 76 ± 6% rise. Thus, LPS induced a similar increase in TF-procoagulant activity in microparticles as in monocytes. When monocytes were pre-treated with insulin, TF activity in microparticles was reduced to 71 ± 12% (P=0.04, n=9) in controls but remained unchanged in patients (109 ± 10%, P=0.4 by Student's test; Fig 2b).

In monocytes from healthy subjects, insulin interferes with LPS signaling through receptor binding, association with Giα–2 and inactivation of the G-protein subunit. This inhibitory G-protein of adenylyl cyclase attenuates formation of cAMP, which is an inhibitor of TF synthesis [15]. To investigate this step in insulin signaling to TF production, monocytes were incubated with insulin and formation of an INS-R - Giα–2 complex detected by immunoprecipitation with anti-Giα–2 antibody coupled to G-sepharose. Semi-quantification and correction for differences in lane loading based on F-actin revealed a significantly reduced complex formation of INS-R and Giα–2 in type 2 diabetes patient compared to controls (Fig 2c,d). Therefore, the finding that insulin fails to suppress TF synthesis in monocytes from type 2 diabetes patients is caused by a defect in IRS-R association with Giα–2.

Discussion

Here we show that the inhibition of TF synthesis in monocytes by insulin has disappeared in type 2 diabetes patients. The cause is impaired formation of an INS-R - Giα–2 complex, which is normal monocytes interferes with the Gi-mediated suppression of cAMP, an inhibitor of TF synthesis [15]. A similar loss of sensitivity to insulin is observed in platelets. In healthy subjects, insulin suppresses agonist induced platelet aggregation, secretion and the generation of a procoagulant surface [17,18]. Platelets from type 2 diabetes patients have become insulin resistant and lack the reduction in platelets functions seen when normal platelets make contact with insulin. Here, the cause of impaired insulin signaling is a defect in IRS-1 regulation since the downstream activation of protein kinase B is disturbed. Interestingly, suppression of cAMP formation in monocytes is independent of IRS-1, which points at a second defect that disturbs insulin signaling to cAMP in type 2 diabetes patients.

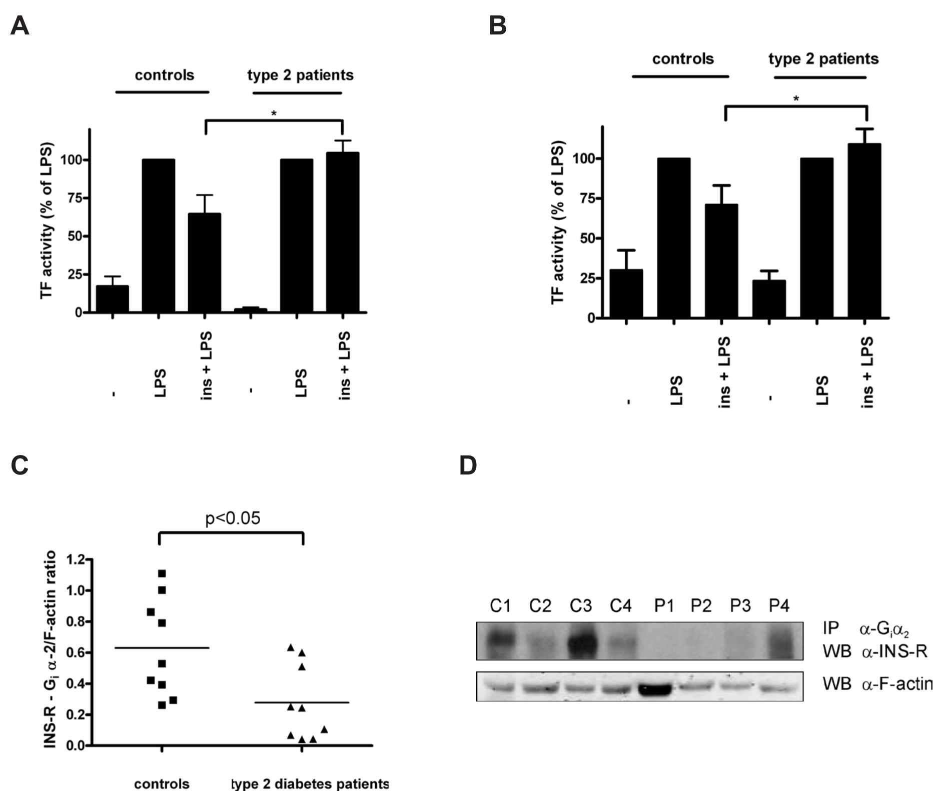


Figure 2. Insulin resistant monocytes in type 2 diabetes patients

(a) TF synthesis induced by LPS is inhibited by insulin in normal but not in type 2 diabetes monocytes. Monocytes from healthy individuals or type 2 diabetes patients were pre-incubated with insulin (100 nmol/L, 15 min, 37°C). TF activity was measured by FXa assay. LPS-induced TF activity was expressed as 100% and was not significantly different between controls and type 2 diabetes patients. (b) Monocyte-derived microparticles show the same changes in TF activity as the cells. Incubations were as in (a), but after 5 hrs stimulation with LPS, microparticles were isolated and TF activity was measured. (c) Monocytes from 9 healthy individuals and type 2 diabetes patients were incubated with insulin (100 nmol/L, 15 min, 37°C) and complex formation of INS-R and Giα-2 was quantified and corrected for F-actin. (d) A representative blot of n=4 for both groups is shown. Data is expressed as mean ± SEM with n=9 and was analyzed with Student' test (a,b) and Mann Whitney U test (c). * denotes a significant difference ($P<0.05$).

Recent findings demonstrate that platelets adhering to a thrombotic surface are capable of synthesizing TF [19]. The anucleate platelet has long been considered incapable of synthesizing proteins, but the presence of a splicing machinery to-

gether with pre-mRNAs for a number of proteins including TF make them a source for TF synthesis. Consistent with the data found in monocytes, we found that type 2 diabetes is accompanied by a loss of insulin's inhibitory effect on TF production in platelets (submitted for publication: Enhanced platelet tissue factor synthesis in type 2 diabetes patients, A.J.G., C.A.K., T.W.H., J.W.A., 2009).

Monocytes participate in plaque formation at the inner lining of arteries, and in atherogenesis by supporting coagulation and inflammation [20-22]. Acute atherothrombotic complications are caused by rupture of advanced atherosclerotic lesions, thereby exposing TF and initiating coagulation. Insulin also reduces TF expression in monocyte-derived microparticles, which are vehicles for so called blood-born TF that supports the propagation of the thrombus.

Together these observations reveal different sources for TF production, each being inhibited by insulin in healthy subjects but not in the patients. Together with the disappearance of suppression of platelet functions, the upregulated TF synthesis might contribute to the atherothrombotic complications observed in type 2 diabetes patients.

Table 1. Characteristics of the study population

	Control group (n=9)	Type 2 diabetes patients (n=9)	P
Age (years)	53 ± 3	58 ± 3	0.2
BMI (kg/m^2)	25 ± 0.7	28 ± 1	0.17
Systolic blood pressure (mmHg)	134 ± 6	131 ± 3	0.6
Diastolic blood pressure (mmHg)	81 ± 3	78 ± 4	0.6
HbA1c (%)	5.4 ± 0.07	7.2 ± 0.4	0.0005
Cholesterol (mmol/L)	6.2 ± 0.4	4 ± 0.3	0.0003
Triglycerides (mmol/L)	1.6 ± 0.3	1.9 ± 0.3	0.5
HDL-cholesterol	1.3 ± 0.07	1.1 ± 0.1	0.06
Creatinine (mmol/L)	100 ± 5.1	102 ± 6	0.8
Management with insulin	-	9	-
Duration of diabetes (years)	-	15 ± 2	-

Acknowledgements

This work was supported by the Dutch Diabetes Research Foundation (no. 2004.00.029) and the Netherlands Thrombosis Foundation (2006-1). The authors

acknowledge the cooperation of the patients and control subjects.

References

1. Kannel WB, McGee DL. Diabetes and cardiovascular risk factors: the Framingham study. Circulation 1979; 59:8-13.
2. Stamler J, Vaccaro O, Neaton JD, Wentworth D. Diabetes, other risk factors, and 12-yr cardiovascular mortality for men screened in the Multiple Risk Factor Intervention Trial. Diabetes Care 1993; 16:434-444.
3. Grundy SM, Howard B, Smith S Jr, Eckel R, Redberg R, Bonow RO. Prevention Conference VI: Diabetes and Cardiovascular Disease: executive summary: conference proceeding for healthcare professionals from a special writing group of the American Heart Association. Circulation 2002; 105:2231-2239.
4. Collier A, Rumley A, Rumley AG, Paterson JR, Leach JP, Lowe GD, Small M. Free radical activity and hemostatic factors in NIDDM patients with and without microalbuminuria. Diabetes 1992; 41:909-913.
5. Carr ME. Diabetes mellitus: a hypercoagulable state. J Diabetes Complications 2001; 15:44-54.
6. Zumbach M, Hofmann M, Borcea V, Luther T, Kotzsch M, Muller M, Hergesell O, Andrassy K, Ritz E, Ziegler R, Wahl P, Nawroth PP. Tissue factor antigen is elevated in patients with microvascular complications of diabetes mellitus. Exp Clin Endocrinol Diabetes 1997; 105:206-212.
7. Lim HS, Blann AD, Lip GY. Soluble CD40 ligand, soluble P-selectin, interleukin-6, and tissue factor in diabetes mellitus: relationships to cardiovascular disease and risk factor intervention. Circulation 2004; 109:2524-2528.
8. Sambola A, Osende J, Hathcock J, Degen M, Nemerson Y, Fuster V, Crandall J, Badimon JJ. Role of risk factors in the modulation of tissue factor activity and blood thrombogenicity. Circulation 2003; 107:973-977.
9. Abdel-Hafiz E, Vaidyula VR, Bagga S, London FS, Boden G, Rao AK. Elevated whole blood tissue factor procoagulant activity in diabetes mellitus. Vitamin E inhibits glucose induced tissue factor activity in vitro (Abstract). Blood 2002; 100: 496a.
10. Reverter JL, Reverter JC, Tassies D, Rius F, Monteagudo J, Rubies-Prat J, Escolar G, Ordinas A, Sanmarti A. Thrombomodulin and induced tissue factor expression on monocytes as markers of diabetic microangiopathy: a prospective study on hemostasis and lipoproteins in insulin-dependent diabetes mel-

litus. Am J Hematol 1997; 56:93-99.

11. Buchs AE, Kornberg A, Zahavi M Aharoni D, Zarfati C, Rapoport MJ. Increased expression of tissue factor and receptor for advanced glycation end products in peripheral blood mononuclear cells of patients with type 2 diabetes mellitus with vascular complications. Exp Diabesity Res 2004; 5:163-169.
12. Diamant M, Nieuwland R, Pablo RF, Sturk A, Smit JW, Radder JK. Elevated numbers of tissue-factor exposing microparticles correlate with components of the metabolic syndrome in uncomplicated type 2 diabetes mellitus. Circulation 2002; 106:2442-2447.
13. Sommeijer DW, Hansen HR, van Oerle R, Hamulyak K, van Zanten AP, Meesters E, Spronk HM, ten Cate H. Soluble tissue factor is a candidate marker for progression of microvascular disease in patients with Type 2 diabetes. J Thromb Haemost 2006; 4:574-580.
14. Sambola A, Osende J, Hathcock J, Degen M, Nemerson Y, Fuster V, Crandall J, Badimon JJ. Role of risk factors in the modulation of tissue factor activity and blood thrombogenicity. Circulation 2003; 107:973-977.
15. Gerrits AJ, Koekman CA, Yildirim C, Nieuwland R, Akkerman JW. Insulin inhibits tissue factor expression in monocytes. J Thromb Haemost 2009; 7:198-205.
16. Hackeng TM, Hessing M, van 't Veer C, Meijer-Huizinga F, Meijers JC, de Groot PG, van Mourik JA, Bouma BN. Protein S binding to human endothelial cells is required for expression of cofactor activity for activated protein C. J Biol Chem 1993; 268:3993-4000.
17. Ferreira IA, Eybrechts KL, Mocking AI, Kroner C, Akkerman JW. IRS-1 mediates inhibition of Ca2+ mobilization by insulin via the inhibitory G-protein Gi. J Biol Chem 2004; 279:3254-3264.
18. Ferreira IA, Mocking AI, Feijge MA, Gorter G, van Haeften TW, Heemskerk JW, Akkerman JW. Platelet inhibition by insulin is absent in type 2 diabetes mellitus. Arterioscler Thromb Vasc Biol 2006; 26:417-422.
19. Schwertz H, Tolley ND, Foulks JM, Denis MM, Risenmay BW, Buerke M, Tilley RE, Rondina MT, Harris EM, Kraiss LW, Mackman N, Zimmerman GA, Weyrich AS. Signal-dependent splicing of tissue factor pre-mRNA modulates the thrombogenicity of human platelets. J Exp Med 2006; 203:2433-2440.
20. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993; 362:801-809.
21. Faruqi RM, DiCorleto PE. Mechanisms of monocyte recruitment and accumulation. Br Heart J 1993; 69:S19-S29.

22. Beekhuizen H, van Furth R. Monocyte adherence to human vascular endothelium. J Leukoc Biol 1993; 54:363-378.

Chapter 6

Insulin inhibition of platelet-endothelial interaction is mediated by insulin effects on endothelial cells without direct effects on platelets - A rebuttal

JW Akkerman[1]
AJ Gerrits[1]
IA Ferreira[2]
JW Heemskerk[3]

[1]Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht, the Netherlands
[2]Department of Cardiology, Isala Clinics, Zwolle, the Netherlands
[3]Department of Biochemistry, University of Maastricht, Maastricht, the Netherlands

J Thromb Haemost 2009; 7:369-71

In a recent issue of this journal, Rauchfuss and colleagues addressed the question whether human platelets possess a functional insulin receptor (INS-R) that changes platelet behavior [1].

In this report, the authors refute the conclusions of our earlier work [2-4] on this issue and conclude that although platelets have INS-R protein, there is little evidence for receptor-mediated signaling that interferes with aggregation, secretion and pro-coagulant activity. The concept that platelet hyperreactivity in type 2 diabetes mellitus patients is caused by impaired insulin signaling is equally discredited. Since the study by the Rauchfuss group appears work performed with great care, a reaction from our group is appropriate.

In the published work, we claim that human platelets respond to insulin with signaling to insulin receptor substrate-1 (IRS-1) inducing the association between IRS-1 and Giα–2, the most abundant member of the family of G-proteins that inhibits adenylyl cyclase and thereby production of cAMP. The evidence for signaling is based on a series of consecutive Tyr phosphorylations and the assumption that Tyr phosphorylated Giα–2 is inactive [2]. The result is interference of P2Y12-mediated suppression of cAMP production with a final effect very much alike interference at the receptor level by clopidogrel.

Interference of insulin with signaling to cAMP is the crux to this story. Only when during the isolation procedure platelets have preserved responsive P2Y12-Rs, insulin shows this effect (Fig. 1). *In vivo*, this condition is met since mice lacking either Giα–2 or P2Y12-R show greatly diminished thrombus formation [5,6]. *In vitro*, the interference by insulin is a transient phenomenon and depends on the association of IRS-1 with Giα–2. Association is optimal at 5 min (1 nM insulin) to 10 min (100 nM insulin) and followed by dissociation 20 min later. It follows that simultaneous addition of insulin and platelet activators has no effect and that after 20 min incubation interference by insulin has disappeared.

This relative narrow window of insulin interference with cAMP control under *in vitro* conditions is an obvious handicap for studies of insulin's impact on platelet functions. Conditions that determine the optimum of IRS-1 with Giα–2 association greatly depend on the platelet isolation procedure and may vary between laboratories. Chances to miss an effect of insulin *in vitro* are greater than chances to see inhibition of platelets

A complete list of methodological differences between the studies of the two laboratories is a boring read but a few discrepancies deserve attention. We found it important to use 1, an isolation procedure with prostacyclin to prevent ADP secretion and not more than 10 mM glucose to keep its activating properties to

a minimum, 2, a stock solution of 100 μM insulin in 10 mM acetic acid, 100 mM NaCl, 0.01% BSA from which dilutions were prepared immediately before use,

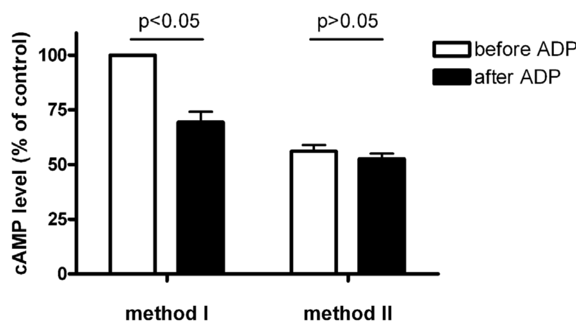


Figure 1. cAMP concentration in platelets isolated according to refs. 2-4 (method I) and ref 1 (method II)

Maximal reduction of the cAMP concentration during platelet isolation (white bars) limits a further reduction by ADP addition (10 μM, final concentration) through P2Y12-R signaling (black bars). For method I: blood from healthy, non-diabetic medication-free volunteers was collected with informed consent into 0.1 volume of 130 mM Na_3 citrate. Platelet-rich plasma (PRP) was prepared by centrifugation (150 x g, 15 min, 20 °C), supplemented with 0.1 volume of ACD (80 mM citric acid, 120 mM Na_3 citrate, 110 mM D-glucose) for acidification to pH 6.5 and centrifuged (330 x g, 15 min, 20 °C). Platelets were resuspended in HEPES/Tyrode buffer (145 mM NaCl, 5 mM KCl, 0.5 mM Na_2HPO_4, 1 mM $MgSO_4$, 10 mM HEPES, pH 6.5) containing 5 mM D-glucose and 10 ng mL-1 PGI_2. After a second centrifugation (330 x g, 15 min, 20 °C), platelets were resuspended in PGI_2-free HEPES/Tyrode buffer, pH 7.25 containing 5 mM D-glucose to a final concentration of 2 x 10^{11} cells L-1. Prior to the experiments, platelets were kept at 20 °C for 45 min to ensure a resting state. For method II: blood was collected under the same conditions into ACD (12 mM citric acid, 15 mM sodium citrate, 25 mM D-glucose, final concentrations). PRP was prepared by centrifugation (330 x g, 15 min, 20 °C). Platelets were pelleted (400 x g, 5 min, 20 °C), washed in citrate-glucose-saline buffer (120 mM NaCl, 12.9 mM Na_3 citrate, 30 mM D-glucose, pH 6.5), and resuspended in HEPES buffer (150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM D-glucose, 10 mM HEPES, pH 7.4) to a final concentration of 2 x 10^{11} cells L-1. Prior to the experiments, platelets were kept at 37 °C for 60 min to ensure a resting state.100% = 20.0 ± 4.2 nmol 10^{11} platelets. Data are means ± SEM, n=6; differences were analysed by t-test.

3, pre-incubation times based on an optimal association between IRS-1 and Giα–2 , 4, antibodies against phosphorylated INS-R Tyr1158 (Biosource) and protein kinase B Ser^{473} (Santa Cruz), 5, blots prepared with exposure times for cells with low INS-R copy number, 6, Ca^{2+} mobilization without concurrent influx as an indicator of cAMP changes, 7, inhibition by the clopidogrel-like inhibitor AR-C69931MX as a control for the presence of fully sensitized P2Y12-R signaling. Unfortunately, the study by Rauchfuss and colleagues lacks these requirements

and controls.

ADP liberated during blood handling will not only desensitize ADP receptor function, but also lower cAMP through P2Y12-R signaling and, hence, inactivate this part of Gi-mediated signaling [7-9]. This is why prostacyclin which prevents ADP secretion and the ADP-removing enzyme apyrase are often included in platelet isolation procedures [2-4,7-9]. The state of sensitization of P2Y12-Rs and the actual cAMP concentration determines signaling to Ca^{2+} [9,10] and is a decisive factor in rate and extend of platelet aggregation [7,11,12].

The presence of INS-R on platelets which is Tyr-phosphorylated upon ligand binding [2] triggering IRS-1 mediated signaling to Giα–2 [2], protein kinase Bα,β [3,4], an affinity increase in the glucose transporter GLUT3 [3] and apoptosis suppression [13] are common features of many cell types and explains the requirement for insulin in megakaryocyte cultures [14]. The upregulation of P2Y12 signaling in type 2 diabetics seen *in vitro*, which goes together with reduced responsiveness to pharmacological blockade [4], translates to increased platelet responses ex vivo [15] and impaired sensitivity to clopidogrel in a large cohort of type 2 diabetics [16].

There remains the issue whether the relative weak effect of insulin significantly contributes to the control of platelet responsiveness in the circulation. The postprandial rise of insulin in healthy individuals ranges between 0.2 and 1.0 nM and lasts 3-4 h after intake of a meal [17,18]. This is a period in which the glucose concentration rises to 7-8 mM in healthy individuals and higher in diabetics. A high glucose concentration enhances agonist-induced aggregation [19]. Under these conditions, activation of platelets may well be suppressed by insulin interference with the P2Y12-mediated fall in cAMP.

References

1. Rauchfuss S, Geiger J, Walter U, Renne T, Gambaryan S. Insulin inhibition of platelet-endothelial interaction is mediated by insulin effects on endothelial cells without direct effects on platelets. J Thromb Haemost 2008; 6:856-64.
2. Ferreira IA, Eybrechts KL, Mocking AI, Kroner C, Akkerman JW. IRS-1 mediates inhibition of Ca2+ mobilization by insulin via the inhibitory G-protein Gi. J Biol Chem 2004; 279:3254-64.
3. Ferreira IA, Mocking AI, Urbanus RT, Varlack S, Wnuk M, Akkerman JW. Glucose uptake via glucose transporter 3 by human platelets is regulated by pro-

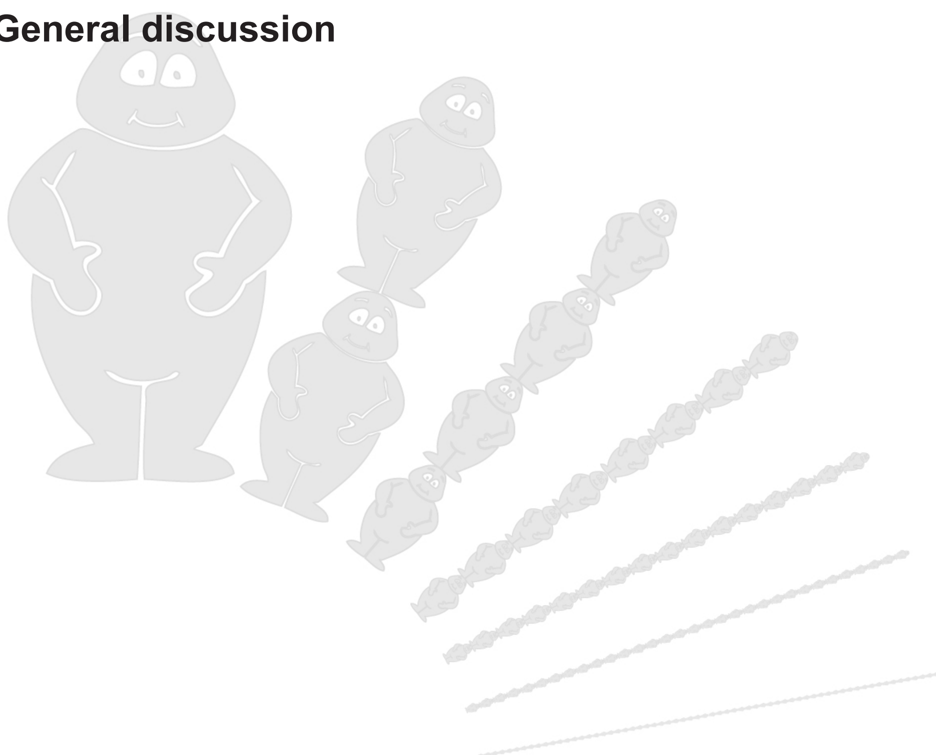
tein kinase B. J Biol Chem 2005; 280:32625-33.

4. Ferreira IA, Mocking AI, Feijge MA, Gorter G, van Haeften TW, Heemskerk JW, Akkerman JW. Platelet inhibition by insulin is absent in type 2 diabetes mellitus. Arterioscler Thromb Vasc Biol 2006; 26:417-22.
5. Jantzen HM, Milstone DS, Gousset L, Conley PB, Mortensen RM. Impaired activation of murine platelets lacking G alpha(i2). J Clin Invest 2001; 108:477-83.
6. Andre P, Delaney SM, LaRocca T, Vincent D, DeGuzman F, Jurek M, Koller B, Phillips DR, Conley PB. P2Y12 regulates platelet adhesion/activation, thrombus growth, and thrombus stability in injured arteries. J Clin Invest 2003; 112:398-406.
7. Keularts IM, van Gorp RM, Feijge MA, Vuist WM, Heemskerk JW. alpha(2A)-adrenergic receptor stimulation potentiates calcium release in platelets by modulating cAMP levels. J Biol Chem 2000; 275:1763-72.
8. Baurand A, Eckly A, Bari N, Leon C, Hechler B, Cazenave JP, Gachet C. Desensitization of the platelet aggregation response to ADP: differential down-regulation of the P2Y1 and P2cyc receptors. Thromb Haemost 2000; 84:484-91.
9. Hardy AR, Conley PB, Luo J, Benovic JL, Poole AW, Mundell SJ. P2Y1 and P2Y12 receptors for ADP desensitize by distinct kinase-dependent mechanisms. Blood 2005; 105:3552-60.
10. Storey RF, Sanderson HM, White AE, May JA, Cameron KE, Heptinstall S. The central role of the P(2T) receptor in amplification of human platelet activation, aggregation, secretion and procoagulant activity. Br J Haematol 2000; 110:925-34.
11. Cooper DM, Rodbell M. ADP is a potent inhibitor of human platelet plasma membrane adenylate cyclase. Nature 1979; 282: 517-8.
12. Labarque V, Freson K, Thys C, Wittevrongel C, Hoylaerts MF, De Vos R, Goemans N, Van Geet C. Increased Gs signalling in platelets and impaired collagen activation, due to a defect in the dystrophin gene, result in increased blood loss during spinal surgery. Hum Mol Genet 2008; 17:357-66.
13. Kozuma Y, Kojima H, Yuki S, Suzuki H, Nagasawa T. Continuous expression of Bcl-xL protein during megakaryopoiesis is post-translationally regulated by thrombopoietin-mediated Akt activation, which prevents the cleavage of Bcl-xL. J Thromb Haemost 2007; 5:1274-82.
14. Watanabe Y, Kawada M, Kobayashi B. Effect of insulin on murine megakaryocytopoiesis in a liquid culture system. Cell Struct Funct 1987; 12:311-6.

15. Angiolillo DJ, Bernardo E, Ramirez C, Costa MA, Sabate M, Jimenez-Quevedo P, Hernandez R, Moreno R, Escaned J, Alfonso F, Banuelos C, Bass TA, Macaya C, Fernandez-Ortiz A. Insulin therapy is associated with platelet dysfunction in patients with type 2 diabetes mellitus on dual oral antiplatelet treatment. J Am Coll Cardiol 2006; 48:298-304.
16. Angiolillo DJ, Shoemaker SB, Desai B, Yuan H, Charlton RK, Bernardo E, Zenni MM, Guzman LA, Bass TA, Costa MA. Randomized comparison of a high clopidogrel maintenance dose in patients with diabetes mellitus and coronary artery disease: results of the Optimizing Antiplatelet Therapy in Diabetes Mellitus (OPTIMUS) study. Circulation 2007; 115:708-16.
17. Malmquist J, Birgerstam G. Assays of pancreatic B cell secretory products: utility in investigative and clinical diabetology. Scand J Clin Lab Invest 1986; 46:705-13.
18. Gerich JE. The genetic basis of type 2 diabetes mellitus: impaired insulin secretion versus impaired insulin sensitivity. Endocr Rev 1998; 19:491-503.
19. Sudic D, Razmara M, Forslund M, Ji Q, Hjemdahl P, Li N. High glucose levels enhance platelet activation: involvement of multiple mechanisms. Br J Haematol 2006; 133:315-22.

Chapter 7

General discussion



The aim of the various studies described in this thesis was to elucidate the cause of the prothrombotic state in type 2 diabetes patients. The studies demonstrate that in type 2 diabetes patients platelets and monocytes, which both play an important role in arterial thrombosis, are insulin-resistant. This results in hyperactive platelets and increased levels of Tissue Factor (TF) produced by both platelets and monocytes. In this chapter these findings are discussed in a broader perspective.

ADIPOKINES INDUCE INSULIN RESISTANCE

About 80 to 85% of all type 2 diabetes patients are overweight or obese. An excess of visceral adipose tissue plays an important role in the pathogenesis of this disease. Adipocytes and inflammatory cells such as macrophages present in adipose tissue produce and secrete adipokines, and in overweight or obese subjects levels of most adipokines are increased compared to lean subjects. More than 50 adipokines have been identified so far with a wide range of physiological functions, such as in body weight homeostasis, inflammation, coagulation, fibrinolysis, insulin sensitivity and angiogenesis. In the obese subjects, de-regulation of secretion of adipokines is the basis of the increased risk for diseases.

Adipokines and phosphorylation of IRS-1

Of specific interest are the effects of adipokines on insulin sensitivity, since they are related to the increased risk for type 2 diabetes and cardiovascular diseases (CVD) in obese subjects. Insulin sensitivity is determined by IRS-1 functioning. Tyr phosphorylation of the insulin receptor (INS-R) and IRS proteins play a role in insulin sensitivity. Protein-tyrosine phosphatase 1B (PTP1B) dephosphorylates Tyr residues in the INS-R and possibly in IRS-1. PTP1B is widely expressed and has an important role in the negative regulation of insulin signaling. A general deficiency of PTP1B or a deficiency restricted to myocytes results in increased insulin sensitivity and protection against adipokine-induced insulin resistance [1,2]. This suggests that PTP1B is an attractive target for prevention and treatment of type 2 diabetes. However, our study described in Chapter 2 shows that restoration of Ser phosphorylation with the Ser phosphatase inhibitor cantharidin completely restores insulin sensitivity in human megakaryocytes, making this protein an even better target in megakaryocytes. In general, it is unclear whether phosphorylation of IRS-1 on Ser residues enhances or suppresses insulin signaling. Ser phospho-

rylation sites are involved in both negative and positive feedback loops. Insulin induces phosphorylation of Ser^{307} making this site an important regulator of IRS-1 activity. In mouse embryo fibroblasts, 3T3-L1 adipocytes and 32DIR cells, Ser^{307} of IRS-1 is phosphorylated by a negative feed-back loop through Jun NH_2-terminal kinase (JNK). This reduces IRS-1 interaction with the INS-R and impairs insulin signaling [3]. In Chinese hamster ovary cells TNF-α activates JNK and thereby induces Ser^{307} phosphorylation of IRS-1 and inhibition of insulin-stimulated Tyr phoshorylation of IRS-1 [4]. In contrast and consistent with our findings in Chapter 2, in Rat hepatoma Fao cells and CHO cells overexpressing the INS-R, insulin-induced Ser^{307} phosphorylation protects active IRS-1 against Tyr phosphatases. In human adipocytes, phosphorylation of Ser^{307} appears to be part of a positive feed back loop.
Although disturbances in phosphorylation of IRS-1 can induce insulin resistance, it is clear that the effect lasts only for a short time and does not explain persistent insulin resistance. Other mechanisms are responsible for insulin resistance for a longer period. Signal downregulation can occur through internalization and loss of the INS-R from the cell surface and degradation of IRS proteins. Members of the suppressor of cytokine signaling (SOCS) family of proteins participate in IRS protein degradation through an ubiquitin-proteosomal pathway, as described in Chapter 2. Mutations in the conserved SOCS box of SOCS-1 abrogate the interaction with the elongin BC ubiquitin-ligase complex and ubiquitination and degradation of IRS-1 and -2 was prevented, illustrating that the importance of SOCS proteins in insulin resistance [1].

Direct effect of adipokines on platelet activation?

In addition to changing the insulin sensitivity, our study shows that incubation with adipokines for two hours or more has an effect on megakaryocytes as demonstrated by the increased aggregation in the presence of resistin (Chapter 2). Other studies with leptin and adiponectin show that these adipokines have direct effect on platelets and megakaryocytes. Leptin promotes agonist-induced platelet aggregation [6,7] and leptin-induced platelet activation requires activation of a signaling cascade that includes Ob-R, JAK2, PI3-K, PKB, IRS-1 and cAMP phosphodiesterase 3A [8]. Others claim that downstream elements of JAK2 signaling include phospholipase Cγ2, PKC as well as p38 MAPK-phospholipase A_2 [9]. In the megakaryocytic cell line MEG-01, leptin enhances ADP-induced $[Ca^{2+}]_i$ increases via JAK2 and Tyr kinases [10]. A study with globular adiponectin showed that it binds to GPVI and stimulates Tyr kinase-dependent platelet aggregation [11].

This result seems counterintuitive, because adiponectin has anti-diabetic, anti-inflammatory and vascular protective properties and levels are decreased in obesity [12]. Consistent with this latter idea, Kato *et al* showed that adiponectin deficiency leads to enhanced thrombus formation and platelet aggregation, revealing a role of adiponectin as endogenous antithrombotic factor [13].

Opposite to findings that leptin increases platelet reactivity is the failure of a four-days leptin infusion in healthy volunteers to change aggregation [2]. In addition, the observation that leptin deficient subjects show increased aggregation by platelets suggests that leptin does not change platelet behaviour through direct interaction [3]. Findings with adiponectin are equally contradictory. Studies in knockout mice suggest that adiponectin is an antithrombotic factor in wild-type mice [4], but adhesion and aggregation of human platelets are not changed upon incubation with a supra-physiological adiponectin concentration [16].

Visceral versus subcutaneous adipose tissue

Subcutaneous and visceral obesity are epidemiologically associated with different relative risks of the metabolic syndrome and diabetes. Increased prevalence of excessive visceral obesity is closely associated with rising incidence of CVD and type 2 diabetes. It has been suggested that the unique location of the visceral adipose tissue plays an important role. Visceral adipose tissue releases secreted factors directly into the portal vein and may thereby affect synthesis of coagulation factors in the liver and induce insulin resistance in hepatocytes. Studies with transplantation of either subcutaneous or visceral fat from donor mice into either subcutaneous or visceral regions of recipient mice suggest that subcutaneous fat is intrinsically different from visceral fat. Mice with subcutaneous fat transplanted into the visceral region lost body weight, total fat mass, while plasma glucose and insulin levels were decreased and insulin sensitivity was improved [17]. Apparently one or more products from the subcutaneous fat tissue has beneficent effects on obesity and on insulin sensitivity presumably via an action on the liver. Identification of these factors may provide new targets for the prevention and/or treatment of obesity-related complications. However, these data must be interpreted with caution. There are differences in visceral fat between human and mice. A large proportion of human visceral fat is omental, a depot that is insignificant in mice and in turn in man no fat tissue corresponding to mouse epididymal fat has been uncovered [18].

Functional differences between subcutaneous and visceral adipose tissue have been observed, for example in the expression of leptin which is mainly produced

by human subcutaneous adipose tissue. More macrophages infiltrate into visceral adipose tissue during the development of obesity than into subcutaneous adipose tissue [19]. Visceral fat is the main contributor of plasma IL-6 as observed in a study in extremely obese patients [20]. IL-6 is an inducer of C-reactive protein (CRP) and proteins involved in hemostasis (PAI-1, fibrinogen, tissue plasminogen activator) and production of visceral IL-6 directly affects the expression of proteins in the liver.

Tissue factor expression in platelets

New platelet proteins with a role in haemostasis

For decades, platelets have been considered incapable of protein synthesis because they lack nuclei. However there were several investigators that independently demonstrated the opposite. Already in the late 1960's it was shown that platelets incorporate amino acids, which was blocked by the protein synthesis inhibitor puromycin [21,22]. In the following years, studies were expanded and the presence of ribosomes and other constituents necessary for protein synthesis was demonstrated [23,24]. Due to lack of techniques that completely eliminate leukocytes from platelets suspensions and mechanisms of protein synthesis by platelets were not elucidated, it took until the last decade for this topic to bloom again. New proteins (and their function) were identified with the characterization of mechanisms that regulate synthesis and their function. One could argue that protein expression in platelets does not contribute to processes such as thrombosis because it is rather slow and limited in quantity. Nevertheless, an example of a protein produced by platelets is B-cell lymphoma 3 (Bcl-3), which binds the SH3 domain of Fyn and thereby regulates platelet-dependent clot retraction [25,26]. Synthesis of Il-1β protein in platelets increases the adhesiveness of endothelial cells for polymorphonuclear leukocytes [27]. In addition, PAI-1 and COX-1 synthesis in platelets have been reported [28,29], which play a role in fibrinolysis and the production of TxA_2 respectively. In 2006 the mechanism of TF expression in platelets was discovered. Schwertz *et al* [30] demonstrated that TF pre-mRNA splicing was controlled by Clk1, which induces phosphorylation of the splicing factor SF2. Further characterization of the extra- and intracellular signaling pathway described in Chapter 3 gives more insight in TF expression in platelets. AR-C699331 MX, which mimics blockade of the P2Y12-receptor by the active metabolite of clopidogrel and indomethacin (an easily soluble COX-1 inhibitor which mimics the

action of aspirin) strongly inhibits TF expression and the combination of these two results in an almost complete inhibition. This suggests that a combination of aspirin and clopidogrel might be beneficial in patients suffering from CVD due to increased platelet TF levels.

Interference by contaminating monocytes

The discussion whether human platelets are capable of regulated protein synthesis has been ongoing for over half a century. Pillitteri *et al* suggest that any IL-1β synthesis detected is a by-product of leukocytes contaminating the platelet preparations and that platelets do not produce IL-1β [31]. In contrast, more reports of different research groups on platelet protein expression appear, thereby independently confirming this process. Most studies take great care in elimination of monocytes and demonstrate pure platelet suspension ([27,30], Chapter 3). Therefore, the critique that TF synthesis by platelets is caused by contaminating leukocytes appears to be unjustified.

In our studies, the CD14 mRNA-free platelet suspensions showed no TF pre-mRNA splicing upon stimulation with LPS, which is a potent inducer of TF synthesis in monocytes (Chapter 3). Recent studies show that platelets and megakaryocytes express the receptor for LPS, Toll-like receptor 4 ([32,33]). Although LPS did not generally activate platelets (no increase in P-selectin expression), platelets bind to fibrinogen under flow conditions in a TLR4-dependent manner, which indicates that LPS signals to $\alpha_{IIb}\beta_3$ [32]. Shashkin *et al* [33] showed that LPS initiated splicing of IL-1β and COX-2 pre-mRNA in platelets resulting in accumulations of these proteins. Yet, they mention that LPS induced COX-2 expression was extremely variable. Likely, splicing of TF pre-mRNA is regulated differently from that of IL-1β (and COX-2).

Elevated levels of platelet TF in type 2 diabetes patients

The question whether elevated levels of platelet TF play a role in a pathological situation needs further examination. Platelets produce less TF than monocytes (0.24 ± 0.04 ng TF/ 2 × 10^8 fibrinogen adhered platelets versus 1075 ± 139 ng membrane associated TF 10^{-7} LPS-induced monocytes, Chapter 3, 4) and it takes 4 hours for platelets to express TF protein. An animal model of laser induced vessel wall injury [35] shows the first appearance of TF at about 100 sec after induction of vessel wall damage, which is well before the first sign of platelet TF synthesis. It is therefore not very likely that platelet TF plays a role in acute injury where it would have to start the coagulation cascade. However, one could argue that

platelet TF could contribute to the stability of a platelet plug due to its location at the inside of the thrombus. In addition, slowly expressed TF might contribute to the formation of a platelet plug over a longer period and might play a role in chronic pathological conditions, such as atherosclerosis. Diabetes increases the risk of developing lower extremity arterial disease 2-5 fold [36,37], which is one of the first signs of a generalized atherosclerotic disease. It is a major risk factor for lower-extremity amputation and cardiovascular events such as myocardial infarction, stroke and death. A key event in the pathogenesis of vascular complications of type 2 diabetes is loss of atherosclerotic plaque stability. When the fibrous cap is disrupted, proaggregatory substances in the subintima are exposed and a platelet plug forms. When the platelet plug is firmly attached to the vessel wall it can continuously increase in size until the lumen is completely obstructed by the platelet-rich thrombus. In other cases, it detaches before it occludes the vessel and flows downstream to peripheral vessels causing clinical events such as stroke. In the chronic process of arterial occlusive disease, the slow but consistent production of TF by platelets may well contribute to the propagation and stabilization of a thrombus. Indeed, previous studies have detected TF antigen and activity in the atherosclerotic plaque [38], which may be derived from platelets. The elevated TF synthesis in platelets from type 2 diabetes patients might result in a more stable clot, inducing occlusion of an artery at sites where atherosclerotic lesions have developed.

TISSUE FACTOR EXPRESSION IN MONOCYTES

Disturbed INS-R – Giα-2 complex formation in type 2 diabetes patients

In megakaryocytes, the induction of insulin resistance by adipokines was studied. These studies indicated that specific adipokines were capable of either disturbing phosphorylation or inducing degradation of IRS-1.

In contrast to previous literature [39], IRS-1 was detected in monocytic THP-1 cells and Tyr phosphorylated after stimulation with insulin, but no insulin-induced complex formation between IRS-1 and Giα-2 was found. In response to insulin, the INS-R and Giα-2 directly associated, with an optimum after 15 minutes. This finding suggests that insulin regulates cAMP control in monocytes through direct receptor - Giα-2 contact [40]. Other studies demonstrate in addition a direct association between INS-R and Giα-2. In adipocytes and rat hepatoma cell membranes,

heterotrimeric G-proteins, typical transducers of seven-helix receptors, are associated with the INS-R and involved in insulin signaling [41-43].
Complex formation was optimal after 15 minutes incubation with insulin and disturbed in monocytes from type 2 diabetes patients (Chapter 5). These findings suggest that the cause of insulin resistance in monocytes must be found in disturbed INS-R – Giα-2 association. SOCS proteins are a family of proteins capable of inhibiting JAK-STAT signaling in various tissues. Eight members have been identified so far. They have a central SH2 domain and SOCS-1 and SOCS-3 contain a kinase inhibitory region. SOCS-1 and -3 mediate downregulation of cytokine-induced JAK-STAT signaling. In addition to the JAK-STAT pathway, SOCS proteins also inhibit other signaling pathways, such as insulin signaling. SOCS proteins might present the link between elevated levels of adipokines and insulin resistance. Besides targeting IRS proteins for ubiquitination and degradation via the proteasomal complex, SOCS-1 and -3 interact with the phosphorylated INS-R, thereby preventing binding and activation of the IRS proteins (Figure 1).

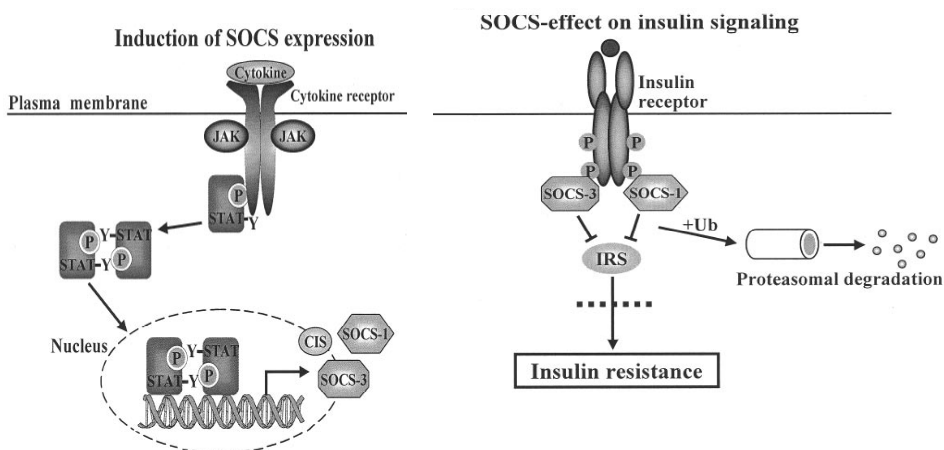


Figure 1. (a) Cytokine (adipokine)-induced activation of the JAK-STAT pathway results in expression of the family of SOCS proteins. (b). SOCS-1 and -3 inhibit insulin signaling. Adipokine-induced SOCS-1 and -3 interact with the phosphorylated INS-R, thereby preventing binding and activation of the IRS proteins. Moreover, the SOCS proteins target IRS-proteins for ubiquitination (Ub) and degradation via the proteasomal complex. Insulin signaling is thereby inhibited and insulin resistance induced (Source: Ronn *et al*, Diabetes, 2007).

SOCS-3 binds Tyr 960 on the INS-R, which is important for IRS-1 binding [44]. Inhibition by SOCS-1 is probably mediated through binding to the kinase domain of the INS-R, preventing further phosphorylation [45]. SOCS-1 and -3 expression in monocytes and macrophages has been reported [46,47], although not in relation with

insulin resistance. Because monocytes express functional Ob-R [48] it is likely that in these cells leptin induces SOCS expression, which disturbs the insulin-induced binding between the INS-R and Giα-2.

Obesity, adipokines and TF expression

Recent studies show that adipokines interfere with TF expression in monocytes and endothelial cells [49,50]. Incubation of monocytes with leptin for 6 hours induces TF activity and antigen in a dose-dependent fashion. In addition, in obese patients, plasma levels of TF and TF expression in resting and LPS-induced monocytes were increased compared to healthy donors. The effect was reversible; loss of body weight resulted in decreased leptin levels, which was accompanied by a reduction in plasma TF and TF expression in monocytes [49]. TF expression by leptin was mediated through JAK2 and PI3-K and involved TNF-α [51]. In endothelial cells, adiponectin inhibits TNF-α-induced TF expression which is accompanied by cAMP accumulation and blocked by PKA inhibition [50,52]. This suggests that a similar mechanism could be present in monocytes, but it has not been studied so far. In addition, leptin induces the production of proinflammatory cytokines, suggesting that adipokines induce hyperactivity observed in other responses than TF synthesis [48].

In addition to monocytes and platelets, other tissues could contribute to increased TF levels. Samad *et al* showed that TF gene expression was elevated in the brain, lung, kidney, heart, liver and adipose tissue of obese mice (ob/ob and db/db mice) compared to lean counterparts [53,54]. It is likely that under normal conditions insulin inhibits TF expression in these tissues by a similar mechanism as in platelets and monocytes and that in insulin-resistant subjects loss of insulin inhibition results in higher TF levels. Gi protein and cAMP are expressed in kidney epithelial cells, hippocampus and frontal cortex, lung fibroblasts, adipocytes, heart and liver and these proteins might regulate TF expression in reponse to insulin [55-59]. However, the increase in TF expression by extravascular cells does not contribute to a prothrombotic condition.

In the study described in Chapter 4 and 5, LPS is used as agonist to induce TF expression. In general, stimulation of monocytes with LPS induces TF surface expression and procoagulant activity. However, studies should be extended with other agonists, which play a role in the complications of diabetes. The effect of advanced glycosylation end products (AGEs) on TF expression and its mechanisms were studied by Ichikawa *et al* [60]. Incubation of human macrophage-like U937 cells and monocytes with AGE-albumin induced TF in a dose-dependent

manner. TF expression in monocytes from diabetes patients with increased levels of circulating AGEs was higher than in normal controls. This suggests that *in vivo* AGEs induce TF expression in monocytes in diabetes patients and thereby promote thrombosis.

DIFFERENCES IN TISSUE FACTOR REGULATION IN PLATELETS AND MONOCYTES

Differences in inhibiton by insulin

The regulation of TF synthesis by insulin differs between platelets and monocytes. In platelets, insulin regulates the splicing of TF pre-mRNA (Chapter 3), whereas in monocytes inhibition is regulated at the level of transcription (Chapter 4).

In platelets, insulin regulates the inhibition of TF pre-mRNA splicing by two different mechanisms. In addition to a direct effect of insulin on the release of ADP/TxA_2, inhibition of TF activity also takes place after the released products start further signaling through the P2Y12- and TxA_2 receptors, mediated through cAMP. Regulation of splicing by cAMP has been described, which could suggest a mechanism for insulin inhibition. PKAα and PKAβ, which are activated by an increase in cAMP concentration, phosphorylate SR proteins *in vitro* and change splice site selection *in vivo* [61]. More specifically, PKA phosphorylates polypyrimidine-tract binding (PTB) protein, an important regulator of alternative pre-mRNA splicing and mRNA localization, but so far the presence of PTB in platelets has not been described.

In monocytes, PKA induces the expression of several genes by phosphorylation of the cAMP response element-binding protein (CREB) and the subsequent recruitment of the co-activator CREB-binding protein (CBP). Besides association with CREB, CBP bind to the NF-κB subunit p65 *in vitro* and *in vivo*. Functional assays suggest that a competition occurs between CREB and p65 for limiting amounts of CBP, which can lead to a decrease in association of CBP with p65, thereby inhibiting NF-kB-dependent transcription [62]. In addition, insulin inhibition of TF in monocytes might be mediated through lowering of $[Ca^{2+}]_i$, while Ca^{2+} plays a role in the activation of NF-kB in RAW 264.7 cells and dendritic cells [63,64].

Differences in regulation by PKB

PKB contributes to phosphorylation of SR proteins, specifically SF2 [65], which

potentiate their binding to recently transcribed mRNA and/or directly stimulate SR protein activities. Interestingly, in monocytes TF expression is under negative control by the PKB pathway [66,67]. Here PKB inhibits NF-κB activity [67], such in contrast to anucleated platelets where PKB activates the splicing mechanism.

Clinical implications and future directions

The results in this study show that platelets and monocytes from type 2 diabetes patients are insulin-resistant, which might explain the prothrombotic situation in these patients. Type 2 diabetes patient have hyperactive platelets and increased levels of TF expressed by platelets and monocytes compared to healthy controls. The mechanism by which insulin resistance in platelets and monocytes develops has been characterized. Yet, the link between insulin resistance and hyperactivity remains unclear.

This study demonstrates that several adipokines play a role in the insulin resistance and hyperactivity of platelets. This suggests that in obese subjects, who have high adipokine levels, platelets are hyperactive. Indeed, obesity is associated with an elevated risk of cardiovascular morbidity and mortality due to atherothrombotic events [68,69]. Subjects affected by obesity and insulin resistance are at high risk of coronary, cerebrovascular and peripheral artery disease [70]. Prevention and treatment of cardiovascular events are a major challenge in these patients. Antiplatelet drugs could be of great benefit in these patients, but a decreased sensitivity to the platelet inhibitory effect of aspirin and clopidogrel in type 2 diabetes patients has been described.

Therefore the focus should be at changes of lifestyle habits, such as diet and physical exercise. This reduces visceral adipose tissue and improves insulin resistance and prevents or at least delayes the onset of type 2 diabetes, arterial hypertension and CVD [71-74]. Weight loss reduces chronic inflammation, oxidative stress and platelet activation [75,76].

The effects of adipokines on monocytes were not studied yet, but it is possible that insulin resistance and hyperactivity in monocytes from type 2 diabetes patients is caused by increased levels of adipokines. Interestingly, improvement of glycemic control, for example by weight reduction, induces a 1.5-fold reduction in circulating TF [77]. Therefore, weight loss in obese patients can be considered a very effective strategy to improve the platelet and monocyte abnormalities linked

to insulin resistance.

References

1. Nieto-Vazquez I, Fernandez-Veledo S, de Alvaro AC, Rondinone CM, Valverde AM, Lorenzo M. Protein-tyrosine phosphatase 1B-deficient myocytes show increased insulin sensitivity and protection against tumor necrosis factor-alpha-induced insulin resistance. Diabetes 2007; 56:404-413.
2. Xue B, Kim YB, Lee A, Toschi E, Bonner-Weir S, Kahn CR, Neel BG, Kahn BB. Protein-tyrosine phosphatase 1B deficiency reduces insulin resistance and the diabetic phenotype in mice with polygenic insulin resistance. J Biol Chem 2007; 282:23829-23840.
3. Lee YH, Giraud J, Davis RJ, White MF. c-Jun N-terminal kinase (JNK) mediates feedback inhibition of the insulin signaling cascade. J Biol Chem 2003; 278:2896-2902.
4. Aguirre V, Uchida T, Yenush L, Davis R, White MF. The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). J Biol Chem 2000; 275:9047-9054.
5. Rui L, Yuan M, Frantz D, Shoelson S, White MF. SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2. J Biol Chem 2002; 277:42394-42398.
6. Nakata M, Yada T, Soejima N, Maruyama I. Leptin promotes aggregation of human platelets via the long form of its receptor. Diabetes 1999; 48:426-429.
7. Konstantinides S, Schafer K, Koschnick S, Loskutoff DJ. Leptin-dependent platelet aggregation and arterial thrombosis suggests a mechanism for atherothrombotic disease in obesity. J Clin Invest 2001; 108:1533-1540.
8. Elbatarny HS, Maurice DH. Leptin-mediated activation of human platelets: involvement of a leptin receptor and phosphodiesterase 3A-containing cellular signaling complex. Am J Physiol Endocrinol Metab 2005; 289:E695-E702.
9. Dellas C, Schafer K, Rohm IK, Lankeit M, Leifheit M, Loskutoff DJ, Hasenfuss G, Konstantinides SV. Leptin signalling and leptin-mediated activation of human platelets: importance of JAK2 and the phospholipases Cgamma2 and A2. Thromb Haemost 2007; 98:1063-1071.
10. Nakata M, Maruyama I, Yada T: Leptin potentiates ADP-induced [Ca(2+)](i) increase via JAK2 and tyrosine kinases in a megakaryoblast cell line. Diabetes

Res Clin Pract 2005; 70:209-216.

11. Riba R, Hughes CE, Graham A, Watson SP, Naseem KM. Globular adiponectin induces platelet activation through the collagen receptor GPVI-Fc receptor gamma chain complex. J Thromb Haemost 2008; 6:1012-1020.
12. Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta K, Shimomura I, Nakamura T, Miyaoka K, Kuriyama H, Nishida M, Yamashita S, Okubo K, Matsubara K, Muraguchi M, Ohmoto Y, Funahashi T, Matsuzawa Y. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. Biochem Biophys Res Commun 1999; 257:79-83.
13. Kato H, Kashiwagi H, Shiraga M, Tadokoro S, Kamae T, Ujiie H, Honda S, Miyata S, Ijiri Y, Yamamoto J, Maeda N, Funahashi T, Kurata Y, Shimomura I, Tomiyama Y, Kanakura Y. Adiponectin acts as an endogenous antithrombotic factor. Arterioscler Thromb Vasc Biol 2006; 26:224-230.
14. Canavan B, Salem RO, Schurgin S, Koutkia P, Lipinska I, Laposata M, Grinspoon S. Effects of physiological leptin administration on markers of inflammation, platelet activation, and platelet aggregation during caloric deprivation. J Clin Endocrinol Metab 2005; 90:5779-5785.
15. Ozata M, Avcu F, Durmus O, Yilmaz I, Ozdemir IC, Yalcin A. Leptin does not play a major role in platelet aggregation in obesity and leptin deficiency. Obes Res 2001; 9:627-630.
16. Elbatarny HS, Netherton SJ, Ovens JD, Ferguson AV, Maurice DH. Adiponectin, ghrelin, and leptin differentially influence human platelet and human vascular endothelial cell functions: implication in obesity-associated cardiovascular diseases. Eur J Pharmacol 2007; 558:7-13.
17. Tran TT, Yamamoto Y, Gesta S, Kahn CR. Beneficial effects of subcutaneous fat transplantation on metabolism. Cell Metab 2008; 7:410-420.
18. Harris RB, Leibel RL: Location, location, location.. Cell Metab 2008; 7:359-361.
19. Cancello R, Tordjman J, Poitou C, Guilhem G, Bouillot JL, Hugol D, Coussieu C, Basdevant A, Bar HA, Bedossa P, Guerre-Millo M, Clement K. Increased infiltration of macrophages in omental adipose tissue is associated with marked hepatic lesions in morbid human obesity. Diabetes 2006; 55:1554-1561.
20. Fontana L, Eagon JC, Trujillo ME, Scherer PE, Klein S. Visceral fat adipokine secretion is associated with systemic inflammation in obese humans. Diabetes 2007; 56:1010-1013.
21. Warshaw AL, Laster L, Shulman NR. The stimulation by thrombin of glucose oxidation in human platelets. The Journal of Clinical Investigation 1966;

45:1923-34.

22. Weyrich AS, Lindemann S, Tolley ND, Kraiss LW, Dixon DA, Mahoney TM, Prescott SM, McIntyre TM, Zimmerman GA. Change in protein phenotype without a nucleus: translational control in platelets. Seminars in Thrombosis and Hemostasis 2004; 30:493-500.
23. Booyse FM, Rafelson ME Jr. Studies on human platelets. I. synthesis of platelet protein in a cell-free system. Biochim Biophys Acta 1968; 166:689-97.
24. Steiner M. Platelet protein synthesis studied in a cell-free system. Experientia 1970; 26:786-9.
25. Weyrich AS, Denis MM, Schwertz H, Tolley ND, Foulks J, Spencer E, Kraiss LW, Albertine KH, McIntyre TM, Zimmerman GA. mTOR-dependent synthesis of Bcl-3 controls the retraction of fibrin clots by activated human platelets. Blood 2007; 109:1975-1983.
26. Weyrich AS, Dixon DA, Pabla R, Elstad MR, McIntyre TM, Prescott SM, Zimmerman GA. Signal-dependent translation of a regulatory protein, Bcl-3, in activated human platelets. Proc Natl Acad Sci U S A 1985; 95:5556-5561.
27. Lindemann S, Tolley ND, Dixon DA, McIntyre TM, Prescott SM, Zimmerman GA, Weyrich AS. Activated platelets mediate inflammatory signaling by regulated interleukin 1beta synthesis. J Cell Biol 2001; 154:485-490.
28. Evangelista V, Manarini S, Di SA, Capone ML, Ricciotti E, Di FL, Tacconelli S, Sacchetti A, D'Angelo S, Scilimati A, Sciulli MG, Patrignani P. De novo synthesis of cyclooxygenase-1 counteracts the suppression of platelet thromboxane biosynthesis by aspirin. Circ Res 2006; 98:593-595.
29. Brogren H, Karlsson L, Andersson M, Wang L, Erlinge D, Jern S. Platelets synthesize large amounts of active plasminogen activator inhibitor 1. Blood 2004; 104:3943-3948.
30. Schwertz H, Tolley ND, Foulks JM, Denis MM, Risenmay BW, Buerke M, Tilley RE, Rondina MT, Harris EM, Kraiss LW, Mackman N, Zimmerman GA, Weyrich AS. Signal-dependent splicing of tissue factor pre-mRNA modulates the thrombogenicity of human platelets. J Exp Med 2006; 203:2433-2440.
31. Pillitteri D, Bassus S, Boller K, Mahnel R, Scholz T, Westrup D, Wegert W, Kirchmaier CM. Thrombin-induced interleukin 1beta synthesis in platelet suspensions: impact of contaminating leukocytes. Platelets 2007; 18:119-127.
32. Andonegui G, Kerfoot SM, McNagny K, Ebbert KV, Patel KD, Kubes P. Platelets express functional Toll-like receptor-4. Blood 2005; 106:2417-2423.
33. Cognasse F, Hamzeh H, Chavarin P, Acquart S, Genin C, Garraud O. Evidence of Toll-like receptor molecules on human platelets. Immunol Cell Biol 2005;

83:196-198.

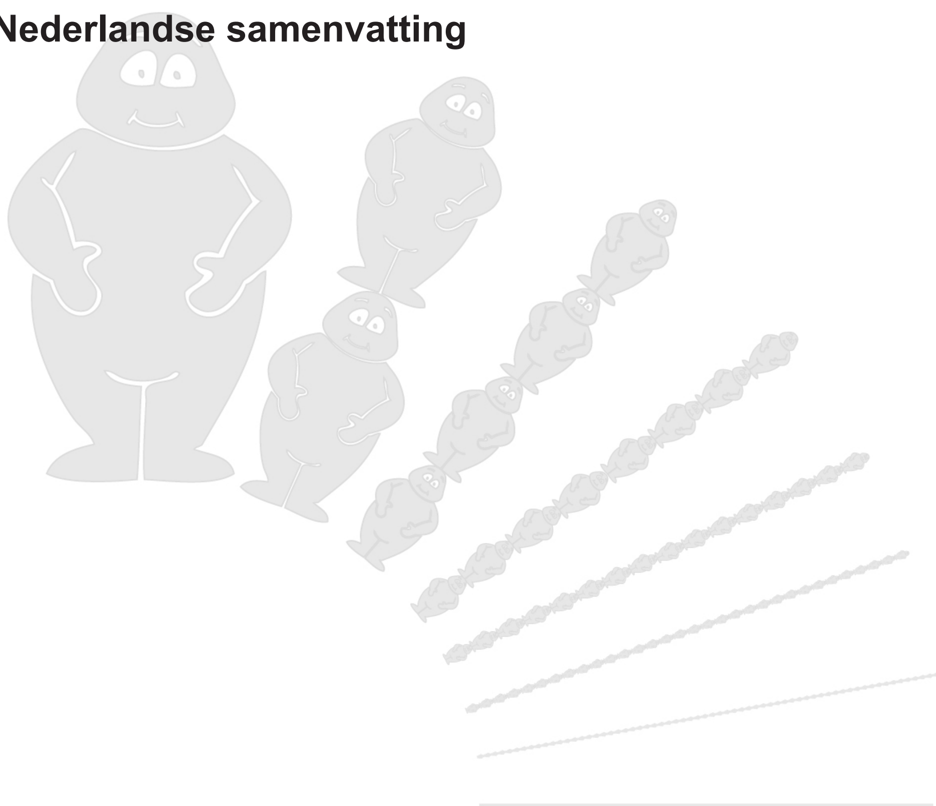
34. Shashkin PN, Brown GT, Ghosh A, Marathe GK, McIntyre TM. Lipopolysaccharide is a direct agonist for platelet RNA splicing. J Immunol 2008; 181:3495-3502.
35. Gross PL, Furie BC, Merrill-Skoloff G, Chou J, Furie B. Leukocyte-versus microparticle-mediated tissue factor transfer during arteriolar thrombus development. J Leukoc Biol 2005; 78:1318-1326.
36. Hirsch AT, Criqui MH, Treat-Jacobson D, Regensteiner JG, Creager MA, Olin JW, Krook SH, Hunninghake DB, Comerota AJ, Walsh ME, McDermott MM, Hiatt WR. Peripheral arterial disease detection, awareness, and treatment in primary care. JAMA 2001; 286:1317-1324.
37. Raffetto JD, Montgomery JE, Eberhardt RT, LaMorte WW, Menzoian JO. Differences in risk factors for lower extremity arterial occlusive disease. J Am Coll Surg 2005; 201:918-924.
38. Marmur JD, Thiruvikraman SV, Fyfe BS, Guha A, Sharma SK, Ambrose JA, Fallon JT, Nemerson Y, Taubman MB. Identification of active tissue factor in human coronary atheroma. Circulation 1996; 94:1226-1232.
39. Iida KT, Suzuki H, Sone H, Shimano H, Toyoshima H, Yatoh S, Asano T, Okuda Y, Yamada N. Insulin inhibits apoptosis of macrophage cell line, THP-1 cells, via phosphatidylinositol -3-kinase-dependent pathway: Arterioscler Thromb Vasc Biol 2002; 22:380-6.
40. Gerrits AJ, Koekman CA, Yildirim C, Nieuwland R, Akkerman JW. Insulin inhibits tissue factor expression in monocytes. J Thromb Haemost 2009; 7:198-205.
41. Krieger-Brauer HI, Medda PK, Kather H. Insulin-induced activation of NADPH-dependent H2O2 generation in human adipocyte plasma membranes is mediated by Galphai2. J Biol Chem 1997; 272:10135-10143.
42. Sanchez-Margalet V, Gonzalez-Yanes C, Santos-Alvarez J, Najib S. Insulin activates G alpha il,2 protein in rat hepatoma (HTC) cell membranes. Cell Mol Life Sci 1999; 55:142-147.
43. Imamura T, Vollenweider P, Egawa K, Clodi M, Ishibashi K, Nakashima N, Ugi S, Adams JW, Brown JH, Olefsky JM. G alpha-q/11 protein plays a key role in insulin-induced glucose transport in 3T3-L1 adipocytes. Mol Cell Biol 1999; 19:6765-6774.
44. Emanuelli B, Peraldi P, Filloux C, Sawka-Verhelle D, Hilton D, Van Obberghen E. SOCS-3 is an insulin-induced negative regulator of insulin signaling. J Biol Chem 2000; 275:15985-15991.

45. Ueki K, Kondo T, Kahn CR. Suppressor of cytokine signaling 1 (SOCS-1) and SOCS-3 cause insulin resistance through inhibition of tyrosine phosphorylation of insulin receptor substrate proteins by discrete mechanisms. Mol Cell Biol 2004; 24:5434-5446.
46. Dickensheets HL, Venkataraman C, Schindler U, Donnelly RP. Interferons inhibit activation of STAT6 by interleukin 4 in human monocytes by inducing SOCS-1 gene expression. Proc Natl Acad Sci U S A 1999; 96:10800-10805.
47. Berlato C, Cassatella MA, Kinjyo I, Gatto L, Yoshimura A, Bazzoni F. Involvement of suppressor of cytokine signaling-3 as a mediator of the inhibitory effects of IL-10 on lipopolysaccharide-induced macrophage activation. J Immunol 2002; 168:6404-6411.
48. Zarkesh-Esfahani H, Pockley G, Metcalfe RA, Bidlingmaier M, Wu Z, Ajami A, Weetman AP, Strasburger CJ, Ross RJ. High-dose leptin activates human leukocytes via receptor expression on monocytes. J Immunol 2001; 167:4593-4599.
49. Napoleone E, Di Santo A, Amore C, Baccante G, Di Febbo C, Porreca E, de Gaetano G, Donati MB, Lorenzet R. Leptin induces tissue factor expression in human peripheral blood mononuclear cells: a possible link between obesity and cardiovascular risk? J Thromb Haemost 2007; 5:1462-1468.
50. Chen YJ, Zhang LQ, Wang GP, Zeng H, Lu B, Shen XL, Jiang ZP, Chen FP. Adiponectin inhibits tissue factor expression and enhances tissue factor pathway inhibitor expression in human endothelial cells. Thromb Haemost 2008; 100:291-300.
51. Rafail S, Ritis K, Schaefer K, Kourtzelis I, Speletas M, Doumas M, Giaglis S, Kambas K, Konstantinides S, Kartalis G. Leptin induces the expression of functional tissue factor in human neutrophils and peripheral blood mononuclear cells through JAK2-dependent mechanisms and TNFalpha involvement. Thromb Res 2008; 122:366-375.
52. Ouchi N, Kihara S, Arita Y, Okamoto Y, Maeda K, Kuriyama H, Hotta K, Nishida M, Takahashi M, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Funahashi T, Matsuzawa Y. Adiponectin, an adipocyte-derived plasma protein, inhibits endothelial NF-kappaB signaling through a cAMP-dependent pathway. Circulation 2002; 102:1296-1301.
53. Samad F, Pandey M, Loskutoff DJ. Regulation of tissue factor gene expression in obesity. Blood 2001; 98:3353-3358.
54. Samad F, Pandey M, Loskutoff DJ. Tissue factor gene expression in the adipose tissues of obese mice. Proc Natl Acad Sci U S A 1998; 95:7591-7596.

55. Valenti G, Procino G, Liebenhoff U, Frigeri A, Benedetti PA, Ahnert-Hilger G, Nurnberg B, Svelto M, Rosenthal W. A heterotrimeric G protein of the Gi family is required for cAMP-triggered trafficking of aquaporin 2 in kidney epithelial cells. J Biol Chem 1998; 273:22627-22634.
56. Maines LW, Polavarapu R, Lakoski JM. Expression of brain Gi protein in the aging F344 rat following exposure to corticosterone. Int J Dev Neurosci 1998; 16:341-346.
57. Liu X, Sun SQ, Ostrom RS. Fibrotic lung fibroblasts show blunted inhibition by cAMP due to deficient cAMP response element-binding protein phosphorylation. J Pharmacol Exp Ther 2005; 315:678-687.
58. Wesslau C, Smith U. The inhibitory GTP-binding protein (Gi) regulates the agonistic property of beta-adrenergic ligands in isolated rat adipocytes. Evidence for a priming effect of cyclic AMP. Biochem J 1992; 288 (Pt 1):41-46.
59. McCloskey DT, Turcato S, Wang GY, Turnbull L, Zhu BQ, Bambino T, Nguyen AP, Lovett DH, Nissenson RA, Karliner JS, Baker AJ. Expression of a Gi-coupled receptor in the heart causes impaired Ca2+ handling, myofilament injury, and dilated cardiomyopathy. Am J Physiol Heart Circ Physiol 2008; 294:H205-H212.
60. Ichikawa K, Yoshinari M, Iwase M, Wakisaka M, Doi Y, Iino K, Yamamoto M, Fujishima M. Advanced glycosylation end products induced tissue factor expression in human monocyte-like U937 cells and increased tissue factor expression in monocytes from diabetic patients. Atherosclerosis 1998; 136:281-287.
61. Kvissel AK, Orstavik S, Eikvar S, Brede G, Jahnsen T, Collas P, Akusjarvi G, Skalhegg BS. Involvement of the catalytic subunit of protein kinase A and of HA95 in pre-mRNA splicing. Exp Cell Res 2007; 313:2795-2809.
62. Parry GC, Mackman N. Role of cyclic AMP response element-binding protein in cyclic AMP inhibition of NF-kappaB-mediated transcription. J Immunol 1997; 159:5450-5456.
63. Martin L, Pingle SC, Hallam DM, Rybak LP, Ramkumar V. Activation of the adenosine A3 receptor in RAW 264.7 cells inhibits lipopolysaccharide-stimulated tumor necrosis factor-alpha release by reducing calcium-dependent activation of nuclear factor-kappaB and extracellular signal-regulated kinase 1/2. J Pharmacol Exp Ther 2006; 316:71-78.
64. Lyakh LA, Koski GK, Telford W, Gress RE, Cohen PA, Rice NR. Bacterial lipopolysaccharide, TNF-alpha, and calcium ionophore under serum-free conditions promote rapid dendritic cell-like differentiation in CD14+ monocytes through distinct pathways that activate NK-kappa B. J Immunol 2000;165:3647-

3655.
65. Blaustein M, Quadrana L, Risso G, Mata ML, Pelisch F, Srebrow A. SF2/ASF regulates proteomic diversity by affecting the balance between translation initiation mechanisms. J Cell Biochem 2009; 107:826-833.
66. Gerrits AJ, Koekman CA, Yildirim C, Nieuwland R, Akkerman JW. Insulin inhibits tissue factor expression in monocytes. J Thromb Haemost 2009; 7:198-205.
67. Guha M, Mackman N. The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. J Biol Chem 2002; 277:32124-32132.
68. National Task Force on the Prevention and Treatment of Obesity. Dieting and the development of eating disorders in overweight and obese adults. Arch Intern Med 2000; 160:2581-2589.
69. Despres JP. Cardiovascular disease under the influence of excess visceral fat. Crit Pathw Cardiol 2007; 6:51-59.
70. Moreno PR, Fuster V. New aspects in the pathogenesis of diabetic atherothrombosis. J Am Coll Cardiol 2004; 44:2293-2300.
71. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). JAMA 2001; 285:2486-2497.
72. Daskalopoulou SS, Mikhailidis DP, Elisaf M. Prevention and treatment of the metabolic syndrome. Angiology 2004; 55:589-612.
73. Getz GS, Reardon CA. Nutrition and cardiovascular disease. Arterioscler Thromb Vasc Biol 2007; 27:2499-2506.
74. Giugliano D, Esposito K. Mediterranean diet and metabolic diseases. Curr Opin Lipidol 2008; 19:63-68.
75. Anfossi G, Russo I, Trovati M. Platelet resistance to the anti-aggregating agents in the insulin resistant states. Curr Diabetes Rev 2006; 2:409-430.
76. Davi G, Guagnano MT, Ciabattoni G, Basili S, Falco A, Marinopiccoli M, Nutini M, Sensi S, Patrono C. Platelet activation in obese women: role of inflammation and oxidant stress. JAMA 2002; 288:2008-2014.
77. Sambola A, Osende J, Hathcock J, Degen M, Nemerson Y, Fuster V, Crandall J, Badimon JJ. Role of risk factors in the modulation of tissue factor activity and blood thrombogenicity. Circulation 2003; 107:973-7.

Nederlandse samenvatting



Achtergrond

Diabetes mellitus

Diabetes mellitus, ook wel suikerziekte genoemd, is een chronische stofwisselingsziekte die wordt gekenmerkt door hoge bloedglucosewaarden. De alvleesklier is niet langer in staat om insuline te produceren (type 1) of de weefsels en cellen van diabetes patiënten worden ongevoelig voor insuline – insuline resistentie (type 2). Patiënten met type 1 hebben een absoluut tekort aan insuline, veroorzaakt door de afbraak van de beta cellen van de alvleesklier door het eigen afweersysteem. Patiënten met type 2 maken meestal in het begin van hun aandoening nog zelf insuline aan, maar door een ongevoeligheid van weefsels voor insuline is het hormoon niet in staat het bloedglucose te verlagen. Type 2 diabetes is ook wel bekend als ouderdomsdiabetes, maar tegenwoordig zijn er al kinderen die te maken hebben met deze vorm van diabetes. Insuline resistentie kan onstaan door obesitas en weinig te bewegen. Andere typen van diabetes zijn zwangerschapsdiabetes en specifieke vormen die een erfelijke achtergrond kunnen hebben zoals MODY (Maturity Onset Diabetes of the Young).

Diabetes wordt een epidemie; wereldwijd zijn er momenteel 246 miljoen diabetes patiënten en de verwachting is dat er in 2025 totaal 380 miljoen patiënten zijn, een stijging van 7 miljoen patiënten per jaar. In Nederland zijn er 740.000 mensen die diabetes hebben en met nog minstens 250.000 mensen bij wie diabetes nog niet is geconstateerd komt het totale aantal diabetes patiënten boven de miljoen uit. Negen op de tien mensen hebben type 2 diabetes. Obesitas is een grote risicofactor voor type 2 diabetes. Met de toename van het aantal mensen met obesitas neemt daarom ook het aantal diabetes patiënten toe. De Wereldgezondheidsorganisatie schat dat er momenteel 400 miljoen mensen met obesitas zijn en dat in 2015 dat aantal is gestegen naar 700 miljoen.

Insuline speelt een belangrijke rol in diabetes. Insuline is een hormoon dat door de alvleesklier wordt uitgescheiden ten gevolge van verhoging van het glucosegehalte in het bloed na het eten van een maaltijd. Insuline verlaagt het bloedglucose doordat het de opname van glucose in weefsel bevordert. Glucose is onmisbaar, want het geeft het lichaam energie om een goede functie van organen te waarborgen. Door het tekort aan insuline en/of de insuline resistentie heeft het lichaam moeite om glucose uit het bloed in de weefsels te laten opnemen.

Diabetes gaat veelal gepaard met aandoeningen van de kleine en grote bloedvaten. Bij stoornissen van de kleine bloedvaten worden met name ogen, zenuwen en nieren aangedaan. Stoornissen van de grote bloedvaten uiten zich in hart-

en herseninfarcten, en perifeer vaatlijden. Dit laatste leidt dikwijls tot amputaties van ledematen. Hart- en vaatziekten zijn de voornaamste doodsoorzaken van patiënten met diabetes.

Trombose

Door te sterk schommelende bloedglucosewaarden en het verminderd werkzaam zijn van insuline in diabetes patiënten kan de bloedstroom belemmerd worden; trombose. In diabetes is de balans tussen handhaving van de bloedstroom en trombose verstoort, waardoor de cellen die verantwoordelijk zijn voor deze balans gemakkelijker bijdragen aan trombose. Deze betrokken cellen zijn bloedplaatjes die na activatie wondjes kunnen afdichten, endotheelcellen die de binnenkant van bloedvaten bekleden en monocyten die een belangrijke bron van het stollingseiwit 'weefselfactor' (tissue factor) zijn. Als plaatjes en stolling actief worden zonder dat er sprake is van een wond spreekt men van trombose. Trombose kan leiden tot acute verstopping van een bloedvat. Bekende voorbeelden hiervan zijn het hart- en herseninfarct.

Bloedplaatjes zijn kleine cellen die in het bloed circuleren en belangrijk zijn bij het stelpen van een bloeding na een beschadiging van een bloedvatwand. Ze worden geproduceerd door megakaryocyten. Bloedplaatjes hebben geen kern en zijn in rustende toestand schijfvormig. Door activatie verandert de schijfvormige gedaante van het bloedplaatje in een vorm met uitsteekels. Onder normale omstandigheden circuleren ze in de bloedbaan zonder vast te plakken aan de cellen van de wand. Wanneer een bloedvat beschadigd raakt, wordt de binnenkant van het bloedvat blootgesteld aan de bloedstroom. Bloedplaatjes hechten zich aan het vat, raken in geactiveerde toestand, en kunnen vervolgens andere bloedplaatjes in het bloed aantrekken en binden. Dit leidt tot verklontering van de bloedplaatjes en resulteert in de vorming van een prop die de wond dicht en bloedverlies voorkomt. Bloedplaatjes worden geholpen door stollingseiwitten. Bloedstolling komt op gang door weefselfactor, een eiwit dat op de buitenkant van witte bloedcellen, monocyten, zit of uit de vaatwand naar buiten lekt. Dit eiwit start de stolling en het eindproduct is fibrine, een polymeer dat door de bloedplaatjesprop geweven wordt voor versteviging. Zowel bloedplaatjes verklontering als bloedstolling zijn belangrijk voor de vorming van een bloedprop (trombus).

Dit proefschrift - samenvatting en conclusies

In een voorgaand project is er een nieuw mechanisme ontdekt waarbij insuline de aktiviteit van bloedplaatjes remt. Bloedplaatjes worden geaktiveerd door stoffen

die vrijkomen na beschadiging van de vaatwand. Insuline remt dit proces door een eiwit (Gi) te remmen, waardoor de aktivatie prikkel minder wordt en de bloedplaatjes minder snel verklonteren. Bloedplaatjes van patiënten met diabetes type 2 zijn ongevoelig geworden voor de remming door insuline en daardoor hyperactief, wat eerder leidt tot verklontering.
In dit proefschrift beschrijven we hoe de ongevoeligheid voor insuline in bloedplaatjes kan ontstaan en onderzoeken we welke gevolgen insuline resistentie in bloedplaatjes en monocyten hebben.

Verlies van gevoeligheid van insuline door stoffen uit vet
Een groot percentage van type 2 diabetes patiënten heeft overgewicht of is obees. Vetweefsel produceert en geeft stoffen en hormonen af genaamd adipokines. Inmiddels zijn er al meer dan 50 adipokines bekend. Adipokines hebben onder normale omstandigheden verschillende functies in het lichaam. Bij verhoogde waarden van deze adipokines, in overgewicht of obesitas, hebben ze ook effecten op andere celtypen en kunnen ze daarbij de functie van de cel veranderen. Incubatie van sommige adipokines leidt tot verlies van insuline gevoeligheid (insuline resistentie) in cellen. We hebben een aantal adipokines getest om te onderzoeken of ze insuline resistentie kunnen induceren in megakaryocyten. Er zijn er een aantal die wel (leptine, resistine, PAI-1 en RBP4) en niet (visfatin, IL-6 en TNF-α) insuline resistentie induceren in megakaryocyten. Na een korte incubatietijd veranderen leptine of resistine de activatiestatus van een belangrijke eiwit in de insuline signaleringsweg genaamd IRS-1, waardoor de insuline weg ‘uit’ staat. Na een lange incubatietijd met leptine of resistine blijkt het eiwit IRS-1 in megakaryocyten afgebroken te zijn. En consistent met deze resultaten vinden we dat in bloedplaatjes van type 2 diabetes patiënten waarden van IRS-1 verlaagd zijn vergeleken met bloedplaatjes van gezonde personen. Deze resultaten suggereren dat in obese, type 2 diabetes patiënten megakaryocyten verkeerde, insuline resistente bloedplaatjes produceren, die te snel actief worden.

Insuline resistentie in bloedplaatjes
Voorheen werd gedacht dat bloedplaatjes geen eiwitten konden produceren omdat ze een kern missen. Echter, kortgeleden is gebleken dat bloedplaatjes weefselfactor aanmaken doordat ze de codering voor het eiwit (messenger RNA) mee-krijgen van de megakaryocyt. Als bloedplaatjes actief worden, wordt deze codering omgezet in het eiwit weefselfactor. Op deze manier kunnen bloedplaatjes die de wond in een vaatwand dichten, er zelf voor zorgen dat de stolling

actief blijft en de stollingsvezels de trombus blijven verstevigen. We hebben de productie van weefselfactor in bloedplaatjes onderzocht en bevestigen dat bloedplaatjes inderdaad weefselfactor aanmaken. Toevoeging van bloedplaatjes remmers die de werking van de medicijnen aspirine en clopidogrel nabootsen remmen de hoeveelheid weefselfactor. Dit wijst erop dat we kunnen interfereren met de hoeveelheid weefselfactor. Experimenten met remmers van belangrijke signaleringswegen in bloedplaatjes wijzen erop dat het eiwit genaamd cyclic AMP betrokken is in de synthese van weefselfactor. Insuline verhoogt de hoeveelheid cyclic AMP in bloedplaatjes, daarom hebben we het effect van insuline op weefselfactor bestudeerd. In gezonde personen remt insuline de hoeveelheid weefselfactor, echter in bloedplaatjes van type 2 diabetes patiënten is de remming door insuline verdwenen, wat resulteert in verhoogde waarden van weefselfactor in bloedplaatjes van type 2 diabetes patiënten. Dit is een mogelijke verklaring voor het verhoogde risico op trombose in type 2 diabetes patiënten. Omdat het lang duurt voordat weefselfactor wordt gemaakt door bloedplaatjes suggeren we dat dit een rol speelt in trombose geinduceerd door chronische processen, die met name plaats vinden in de vaten van de benen.

Insuline resistentie in monocyten

Naar aanleiding van de bevindingen dat insuline de activiteit van bloedplaatjes remt en deze remming is verdwenen in bloedplaatjes van type 2 diabetes patiënten, zijn we gaan onderzoeken of een belangrijke bron van weefselfactor, monocyten, ook geremd kan worden door insuline en of deze remming is verdwenen bij diabetes type 2 patiënten. Monocyten maken bij activering een eiwit aan, weefselfactor. Insuline remt de productie van weefselfactor in monocyten. Na verder onderzoek blijkt dat het effect van insuline is veroorzaakt door binding van insuline aan een specifieke receptor. Deze receptor bindt aan het Gi eiwit en remt de activiteit hiervan. Remming van Gi zorgt ervoor dat het niveau van cyclic AMP, een mediator in monocyten, verhoogd wordt, dat vervolgens resulteert in een remming van de hoeveelheid weefselfactor. Verder heeft het calcium niveau in de cel een effect op de hoeveelheid weefselfactor. Door een verlaging van het calcium niveau zien we een remming van de hoeveelheid weefselfactor. Deze resultaten laten zien dat in monocyten van gezonde personen insuline een remming geeft van weefselfactor synthese. In type 2 patiënten is de remming door insuline verloren wat resulteert in hogere waarden van weefselfactor in monocyten vergeleken met die van gezonde personen. Een verklaring van de insuline resistentie is de verminderde associatie tussen de receptor waar insuline aan bindt en het Gi eiwit.

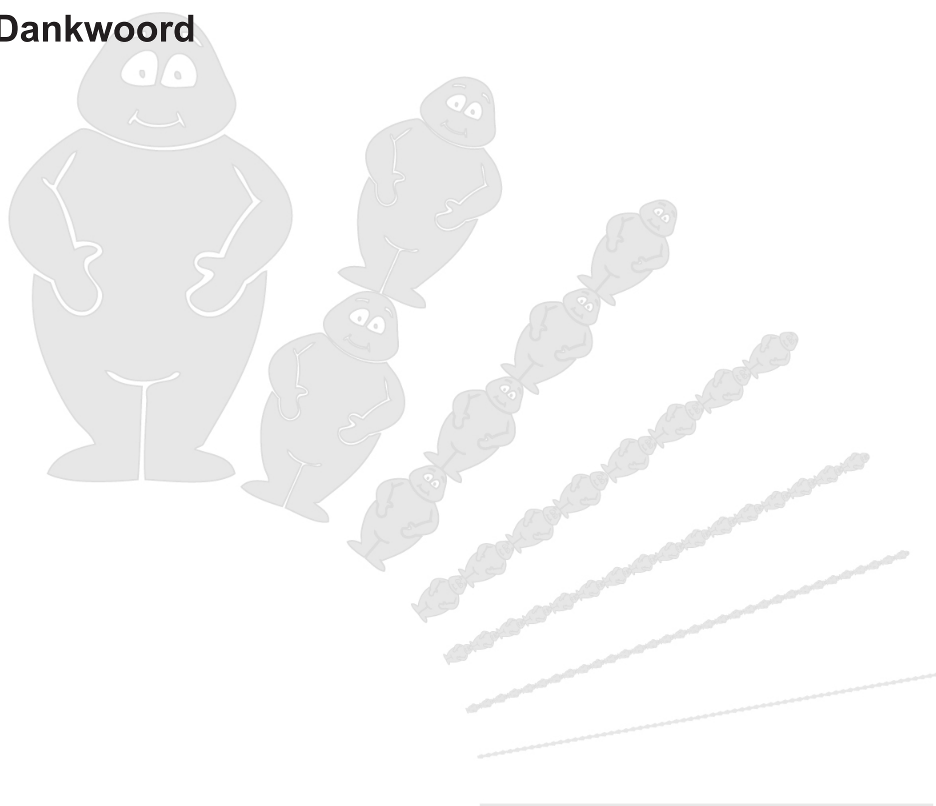
Insuline remt bloedplaatjes activatie

In een respons op een artikel waarin wordt beweerd dat insuline geen directe effecten op bloedplaatjes heeft, laten we zien dat het belangrijk is hoe de bloedplaatjes worden geïsoleerd in het laboratorium en weerleggen we deze bevindingen. Als je bloedplaatjes op de juiste manier isoleert remt insuline de activatie van bloedplaatjes.

Conclusies

Type 2 diabetes patiënten hebben een verhoogd risico op hart- en vaatziekten. Tachtig procent van alle patiënten overlijdt aan de gevolgen van trombose. Dit kan verklaard worden door insuline resistente bloedplaatjes en monocytes in type 2 diabetes patiënten die sneller actief worden. Stoffen uit vet spelen een belangrijke rol bij het insuline resistent worden van bloedplaatjes en vermoedelijk ook van andere cellen. Insuline resistentie in bloedplaatjes en monocyten kan leiden tot hogere waarde van weefselfactor, waardoor er eerder stolling plaatsvindt in type 2 diabetes patiënten.

Dankwoord



Dankwoord

Graag wil ik hierbij iedereen bedanken die op welke manier dan ook heeft bijgedragen aan dit proefschrift. Van een opmerking tijdens een werkbespreking of zomaar in de gang dat leidde tot nieuwe inzichten of praktische tips tot sociale activiteiten waardoor ik juist eens de materie van diabetes, plaatjes en tissue factor kon vergeten. De volgende mensen wil ik met name noemen:

Jan-Willem, onze dinsdagochtend-werkbesprekingen waren elke week weer een feestje. Waar ik in het begin nog ietwat verlegen je kamer binnenstapte, was ik op het eind niet te stoppen in discussies die menigmaal bijna de lunch haalden. De onderwerpen gingen alle kanten op, van een half uurtje nonsense verhalen tot bijna niet te bevatten signaleringswegen. Behalve filosoferen over *in vivo* situaties en discussies over experimenten heb ik dikwijls dubbel gelegen van het lachen. Dank dat je me hebt laten zien hoe leuk wetenschap kan zijn.
Timon, wat was uw hulp welkom en wat ging ons onderzoek als een trein opeens. Dank voor al uw patientenwerk, kennis en snelle correcties. En uw grote enthousiasme.
Flip, dank voor een plek op je afdeling.

Collega's van de afgelopen 4 jaren, dank voor al jullie hulp en gezelligheid. Beginnend op lab II waar ik voluit meezong op de cd's van K's Choice, heb leren meezingen met Queens of the Stone Age en tussen het pipetteren door bakkies op de bench dronk! Het heen en weer gesjouw naar lab III met alle blotapparatuur, waardoor ik toch besmet werd met het plaatjeswerk. En eindigend in de AIO-kamer met zijn kwart voor 3 theepauzes en het oude computertje dat ik me mocht toe-eigenen omdat toch niemand er meer op wilde werken.
Mijn paranimfen Arnold en Eelo. *Arnold*, gezien het feit dat je naam op elk artikel/manuscript staat heb ik het gevoel dat het meer onze promotie is dan alleen de mijne. Feilloos kon je switchen tussen de projecten en het bleef niet alleen bij het praktische werk. Met jouw denkwerk heeft menig project een flinke boost gekregen. Dank voor het altijd rustig blijven terwijl ik heel ongeduldig -en dat heel duidelijk liet merken!- over je schouder meekeek En voor; regeren is vooruitzien!! of is het nu: vooruitzien is regeren?? *Eelo*, tijdens je stage werd het me al snel duidelijk dat we meer aan het samenwerken waren dan ik jou aan het begeleiden. Het project ging als een trein en is nu een pareltje in dit proefschrift. Dank voor je talloze Ca^{2+} metingen op dat oude beestje.

Naast Eelo mijn andere studenten; *Cansu*, dank voor je vele monocyten-blotjes, en *Ellen*, ik heb je het een-en-ander moeten afleren, maar vond onze samenwerking leuk en je zelfstandigheid groot en ik geloof nog steeds in je studentenproject!

Joost, dank voor de dikke mannetjes!

Ester, vriendinnetje van het eerste uur, dankjewel voor je correcties!

Zomaar een hele bijzonder familie erbij krijgen lijkt wel een groot kado. *Marga*, *Amanda* en *Lennart*, wat fijn dat ik jullie in mijn leven heb. En wat was het heerlijk ontspannen met champagne in de zon zo vlak voor mijn grote deadline!

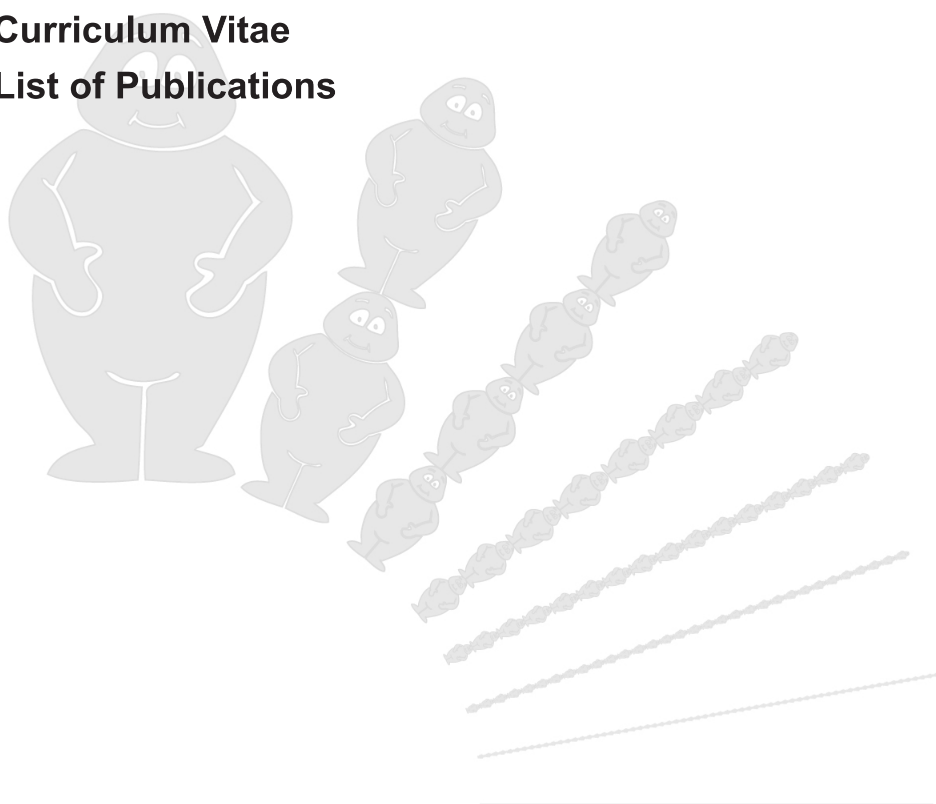
Henri en *Simone*, dank voor een fijn thuisfront en de grootste schatten die ik ken; Silvan en Robine.

Lieve *zus*, altijd een groot voorbeeld. Ik ben je gevolgd naar het VWO, de pianolessen, maar met de studie hield ik op. Nu ben je mij gevolgd. Samen als Gerrits-zusjes naar de EASD. Ik vind je heel bijzonder; jouw kracht en doorzettingsvermogen zijn onvoorstelbaar groot, dat heb je laten zien en nog steeds.

Mijn lieve *ouders*. Onvoorwaardelijk, dat woord komt in me op als ik aan jullie denk. Dat jullie er altijd voor me zijn. Al vroeg in mijn leven werd me dat duidelijk toen ik de keuze moest maken voor de middelbare school. En het is nooit meer anders geweest. Dank voor het altijd meeleven en jullie steun!

Lieve *Ivar*, niemand anders weet hoeveel ik echt kan praten. Dat zegt alles over hoe fijn ik me voel bij jou, maar ik besef me dat getetter over tissue factor, adipokines en submissies anders kan overkomen. En als ik maar even denk dat je niet oplet, kun je me feilloos herhalen. Dank voor je luisterend oor!

Curriculum Vitae
List of Publications



CURRICULUM VITAE

De schrijfster van dit proefschrift werd geboren op 24 september 1980 te Hardenberg. Na het behalen van het VWO diploma aan het Vechtdal College te Hardenberg, begon zij in 1998 met de studie Biomedische Wetenschappen, voorheen Medische Biologie aan de Universiteit Utrecht. Als onderdeel van deze studie werd een onderzoeksstage voltooid bij de afdeling Fysiologische Chemie, Universiteit Utrecht onder begeleiding van Dr. M.A. Essers en Prof. Dr. B.M. Burgering. Vervolgens doorliep zij een stage bij Moleculaire Celbiologie, Neurale ontwikkelingsbiologie, Leids Universitair Medisch Centrum bij Dr. L.G. Fradkin en Prof. Dr. J.N. Noordermeer. Het doctoraal examen werd in 2004 behaald.
Van mei 2005 tot september 2009 was zij werkzaam als Assistent in Opleiding bij de afdeling Klinische Chemie en Haematologie van het Universitair Medisch Centrum Utrecht. Het in dit proefschrift beschreven onderzoek werd uitgevoerd onder leiding van Prof. Dr. J.W. Akkerman.

List of publications

Gerrits AJ, Koekman CA, Yildirim C, Nieuwland R, Akkerman JW. Insulin inhibits tissue factor expression in monocytes. J Thromb Haemost. 2009; 7:198-205

Akkerman JW, Gerrits AJ, Ferreira IA, Heemskerk JW. Insulin inhibition on platelet-endothelial interaction is mediated by insulin effects on endothelial cells without direct effects on platelets: a rebuttal. J Thromb Haemost. 2009; 7:369-71

Gerrits AJ, Koekman CA, van Haeften TW, Akkerman JW. Enhanced platelet tissue factor synthesis in type 2 diabetes patients. Diabetes, accepted pending modifications

Gerrits AJ, Gitz E, Koekman CA, Visseren FL, van Haeften TW, Akkerman JW. The adipokines resistin, leptin, PAI-1 and RBP-4 induce degradation of IRS-1 in megakaryocytes and production of insulin-resistant platelets. Submitted

Gerrits AJ, Koekman CA, van Haeften TW, Akkeman JW. Increased tissue factor expression in type 2 diabetes monocytes in an insulin-rich environment. Submitted

Other publications

Gerrits J, Akkerman J. Insulin inhibits tissue factor expression in monocytes. J Thromb Haemost 2007; 5 Supplement 2: Abstract P-S-646

Gerrits J, Akkerman JW. Insulin inhibits tissue factor expression in monocytes Diabetologia 2007, Volume 50, Supplement 1: Abstract PS128-1247

Gerrits AJ, Gitz E, Koekman CA, Akkerman JWN. Reversible induction of insulin resistance by adipokines in human megakaryocytes Diabetologia 2008, Volume 51, Supplement 1: Abstract OP34-199

Gerrits AJ, Gitz E, Koekman CA, Visseren FL, Akkerman JWN. Induction of insulin resistance by adipokines in human megakaryocytes. Journal of Thrombosis and Haemostasis 2009; Volume 7, Supplement 2: Abstract PP-MO-007

Gerrits AJ, Koekman CA, Akkerman JWN. Tissue factor pre-mRNA splicing in platelets from healthy subjects and type 2 diabetes mellitus patients. Journal of Thrombosis and Haemostasis 2009; Volume 7, Supplement 2: Abstract PP-WE-024

Gerrits AJ, Gitz E, Koekman CA, Visseren FL, de Valk HW, Akkerman JWN. Adipokines induce degradation of IRS-1 in megakaryocytes resulting in insulin-resistant platelets Diabetologia 2009, Volume 52, Supplement 1: Abstract OP6-35

Akkerman JWN, Koekman CA, de Valk HW, Gerrits AJ. Tissue Factor pre-mRNA splicing in platelets from healthy subjects and type 2 diabetes mellitus patients Diabetologia 2009, Volume 52, Supplement 1: Abstract OP14-81