Institute of Physiology

University of Fribourg (Switzerland)

Regulation of Tissue Factor Expression

Implications for Coronary Artery Disease

THESIS

Submitted to the Faculty of Sciences of the University of Fribourg

(Switzerland) to obtain the degree of *Doctor rerum naturalium*

Presented by

Giovanni G. Camici

from

Italy

Dissertation Nr. 1543

Printed by the Studentendruckerei

From the Faculty of Sciences of the Institute of Physiology

University of Fribourg, Switzerland, accepted upon application by:

Prof. Dr. Zhihong Yang, Supervisor of the thesis,

Prof. Dr. Daniel Hayoz

Prof. Dr. Jean-Pierre Montani

Prof. Dr. Thomas F. Lüscher

Prof. Dr. Eric Rouiller, President of the jury

Fribourg, Nov. 7th 2006

The thesis supervisor: The Dean:

(Prof. Dr. Zhihong Yang) (Prof. Dr. Titus Jenny)

Acknowledgments

My first thought goes to the two people who made this work possible. Prof Felix C. Tanner taught me what science is and with how much carefulness research projects must be developed. Prof T. Lüscher, with his endless knowledge and enthusiasm, always pointed to me the right direction and kept my passion for this field alive.

In addition I would like to thank Prof Z. Yang who guided me towards the successful completion of my PhD thesis. My gratitude also goes to my family who supported me through this tough period of my life and was always present when I needed them the most.

Last but not least, I would like to thank my colleagues, in particular Hana Joch, who sustained me in the everyday routine and always assisted me in all my efforts.

Table of Contents

Material and Methods:

Results part I:

Abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
BMS	bare metal stents
CAD	coronary artery disease
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanine monophosphate
c57BL6	c 57 black 6 mice
DES	drug eluting stents
DMEM	dulbecco modified eagle medium
DMSO	dimethyl sulfoxide
EC	endothelial cells
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immuno assay
eNOS	endothelial nitric oxide synthase
ET-1	endothelin-1
ET_A	endothelin receptor A
ET_B	endothelin receptor B
FCS	fetal calf serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HDL	high density lipoprotein
HTP1	human monocytic cell line
i.p	intraperitoneal

LDH	lactate dehydrogenase
LDL	low density lipoprotein
MAPK	mitogen activated protein kinases
MACE	major adverse coronary events
MI	myocardial infarction
mRNA	messenger ribonucleic acid
NO	nitric oxide
p	probability
PAR	protein activated receptor
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PGIS	prostacyclin synthase
PI3K	phosphatidylinositol 3 kinase
PKC	protein kinase C
PTCA	percutaneous transluminal coronary angiography
RT-PCR	real time- polymerase chain reaction
SDS-PAGE	sodium dodecyl-sulphate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
SMC	smooth muscle cells
TF	tissue factor
TNF-α	tumor necrosis factor-alpha
TFPI	tissue factor pathway inhibitor
tPA	tissue plasminogen activator

TVR	target vessel revascularization
TXA2	thromboxane A2
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cells
vWF	Von Willebrand factor
WB	Western blotting

Summary

The innermost layer of a vessel, the endothelium, forms an interface between the circulating blood in the vascular lumen and the vessel wall. The endothelium functions as a.) a physical barrier between the blood and the vessel; b.) a vital source of enzymes activating and deactivating cardiovascular hormones; c.) a site of production of relaxing and contracting factors; and d.) a source of growth inhibitors and promoters.

Risk factors such as diabetes, hypertension, obesity and smoking can disturb the integrity of the endothelium rendering it dysfunctional. A dysfunctional endothelium is characterized by a decreased endothelium-dependent relaxation which in turn facilitates the entry and oxidation of circulating lipoproteins and monocytes through the endothelium into the intima. The advancing accumulation of lipoproteins and monocytes into the intima eventually gives rise to an early atherosclerotic lesion which later develops into a necrotic core and a fibrous cap and ultimately becomes an advanced plaque at risk of rupture. Rupture of unstable plaques is responsible for coronary thrombosis, the main cause of unstable angina, acute myocardial infarction, and sudden cardiac death.

Percutaneous transluminal coronary angioplasty is a clinical procedure routinely employed to revascularise occluded vessels. Following percutaneous transluminal coronary angioplasty a tube-like metal structure, a stent, is inserted into the vessel to prevent it from occluding once again.

Stents are commonly coated with drugs orientated at inhibiting the excessive proliferation of vascular smooth muscle responsible for restenosis.

Unlike for restenosis, recent evidence has shown that the incidence of in stent thrombosis has not decreased following the advent of drug eluting stents. Rapamycin which is widely used for coating stents has been shown to induce the expression of TF, the key initiator of the coagulation cascade. This finding shed new light on the possible causes for the occurrence of stent thrombosis.

In the first part of this thesis we characterised the impact of paclitaxel, the main alternative to rapamycin, on TF expression in human endothelial cells. Indeed, paclitaxel enhanced thrombin-induced endothelial TF protein expression in a concentration- and time-dependent manner. A concentration of 10^{-5} mol/L elicited a 2.1-fold increase in TF protein and a 1.6-fold increase in cell surface TF activity. The effect was similar after a 1 h as compared to a 25 h pretreatment period. Real-time polymerase chain reaction revealed that paclitaxel increased thrombin-induced TF mRNA expression. Paclitaxel potently activated c-Jun terminal NH_2 kinase (JNK) as compared to thrombin alone, while the thrombin-mediated phosphorylation of p38 and extracellular signal-regulated kinase remained unaffected. Similar to paclitaxel, docetaxel enhanced both TF expression and JNK activation as compared to thrombin alone. The JNK inhibitor SP600125 reduced thrombin-induced TF expression by 35%. Moreover, SP600125 blunted the effect of paclitaxel and docetaxel on thrombin-induced TF expression.

Paclitaxel increases endothelial TF expression via selective activation of JNK. This observation provides novel insights into the pathogenesis of

thrombus formation after paclitaxel-eluting stent deployment and may have an impact on drug-eluting stent design.

In the second part of this thesis we directed our efforts at characterising the potential application of Dimethyl sulfoxide (DMSO) as a novel agent to be used for stent coating. DMSO is used for preservation of hematopoietic progenitor cells and infused into patients undergoing bone marrow transplantation. Despite of its intravenous application, the impact of DMSO on vascular cells has not been assessed. In this study we found that DMSO inhibited TF expression in human endothelial cells, monocytes, and VSMC. Real-time PCR revealed that inhibition of TF expression occurred at the mRNA level. This effect was mediated by reduced activation of the MAP kinases JNK and p38, but not ERK. *In vivo*, DMSO treatment suppressed TF activity and prevented thrombotic occlusion in a mouse model of carotid artery photochemical injury. DMSO also inhibited VSMC proliferation and migration in a concentration-dependent manner; moreover, it prevented rapamycin and paclitaxel-induced upregulation of TF expression.

As the use of DMSO is established in different areas of modern medicine, we propose this drug as a novel strategy for treating acute coronary syndromes; in particular, DMSO seems to represent an attractive compound for application on drug-eluting stents, either alone or in combination with rapamycin or paclitaxel.

Zusammenfassung

Das Endothel als innerste Schicht eines Blutgefäßes stellt eine Verbindung zwischen dem Blutstrom und dem Rest der Gefäßwand dar. Es fungiert als eine physikalische Barriere zwischen Blut und Gefäß, dient als Quelle für Wachstumsfaktoren –und Inhibitoren sowie für Enzyme, die kardiovaskuläre Hormone sowohl aktivieren als auch deaktivieren, und ist an der Produktion von Kontraktions- bzw. Relaxations-Faktoren beteiligt.

Risikofaktoren wie Daibetes, Hypertonie, Adipositas und Rauchen stören die Integrität des Endothels und führen zum Verlust der Funktionalität. Eine endotheliale Dysfunktion wird durch eine reduzierte NO Synthese gekennzeichnet, was wiederum die Aufnahme und Oxidierung zirkulierender Lipoproteine und Monozyten durch das Endothel in die Intima erleichtert. Diese vortschreitende prozess führt möglicherweise zu einer frühen arteriosklerotischen Läsion, welche sich mit der Zeit zu einer plaque mit nekrotischem Kern und fibroider Schale entwickelt, woraus im weiteren Verlauf eine plaque mit gefahr der ruptur entstehen kann. Die Ruptur eine instabilen Plaque ist verantwortlich für koronare Thromben, die Hauptursachen für ein akutes coronares syndrom.

Perkutane transluminale koronare Angioplastie ist ein in der Klinik routinemäßig eingesetztes Verfahren zur Revaskularisierung verschlossener Gefäße. Daran angeschlossen ist die Einlage einer als Stent bezeichneten röhrenförmigen Struktur, was das wiedereröffnete Gefäß vor erneutem Verschluß bewahren soll. Stents sind gewöhnlich mit Substanzen beschichtet,

welche die für eine Restenose ursächliche excessive Proliferation glatter Gefäßmuskulatur unterbindet.

Wie neulich gezeigt werden konnte ist, im Gegensatz zu Restenose, die Inzidenz für In-Stent-Thrombosen durch die Anwendung von sogenannten „Drug eluting stents“ nicht gesunken. Es konnte nachgewiesen werden, dass das vielfach zur Beschichtung von Stents verwendete Rapamycin die Expression von TF, Hauptinitiator der Koagulationskaskade, induziert, was ein neues Licht auf mögliche Ursachen für das Auftreten von In-Stent-Thrombosen wirft.

Im ersten teil dieser Arbeit charakterisieren wir die Auswirkungen des als alternativ zu Rapamycin vorwiegend verwendeten Paclitaxel auf die TF-Expresssion in menschlichen Endothelzellen. In der Tat steigert Paclitaxel die durch Thrombin induzierte endotheliale TF Protein Expression sowohl konzentrations -als auch zeitabhängig. Eine Konzentration von 10^{-5} mol/l erbrachte eine 2,1-fache Erhöhung in Bezug auf TF Protein und einen 1,6 fachen Anstieg der TF Oberflächenaktivität. Die Prästimulation für 1h ergab im Vergleich zu einer Vorbehandlung für 25 h keinen wesentlichen Unterschied. Anhand RT-PCR konnte gezeigt werden, dass Paclitaxel die durch Thrombin induzierte TF mRNA Expression ebenfalls erhöht. Darüber hinaus potenzierte Paclitaxel die Aktivierung der c-Jun terminalen NH_2 Kinase (JNK) durch Thrombin, wohingegen die durch Thrombin vermittelte Phosphorilierung von p38 und der extrazellulären signal-regulierten Kinase (ERK) unter Zugabe von Paclitaxel unverändert blieb. Docetaxol steigerte, ähnlich dem Paclitaxel, sowohl TF Expression als auch JNK Aktivierung im

Vergleich zur alleinigen Applikation von Thrombin. Der JNK Inhibitor SP600125 konnte die Thrombin-induzierte TF Expression um 35 % reduzieren. Im weiteren wurde durch SP600125 der Effekt von sowohl Paclitaxel als auch Docetaxel bezüglich TF Expression verschleiert.

Schwerpunkt des zweiten Teils der Arbeit war die Untersuchung von Dimethyl Sulfoxid (DMSO) als potentielle neue Substanz zur coating von Stents. DMSO wird zur Konservierung von hämapoetischen Stammzellen verwendet und Patienten vor Knochenmarktransplantation infundiert. Trotz seiner intravenösen Anwendung wurde die Auswirkung von DMSO auf vaskuläre Zellen noch nicht untersucht. In dieser Studie konnte gezeigt werden, dass DMSO die TF Expression in menschlichen Endothelzellen, Monozyten und VSMC unterdrückt. RT-PCR zeigte die Inhibition die TF Expression auf mRNA Ebene, was durch die reduzierte Aktivierung der MAP Kinasen JNK und p38, jedoch nicht ERK, vermittelt wurde. In vivo unterdrückt DMSO die TF Aktivität und verhinderte im Maus-Modell einen thrombotischen Verschluß. Darüber hinaus inhibierte DMSO konzentrationsabhängig die Proliferation und Migration von glatten Gefäßmuskelzellen, vielmehr es verhindertees die Rapamycin bzw. Paclitaxel induzierte Hochregulation der TF Expression.

Da DMSO auf unterschiedlichem Gebiet der modernen Medizin bereits etabliert zur Anwendung kommt, empfehlen wir diese Substanz als neuwertiges Verfahren zur Behandlung eines Akutem Koronarsyndrom; im speziellen, DMSO scheint ein attraktives Mittel zur Beschichtung von Drug-

eluting Stents zu sein, entweder allein oder in Kombination mit Rapamycin oder Paclitaxel.

Introduction

I: Overview of the cardiovascular system.

The cardiovascular system is an organ system that moves substances to and from cells and is composed by the heart, the vasculature and blood (Figure 1). The first scientific study reporting the existence and function of the cardiovascular system dates back to 1628 and was published by William Harvey.

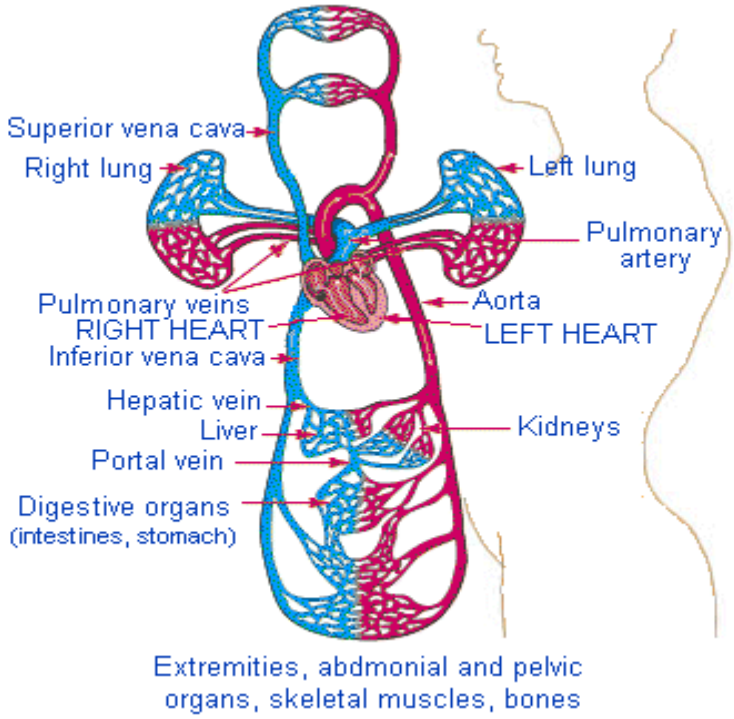


Figure 1: Schematic representation of the pulmonary and systemic circulation

In this scientific report entitled "*Exercitatio Anatomica de Motu Cordis et Sanguinis in Animalibus*" (An Anatomical Exercise on the Motion of the

Heart and Blood in Animals), William Harvey described the complete circulation and looked upon the heart, not as a mystical organ, but as a pump analyzable along mechanical lines. He also measured the amount of blood which it sent out to the body. He observed that with each heart beat two ounces of blood leave the heart. Moreover he described the one-way valves in the heart, like those in the veins, and indicated that, following the pulmonary circulation, the blood goes out to all parts of the body through the arteries and returns by way of the veins. The blood thus makes a complete closed circuit. There was, however, one part of the vasculature which Harvey did not observe -the capillary vessels-. It was in fact not until 1660, that Marcello Malpighi saw the blood moving in the capillary vessels of the frog's lung, and thus supplied the missing link in Harvey's proof of the circulation of the blood.

II: The endothelium.

The endothelium is the layer of thin, flat cells that lines the interior surface of blood vessels, forming an interface between circulating blood in the lumen and the rest of the vessel wall. Not only it functions as a physical barrier between the blood and the vessel wall but it also serves as a.) a physical barrier between the blood and the vessel; b.) a vital source of enzymes activating and deactivating cardiovascular hormones; c.) a site of production of relaxing and contracting factors; and d.) a source of growth inhibitors and promoters.

Nitric oxide is a potent vasodilator and inhibitor of platelet function it is often released together with prostacyclin, which exerts similar effects (Figure 2). Nitric Oxide and prostacyclin play an important protective role in the coronary circulation by mediating vasodilation and inhibiting platelet activation (Figure 2). Moreover, the endothelium also releases contracting factors such as endothelin-1 and thromboxane A_2 (Figure 2); (Luscher, Tanner et al. 1993; Yang, Arnet et al. 1994; Ming, Barandier et al. 2004).

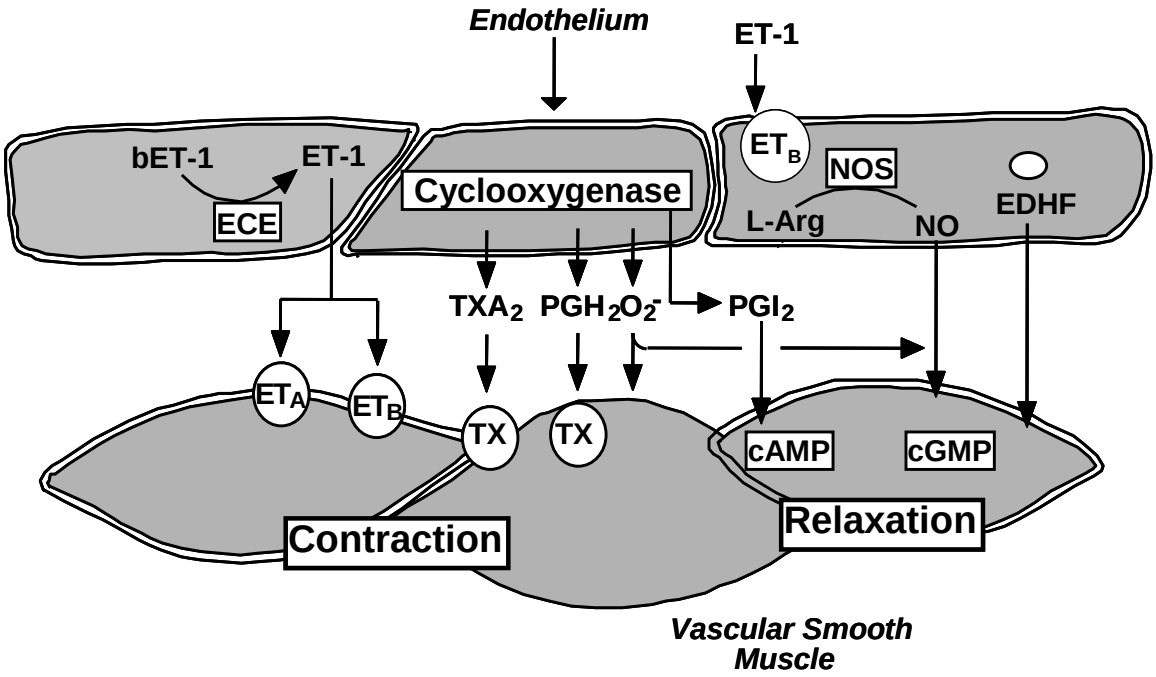


Figure 2: Overview of vasoactive substances. Nitric oxide (NO) produced by nitric oxide synthase (NOS) exerts relaxation and possibly antiproliferation effects by activating cGMP. The products of Cyclooxygenase enzymes elicit both vasoconstriction and vasorelaxation; prostacyclin (PGI_2) causes vasorelaxation via the formation of cAMP, while Thromboxane A_2 (TXA_2) and prostaglandin H_2 (PGH_2) elicit vasoconstriction through the TXA_2/PGH_2 receptor (TX). Cyclooxygenase can also be a source of superoxide (O_2) which scavenges NO. Endothelin (ET) can activate endothelial receptors (ET_B-receptors) in the endothelial cell membrane, which are linked to the production of NO and prostacyclin. Furthermore, endothelin activates vascular smooth muscle cell receptors (ET_A and ET_B receptors), which mediate profound contraction and proliferation under certain conditions. ECE= endothelin converting enzyme; bET-1= big endothelin-1; EDHF= endothelial derived hyperpolarising factor; L-Arg= L-Arginine.

Because of its strategic anatomic position, the endothelium is a primary target for injuries and cardiovascular risk factors. In particular, aging, low density lipoproteins, hypertension, diabetes, and ischemia alter endothelium function. Alterations of endothelial function may contribute to vasospasm, thrombus formation, and vascular proliferation and in turn myocardial ischemia, all common events in patients with coronary artery disease.

An altered or dysfunctional endothelium is characterized by a decreased synthesis of Nitric oxide which in turn facilitates vessel wall entry and oxidation of circulating lipoproteins and monocyte as well as causing smooth cell proliferation (Fuster, Moreno et al. 2005). The growing mass of cells and proteins which accumulates in the intima gives rise to an early atherosclerotic lesion (Figure 3). Continuous exposure to pro-atherogenic environment will increase chemotaxis of monocytes leading to lipid accumulation, necrotic core, and fibrous cap formation, ultimately turning the early plaque into an advanced plaque which is unstable and at risk of rupture (Figure 3). Rupture of unstable plaques is responsible for coronary thrombosis, the main cause of unstable angina, acute myocardial infarction, and sudden cardiac death (Fuster, Moreno et al. 2005).

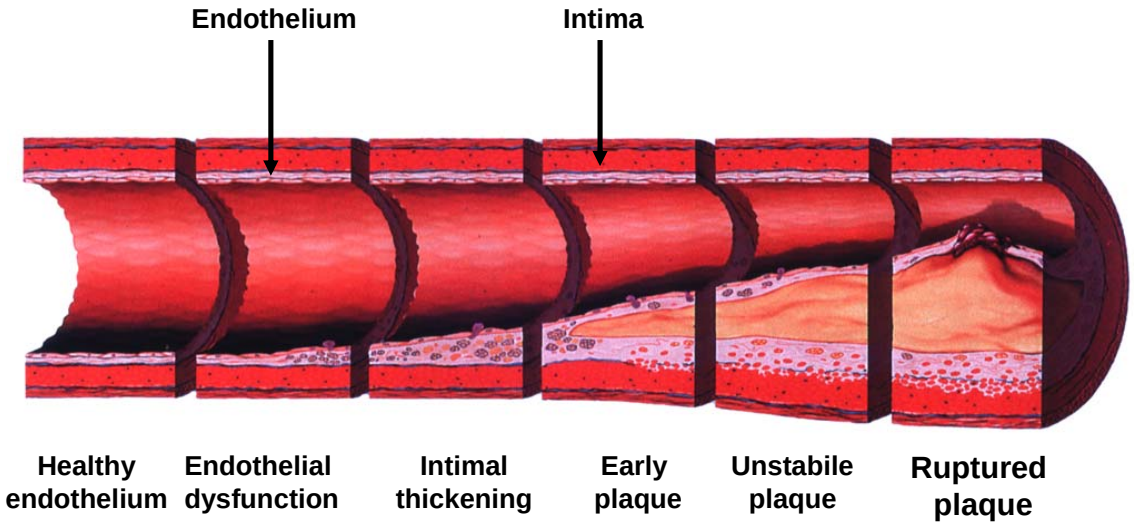


Figure 3: Schematic representation of the evolution of an atherosclerotic plaque. Healthy endothelium becomes dysfunctional and nitric oxide production decreases. Infiltration and oxidation of lipids and lipoproteins through the endothelium occurs and causes intimal thickening. Advancing infiltration of lipids and lipoproteins results in plaque formation which later becomes unstable and eventually ruptures.

III: The role of tissue factor in thrombosis.

The understanding of the mechanisms regulating Tissue Factor (TF) in vascular cells has deepened considerably over the last years. TF has been recognized to be involved in the pathogenesis of cardiovascular diseases and therefore therapeutic strategies to specifically interfere with TF are being developed.

TF has long been known for being the key initiator of the coagulation cascade, it binds factor VIIa resulting in activation of factor IX and factor X, ultimately leading to thrombus generation and fibrin formation (Figure 4).

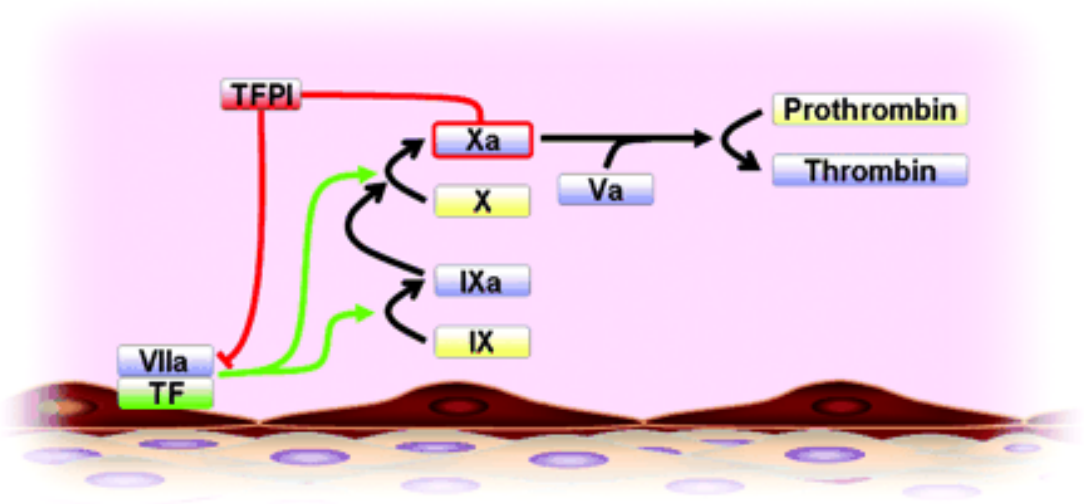


Figure 4: Tissue factor: A key regulator of coagulation. Tissue factor (TF) is a key initiator of the coagulation cascade. Formation of a complex with factor VIIa (FVIIa) leads to activation of factor IX (FIX) and factor X (FX), resulting in thrombin generation and, ultimately, clot formation. Tissue factor pathway inhibitor (TFPI), the endogenous inhibitor of TF activity, is synthesized and secreted mainly by endothelial cells. TFPI binds to FXa and thereby inhibits TF/FVIIa activity (*Steffel J et al Circulation* 2006;113:722-731)

The TF gene consists of 6 exons (Mackman, Morrissey et al. 1989; Morrissey, Gregory et al. 1989) and presents one predominant transcript as well as at least one alternatively spliced form (Bogdanov, Balasubramanian et al. 2003). TF, a 47-kDa protein, is expressed in both vascular and nonvascular cells. Congruent to its physiological role, normally there is no direct contact between TF and the bloodstream. In the vessel wall, TF is constitutively expressed only by vascular smooth muscle cells leading to rapid initiation of coagulation in the event of an injury. In contrast, other vascular cells such as endothelial cells and monocytes only express TF in the presence of specific mediators like thrombin or TNF-α. The coagulation cascade is initiated as soon as TF comes into contact with circulating activated factor VII (VIIa), resulting in the TF-FVIIa complex (Figure 4). Alternatively, TF can bind inactive factor VII and form the TF-FVII complex, which is converted to TF-FVIIa by FVIIa or already formed TF-FVIIa. The TF-VIIa complex 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 calcium, Factor Xa catalyzes the conversion of prothrombin to thrombin, thereby leading to fibrin formation, platelet activation, and, ultimately, generation of a thrombus (Steffel, Luscher et al. 2006).

Increased levels of TF antigen and activity have been detected in atherectomy specimens of patients with unstable angina or myocardial infarction (Annex, Denning et al. 1995). Furthermore, in acute coronary syndromes, plasma concentrations of inflammatory cytokines such as TNF-α and interleukins are indeed increased at the site of coronary artery occlusion to such an extent that TF is induced in vascular cells (Maier, Altwegg et al.

2005). In view of these findings it is reasonable to postulate that the circulating leukocytes and platelets as well as vascular cells may contribute to the heightened levels of TF measured in the plasma of patients with unstable angina (Soejima, Ogawa et al. 1999).

Induction of TF expression occurs through a wide array of mediators; however, most mediators exert their effect on TF via a limited subset of signalling pathways. The MAP kinases p38, p44/42 (ERK), and c-*jun* terminal NH_2-kinase (JNK) mediate TNF-α–induced, histamine-induced, and thrombin-induced TF expression (Mechtcheriakova, Schabbauer et al. 2001; Eto, Kozai et al. 2002; Steffel, Akhmedov et al. 2005; Steffel, Hermann et al. 2005), while the effect of vascular endothelial growth factor (VEGF) is mediated by p38 and ERK (Mechtcheriakova, Schabbauer et al. 2001). In addition, TNF-α and VEGF are known to induce TF expression through activation of protein kinase C as well (Mechtcheriakova, Schabbauer et al. 2001). The small G-protein RhoA and PI3-Akt pathways have also been shown to modulate TF expression in response to specific stimuli (Viswambharan, Ming et al. 2004). In fact reconstituted-HDL inhibits thrombin-induced TF expression via inhibition of RhoA and stimulation of PI3K (Viswambharan, Ming et al. 2004).

Percutaneous coronary intervention is an invasive clinical procedure commonly used to revascularise occluded coronary arteries of patients which suffered an acute myocardial infarction. Following revascularisation stents – cylinder-like shaped “tubes” designed with the intent of preventing or delaying the process of restenosis (Figure 5) – are routinely inserted in the vessel. Initially, stents were only considered as a physical impediment to prevent

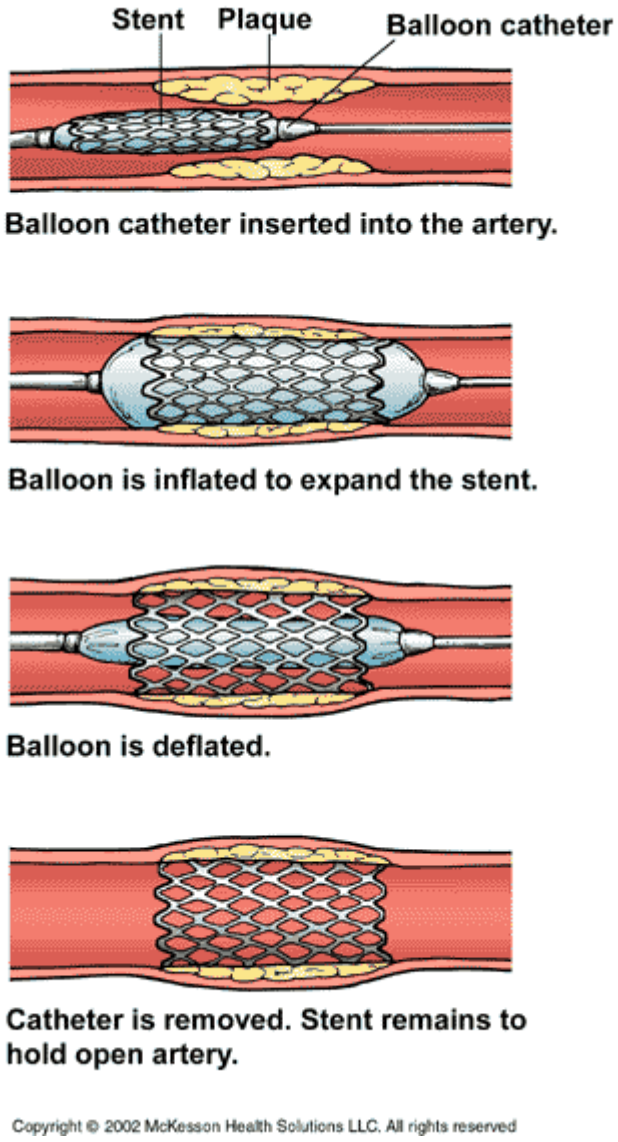


Figure 5: Schematic representation of a stented coronary artery

restenosis; however in the last decade a new concept developed which saw stents as potential reservoir of specific drugs which could be released at the site of injury. This novel concept enabled the coating of stents with drugs such as rapamycin and paclitaxel, aimed at inhibiting the excessive proliferation and migration of vascular smooth muscle cells taking place after

stent deployment. In contrast to reduced restenosis rates, however, the frequency of stent thromboses has not decreased with the advent of drug-eluting stents (DES) as compared with bare metal stents (Figure 6). For this purpose, in-stent thrombosis remains a major concern in modern clinical practice where DES are employed. This clinical setting prompted several investigators to look for the cause and explanation for the lasting occurrence of stent thromboses. Recently rapamycin, which is used for stent coating, was found to increase endothelial TF expression, suggesting a potential role for this drug in the development of subacute stent thrombosis (Steffel, Latini et al. 2005; Zhu, Viswambharan et al. 2005).

Outcome	Bare-metal stent (%)	DES (%)	p
Cardiac death	0	1.2	0.09
Nonfatal MI	1.3	4.1	0.04
Cardiac death / nonfatal MI	1.3	4.9	0.01
Restenosis-related TVR	6.7	4.5	0.21
MACE	7.9	9.3	0.53

Figure 6: Slide presented by Pfisterer ME at the American College of Cardiology 2006. Outcome comparison between bare metal stents and drug eluting stents (DES). MI= myocardial infarction; TVR= target vessel revascularization; MACE= major adverse cardiac events.

Taking into consideration the above mentioned facts, the search for new strategies to inhibit directly or indirectly TF is vigorous. Various agents have been developed to specifically interfere with the action of TF and the TF/FVIIa complex (Steffel, Luscher et al. 2006). In contrast to classic antithrombotic drugs, these agents interfere with the upstream mediators of the coagulation cascade while leaving the downstream effectors intact. In addition, these drugs can target the promigratory and proproliferative effects of TF.

IV: Aims of the thesis.

The aim of this thesis is to:

1. Investigate the impact of paclitaxel –a drug commonly used for coating stents– on TF expression.

2. Evaluate the potential of dimethyl sulfoxide as a novel agent to impair TF expression in vascular cells, inhibit thrombus formation and prevent vascular smooth muscle cells activation.

Material and Methods

I: Cell culture and morphological assessment.

Human aortic endothelial cells (HAEC) and VSMC (Clonetics, Allschwil, Switzerland) were cultured as described (Steffel, Akhmedov et al. 2005). THP-1 cells (LGC Promochem, Molsheim, France) were cultured according to the supplier's recommendation. Adhering cells were grown to confluence in 3 cm dishes and rendered quiescent for 24 hours before stimulation with 5 ng/mL TNF-α or 1 U/ml thrombin (both from Sigma, Basel, Switzerland) for 5 hrs. Cells were pretreated with Paclitaxel (Sigma and Alexis), Rapamycin or DMSO (both from Sigma) for 1 h before stimulation. To block the mitogen-activated protein (MAP) kinase p38, p44/42 (ERK), or c-Jun terminal NH_2 kinase (JNK), cells were treated with SB203580 (Sigma), PD98059 (Cell Signaling, Denvers, MA), or SP600125 (Calbiochem, Lucerne, Switzerland), respectively, for 60 minutes before stimulation. To assess cytotoxicity, a colorimetric assay for detection of lactate dehydrogenase (LDH) was used according to the manufacturer's recommendations (Roche, Basel, Switzerland); in addition, trypan blue exclusion assay (0.4% solution, Sigma) and morphological examination by phase contrast microscopy (Leica, Glattbrugg, Switzerland) was performed.

II: Western blot analysis.

Protein expression was determined by Western blot analysis as described (Steffel, Hermann et al. 2005). Cells were lysed in 50 mmol/L TRIS buffer, 25 µg were loaded per lane, and 10% SDS-PAGE was performed under reducing conditions. Resolved proteins were transferred to PVDF membranes (Millipore) by semidry transfer. The antibody against human TF (American Diagnostica) was used at 1:2'000 dilution; antibodies against phosphorylated p38 MAP kinase (p38), phosphorylated p44/42 MAP kinase (ERK), and phosphorylated c-jun terminal NH_2 kinase (JNK) (all from Cell Signaling) were used at 1:1'000, 1:5'000, and 1:1'000 dilution, respectively. Antibodies against total p38, total ERK, and total JNK (all from Cell Signaling) were used at 1:2'000, 1:5'000, and 1:1'000 dilution, respectively. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used to ensure equal protein loading at an antibody (Chemicon) dilution of 1:20'000. Proteins were detected with a horseradish peroxidase linked secondary antibody (Amersham).

III: Real-time PCR.

Total RNA was extracted with 1 mL TRIzol Reagent (Invitrogen) according to the manufacturer's recommendations. Conversion of total cellular RNA to cDNA was carried out with Moloney murine leukemia virus reverse transcriptase and random hexamer primers (Amersham Bioscience) in a final volume of 33 µL using 4 µg of RNA. Real-time PCR amplification was

performed in an MX3000P PCR cycler (Stratagene) using the SYBR Green JumpStart kit (Sigma) in 25 µL final reaction volume containing 2 µl cDNA, 10 pmol of each primer, 0.25 µl of internal reference dye, and 12.5 µl of JumpStart Taq ReadyMix (buffer, dNTP, stabilizers, SYBR Green, Taq polymerase, and JumpStart Taq antibody).(Bogdanov, Balasubramanian et al. 2003) The total cDNA pool obtained served as template for subsequent PCR amplification with TF (F3)-specific primers (Sense primer: 5'-TCCCCAGAGTTCACACCTTACC–3'; bases 508-529 of *F3* cDNA; NCBI no. NM 001993; antisense primer: 5'–TGACCACAAATACCACAGCTCC–3'; bases 892-913 of *F3* cDNA; NCBI no. NM 001993) using the following cycling parameters: 95° for 10 minutes for 1 cycle; 95° for 30 seconds, 60° for 1 minute, 72° for 1 minute, for a total of 40 cycles. A melting curve analysis was performed after amplification to verify the accuracy of the amplicon. L28 primers served as loading control. Products were separated by electrophoresis on a 1.6% agarose gel and visualized with ethidium bromide.

IV: TF activity.

TF cell surface and tissue activity was analyzed in HAEC and mouse carotid artery homogenates, respectively, using a colorimetric assay (American Diagnostica) according to the manufacturer's recommendations with some modifications as described (Day, Reeve et al. 2005; Steffel, Akhmedov et al. 2005). Cells were grown in 6-well plates; after stimulation, cells were washed twice with PBS followed by incubation with human FVIIa and FX at 37° which allowed the formation of TF/FVIIa complex at the cell surface. Right carotid arteries were homogenized in 50 µl of lysis buffer (50

mmol Tris-HCl, 100 mmol NaCl, 0.1% Triton X-100 pH 7.4) and left to stand on ice for 30 minutes. TF/FVIIa complex converted human factor X to factor Xa, which was measured by its ability to metabolize a chromogenic substrate. A standard curve with lipidated human TF was performed to assure that measurements were taken in the linear range of detection.

V: Carotid artery thrombosis model.

The laser-injury thrombosis model was selected for this investigation since it has numerous advantages for the study of thrombosis *in vivo*. This model enables the examination of thrombus formation in live animals and does not require intravascular intrusion (Furie and Furie 2005). In addition, the precise location and initiation time of the injury is known. Figure 1 shows a schematic diagram of such injury model.

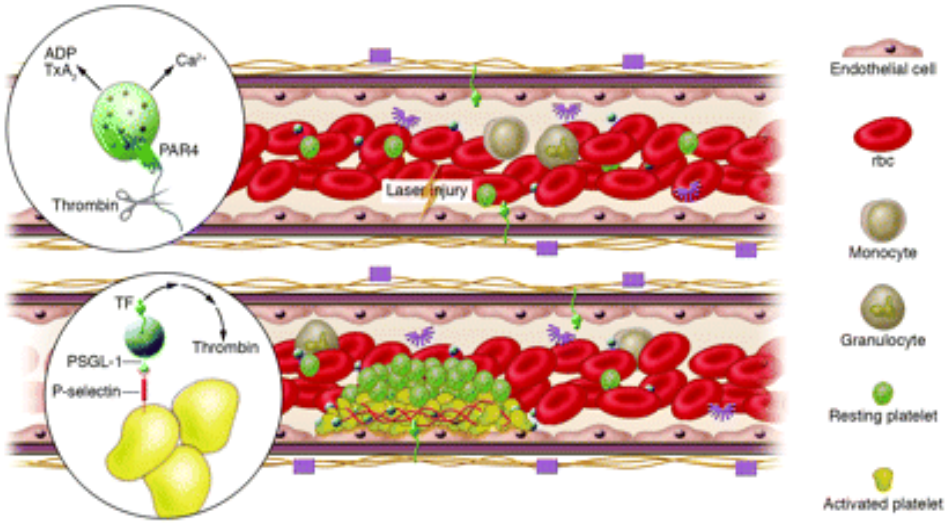
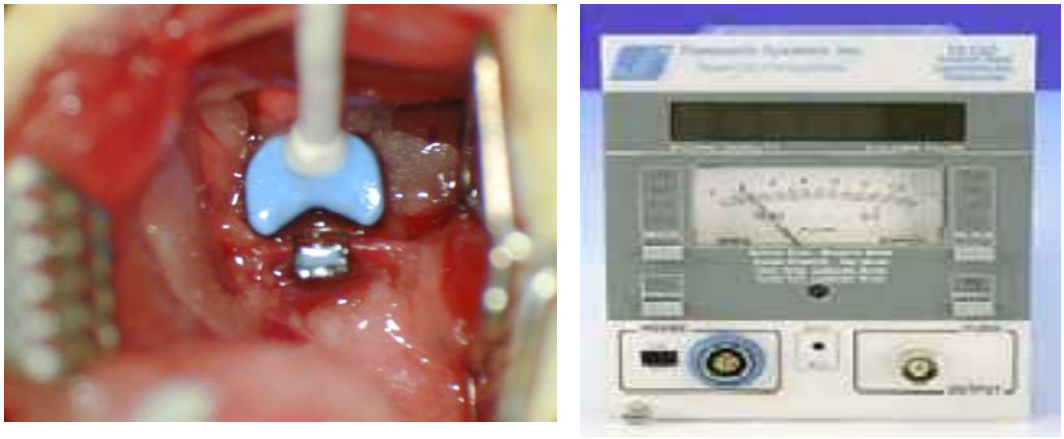


Figure 1: Light-sensitive rose bengal was injected intravenously in the anesthetised mouse. Following midline cervical incision the right carotid artery was then exposed and a green laser

light was directed at it so as to elicit a photochemical reaction with the circulating rose bengal. Upon laser-induced photochemical injury of the vessel wall, vWF mediates the interaction of platelets with the endothelium. Tissue factor in the vessel wall leads to thrombin generation. Activated platelets undergo calcium mobilization and the release of ADP and thromboxane A_2 (TxA_2) to accelerate platelet recruitment and activation and the formation of a platelet thrombus. These platelets express P-selectin, and leukocyte microparticles expressing PSGL-1 and tissue factor accumulate in the. The concentration of tissue factor initiates coagulation, the generation of more thrombin, and the propagation of a fibrin clot (Figure and text adapted from *J. Clin. Invest.* **115**:3355-3362 2005).

C57BL/6 mice (6-8 weeks old) weighing an average of 23±2 g were anesthetized with intraperitoneal injection of 2 mg sodium pentobarbital (Butler, Columbus, OH) as previously described (Eitzman, Westrick et al. 2000). Rose bengal (Fisher Scientific, Fair Lawn, NJ) was diluted to 10 mg/mL in phosphate-buffered saline and then injected into the tail vein in a volume of 0.12 mL at a concentration of 50 mg/kg using a 27-gauge Precision Glide needle with a 1-mL latex free syringe (Becton Dickinson, Franklin Lakes, NJ). Mice were secured in a supine position, placed under a dissecting microscope (Olympus C-4040 Zoom; spatial resolution 4.1 megapixels), and the right common carotid artery was exposed following a midline cervical incision. A Doppler flow probe (Model 0.5 VB, Transonic Systems, Ithaca, NY, Figure 2 left panel) was then applied and connected to a flowmeter (Transonic, Model T106, Figure 2 right panel).



:

Figure 2: On the left, a perivascular doppler flow probe (Model 0.5 VB, Transonic Systems, Ithaca, NY) measuring flow on the right common carotid artery. On the right, a flowmeter (Transonic, Model T106) device.

Six minutes after rose bengal injection, a 1.5-mW green light laser (540 nm) (Melles Griot, Carlsbad, CA) was applied to the desired site of injury at a distance of 6 cm for 60 minutes or until thrombosis occurred (depending on what elapsed first). From the onset of injury, blood flow in the vessel was monitored for 120 minutes, at which time the experiment was terminated. Occlusion was defined as a flow of ≤0.1ml/min for at least 1 minute (Eitzman, Bodary et al. 2003). Mice were divided into 3 groups: DMSO i.p. (1 hour before surgery, 3.8 ml/kg of 40% DMSO in PBS), PBS i.p. (1 hour before surgery, 3.8 ml/kg of PBS), and an additional control without laser injury.

VI: Proliferation and migration of human aortic VSMC.

For proliferation, VSMC were seeded at a density of 10,000 cells per

35-mm dish and cultured for 24 hours in DMEM with 10% FCS. Successively,

cells were serum withdrawn in DMEM with 1% FCS for 48 hours. Cells were then maintained in DMEM with 10% FCS in the presence of different DMSO concentrations. Medium and appropriate DMSO concentrations were replaced daily and cell number was determined after 3 days by using a hemacytometer.

For migration, VSMC were seeded at a density of 250,000 cells per 150-mm dish and cultured for 24 hours in DMEM with 10% FCS. Cells were then harvested and resuspended (500'000 cells/ml) for analysis of migration in response to PDGF 10 ng/ml. Migration was assessed in a modified Boyden chamber system (Neuroprobe Inc., Cabin John, MD) as previously described (Tanner, Largiader et al. 2004). The number of migrated cells was determined by staining (Diff-Quik; Dade Diagnostics Inc., Aguada, Puerto Rico) and counting the cells at 400× magnification (Leica, Glattbrugg, Switzerland) on 4 random microscopic fields per group.

VII: Statistical analysis.

Data are reported as mean ± S.E.M. Unpaired Student *t* test was performed for statistical analysis. A p value <0.05 was considered to indicate a significant difference.

Results part I

Preface.

Percutaneous intervention is a common clinical practice employed for treating acute coronary syndromes (Grines, Cox et al. 1999; Lincoff, Califf et al. 1999). Drug-eluting stents (DES), which are coated with antiproliferative agents, improve the outcome after coronary artery stenting (Babapulle, Joseph et al. 2004). Paclitaxel (Figure 1), a microtubule-stabilizing drug eliciting cell cycle arrest in G2/M phase, is used on DES because it reduces vascular smooth muscle cell proliferation and migration (Sollott, Cheng et al. 1995).

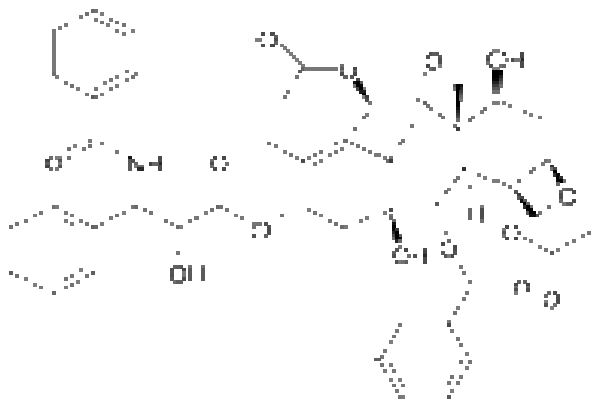


Figure 1: Atomical structure of paclitaxel, molecular weight 853.

Several randomised clinical trials have demonstrated that paclitaxel-

eluting stents decrease restenosis. In contrast the use of DES has not reduced the occurrence of stent thrombosis as compared to bare metal stents (BMS) (Babapulle, Joseph et al. 2004; Eisenberg 2004; McFadden, Stabile et al. 2004; Stone, Ellis et al. 2004; Stone, Ellis et al. 2004). Acute, subacute, and late in-stent thrombosis has been observed in patients treated with paclitaxel-eluting stents and is associated with high morbidity and mortality (Ong, Hoye et al. 2005).

Recently, rapamycin (Figure 2), another drug used for DES, was shown to enhance endothelial TF expression (Steffel, Latini et al. 2005). The effect of paclitaxel on TF expression, however, is not known. The following experiments were therefore designed to investigate the effect of paclitaxel on TF expression in human endothelial cells.

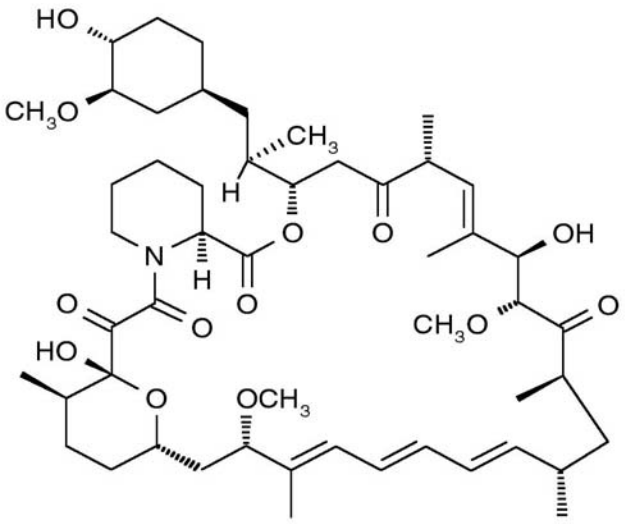


Figure 2: Atomical structure of rapamycin, molecular weight 914.

I: Paclitaxel Enhances Endothelial Tissue Factor Expression.

HAECs were stimulated with thrombin (1 U/ml) for 5 hours in the presence or absence of paclitaxel (10^{-9}-10^{-5} mol/L). Thrombin induced a 20–fold increase in TF expression as compared to baseline (n=7; P<0.0001; Figure 3A). One hour pretreatment with paclitaxel enhanced thrombin-induced TF expression in a concentration-dependent manner; a maximal effect occurred at 10^{-5} mol/L and resulted in a 2.1-fold induction as compared to thrombin alone, corresponding to a 43.3–fold induction as compared to baseline (n=6; P<0.0005; Figure 3A). The effect of paclitaxel was similar after a 1 hour as compared to a 25 hour pretreatment period (n=4; P=n.s. for 1 vs 25 hours; Figure 3B). The effect of paclitaxel was first observed after 3 hours and elicited a significant increase in thrombin-induced TF expression after 5 and 7 hours (n=4; P<0.05; Figure 5B). In another time course analysis, thrombin-induced TF expression was maximal after 6 hours of stimulation and decreased to 50% after 12 hours, 32% after 18 hours, and 27% after 24 hours; paclitaxel significantly enhanced thrombin-induced TF expression by 2.2–fold after 6 hours (n=4; P<0.05) and by 1.4–fold after 12 hours (n=4; P<0.05), while its effect did not reach statistical significance after 18 and 24 hours (n=4; P=n.s.) (data not shown). Consistent with these observations, paclitaxel enhanced thrombin-induced TF surface activity by 56% (n=3; P<0.005; Figure 3C). Paclitaxel alone did not affect basal TF expression (n=7; P=NS; Figure 3A). Paclitaxel did not affect endothelial cell morphology (Figure 4A) or LDH release (Figure 4B) at any concentration used (n≥5; P=NS).

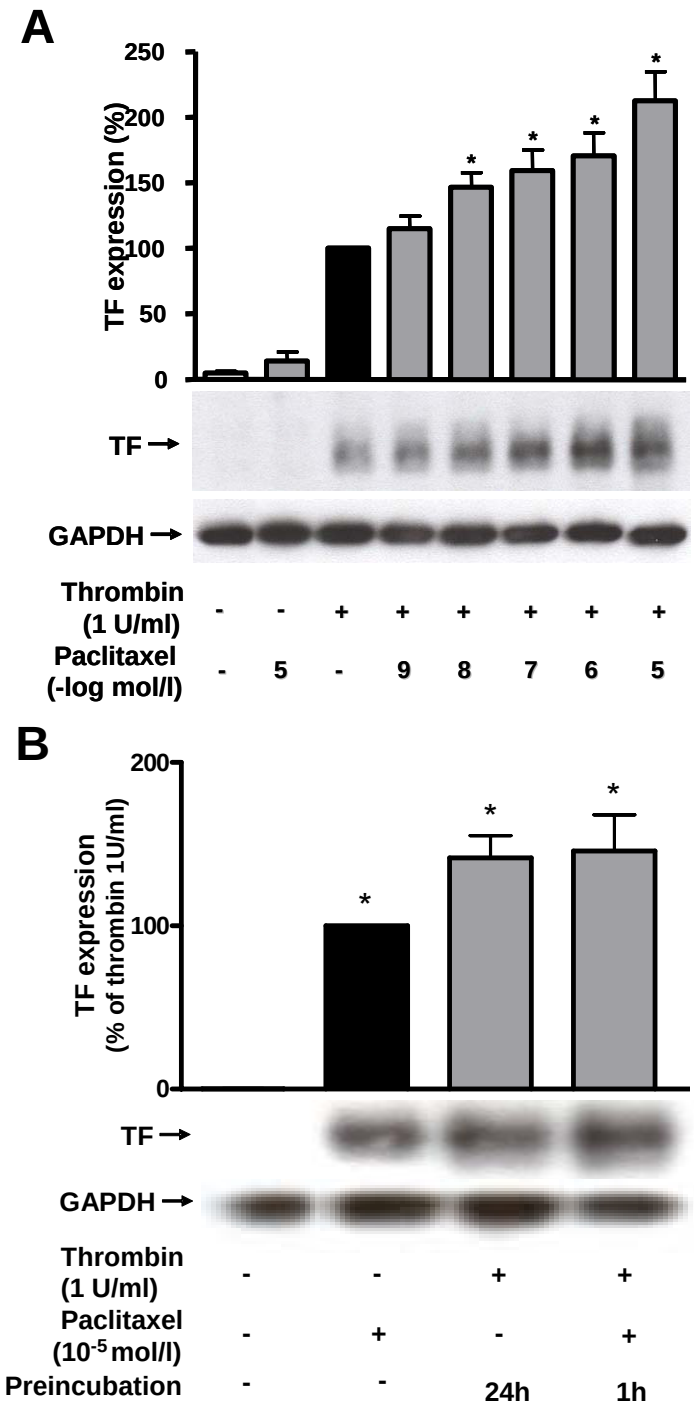


Figure 3. Paclitaxel enhances thrombin-induced TF protein expression.

A. Paclitaxel enhances thrombin-induced TF protein expression in a concentration-dependent manner (n=6; **P*<0.0005 vs thrombin alone). Values are given as percent of TF expression in response to 5 hours thrombin stimulation. Blots are normalized to GAPDH expression. **B.** Similar effect of paclitaxel on thrombin-induced TF protein expression after 1 hour and 25

hours pretreatment (n=4; *P<0.005 vs thrombin alone, P=n.s. for 1 vs 25 hours).

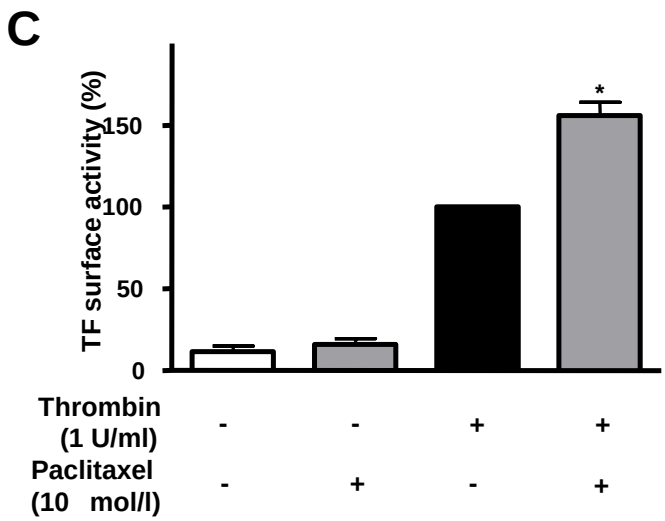


Figure 3. Paclitaxel enhances thrombin-induced TF protein expression.

C. Paclitaxel enhances thrombin-induced TF surface activity (n=3; *P<0.005 vs thrombin alone). Values are given as percent of TF surface activity in response to 5 hours thrombin stimulation.

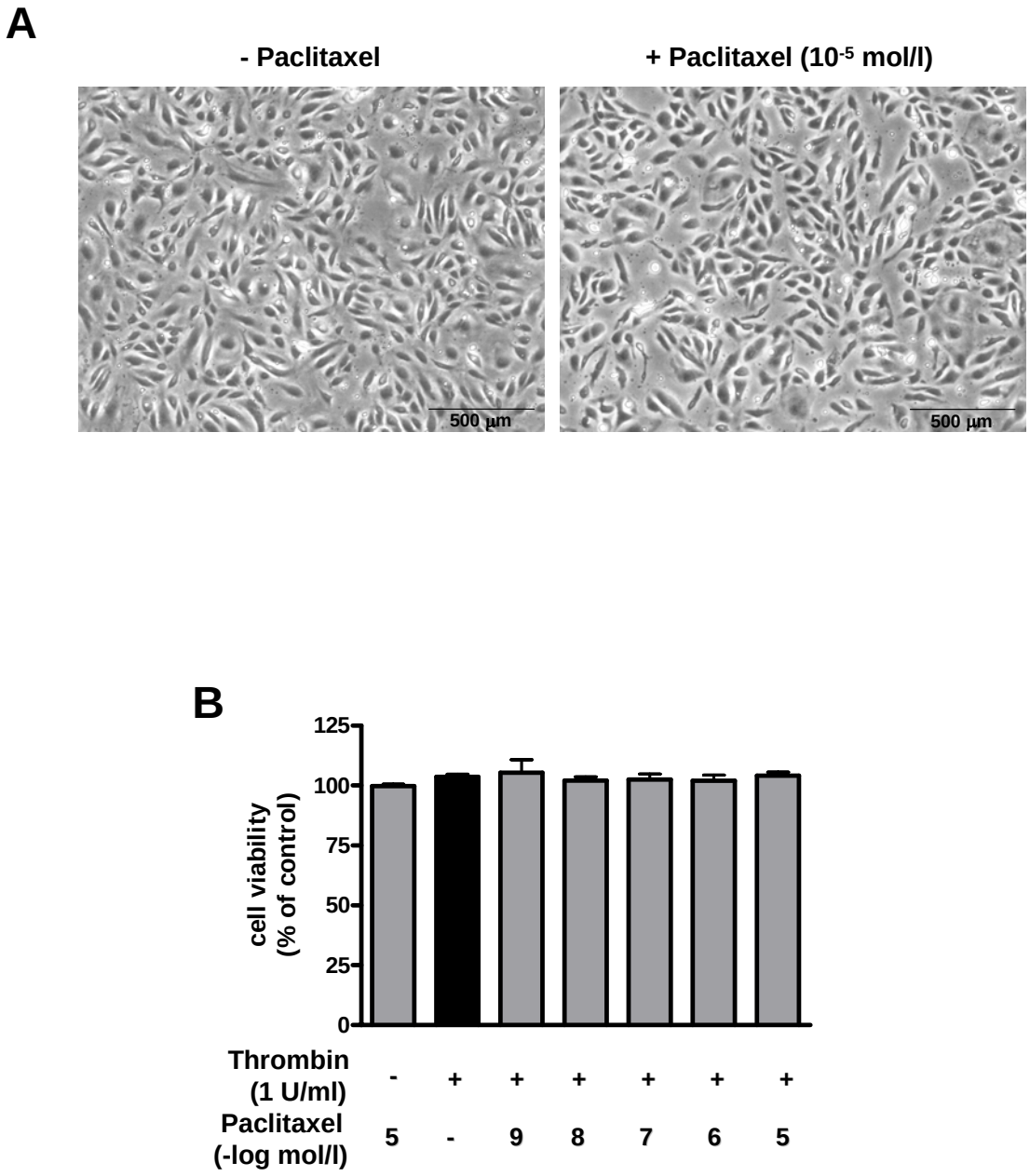


Figure 4. Lack of toxicity of paclitaxel.

A. Morphology of HAECs after 5 hours thrombin stimulation is similar without (left panel) and with (right panel) paclitaxel (10^{-5} mol/L). Magnification x 50. **B.** LDH-release reveals no cytotoxic effect of paclitaxel on HAECs at any concentration used (n≥5; *P*=NS).

II: Paclitaxel enhances TF mRNA expression.

Real-time PCR revealed that thrombin (1 U/ml) induced TF mRNA expression with a maximal effect occurring after 2 hours. Pretreatment with paclitaxel (10^{-5} mol/L) enhanced thrombin-induced TF mRNA expression by 1.6–fold after 1 hour (n=5; P<0.05; Figure 5A), by 1.5–fold after 2 hours (n=5; P=0.09; Figure 5A), and by 1.7– fold after 3 hours (n=5; P=0.21; Figure 5A). The effect of paclitaxel on TF protein expression is time dependent (n=4; **P*<0.05 vs thrombin alone; Figure 5B).

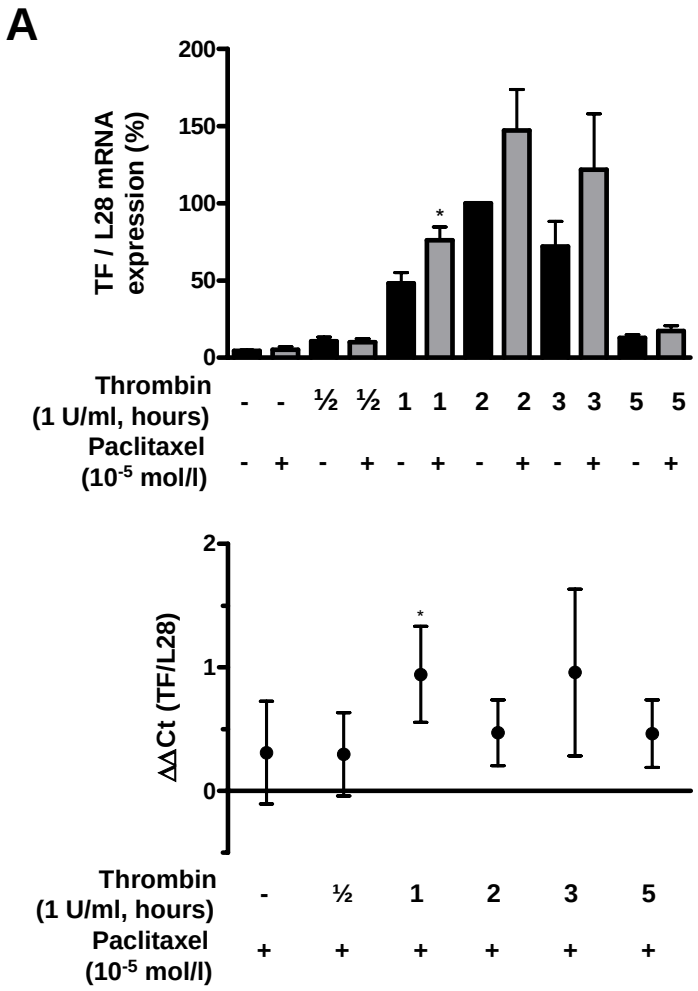


Figure 5. Paclitaxel enhances thrombin-induced TF mRNA expression.

A. Upper panel: Paclitaxel enhances thrombin-induced TF mRNA expression in a time-dependent manner (n=5; *P*<0.05 vs thrombin alone). TF mRNA levels are normalized to L28; values are indicated as percent of TF mRNA expression induced by 2 hours thrombin stimulation. Lower panel: $\Delta\Delta C_T$ values assessing the effect of paclitaxel on thrombin-induced TF mRNA expression at each time point (n=5; **P*<0.05 vs thrombin alone).

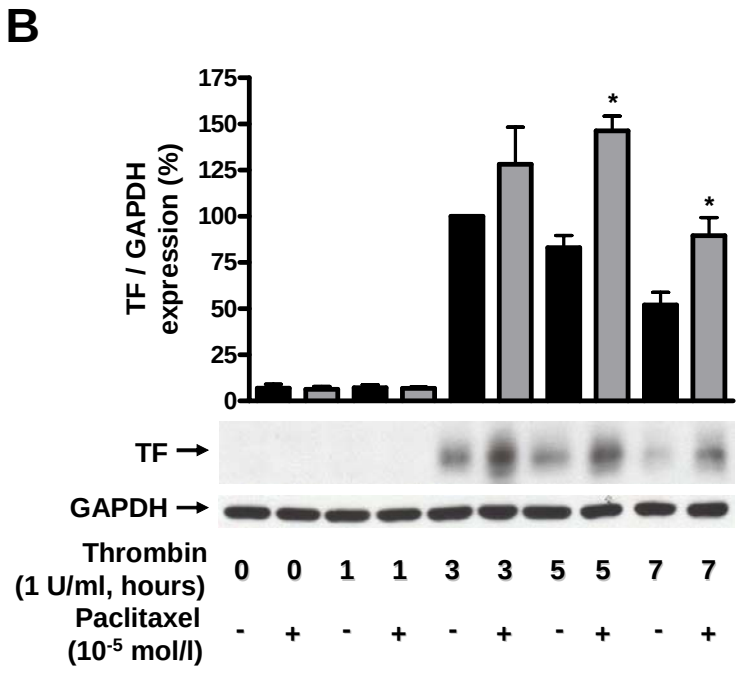


Figure 5. Paclitaxel time dependently enhances thrombin-induced TF expression.

B. Western blot showing that the effect of paclitaxel on thrombin-induced TF protein expression is time-dependent. A significant increase is observed after incubation with paclitaxel for 5 and 7 hours (n=4; **P*<0.05 vs thrombin alone). Values are given as percent of TF expression in response to 3 hours thrombin stimulation. Blots are normalized to GAPDH expression.

III: Paclitaxel selectively activates JNK.

Thrombin 1U/ml induced a transient phosphorylation of the MAP kinases p38, ERK, and JNK. Maximal activation of JNK was observed after 60 minutes, while that of p38 and ERK occurred after 5 minutes. Paclitaxel 10^{-5} mol/L significantly increased JNK phosphorylation after 15, 30, and 60 minutes by 3.7-, 3.2-, and 2.0–fold, respectively, as compared to thrombin alone (n=4; $P<0.05$ for each time-point; Figure 6A). Phosphorylation of p38 was slightly prolonged by paclitaxel 10^{-5} mol/L after 15 minutes of stimulation (n=4; $P<0.005$; Figure 6B), while all the other time-points remained unaltered (n=4; P=n.s.; Figure 6B). Phosphorylation of ERK was not affected by paclitaxel except for a slight decrease at the 5 minute time-point (n=4; $P<0.01$; Figure 6C). Neither thrombin 1U/ml nor paclitaxel 10^{-5} mol/L altered total expression of MAP kinases.

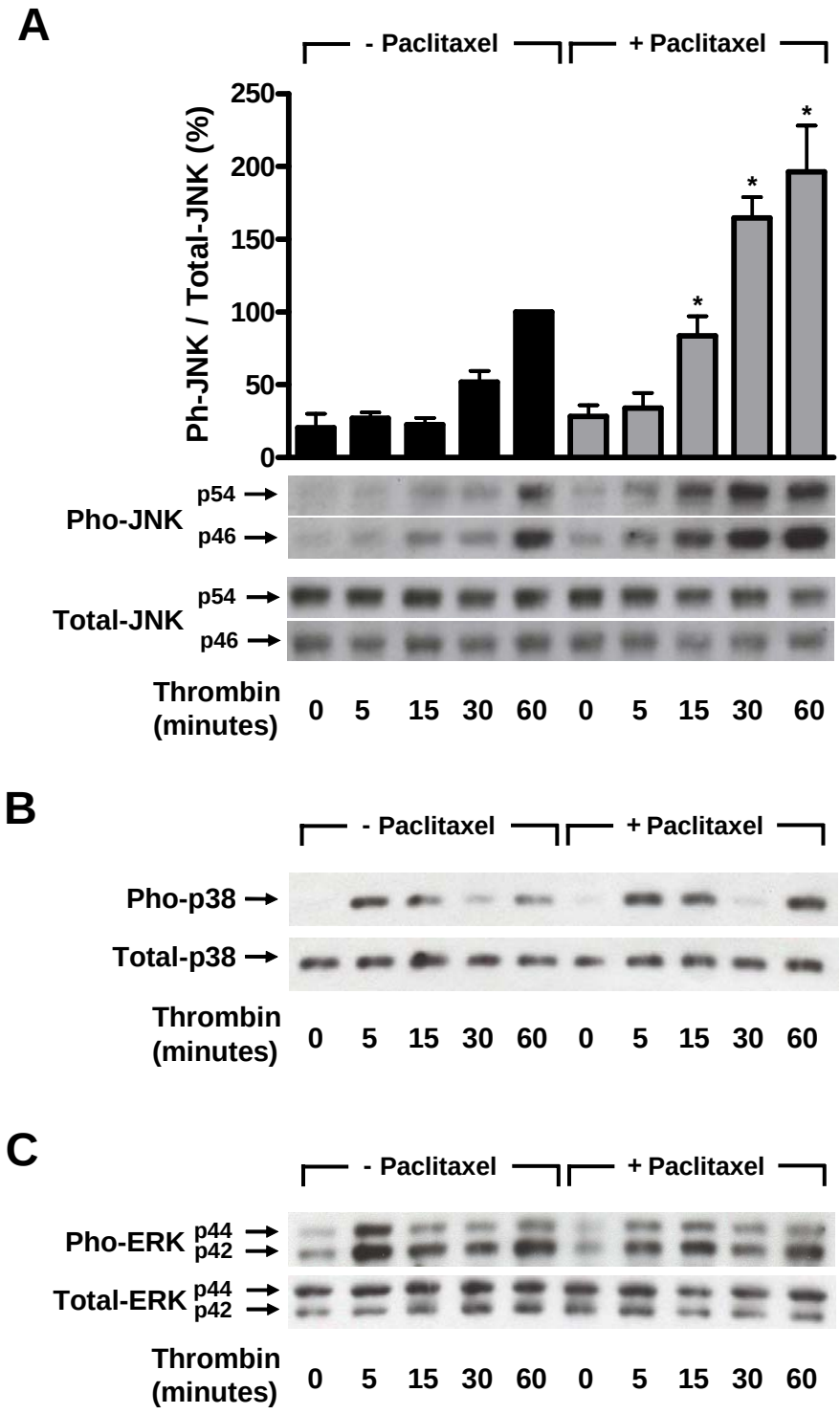


Figure 6. Paclitaxel enhances thrombin-induced JNK activation.

A. Paclitaxel (10^{-5} mol/L) enhances thrombin (1U/ml)-induced JNK activation after 15, 30, and 60 minutes of stimulation (n=4; **P*<0.05 vs thrombin alone). Expression of total JNK is not affected. **B.** Paclitaxel (10^{-5} mol/L) slightly prolongs maximal p38 phosphorylation. Expression of total p38 is not affected. **C.** Paclitaxel (10^{-5} mol/L) slightly decreases maximal ERK phosphorylation. Expression of total ERK is not affected.

IV: Paclitaxel and docetaxel exert similar effects on TF and JNK.

HAECs were stimulated with thrombin (1 U/ml) for 5 hours in the presence or absence of paclitaxel or docetaxel (respectively at 10^{-6} and 10^{-5} mol/L). Similar to paclitaxel, docetaxel enhanced thrombin-induced TF expression by 2.2-fold as compared to thrombin alone (n=4; $P<0.05$ for thrombin plus paclitaxel vs thrombin alone; $P<0.005$ for thrombin plus docetaxel vs thrombin alone; P=NS for thrombin plus paclitaxel vs thrombin plus docetaxel; Figure 7A). Docetaxel did not affect endothelial cell morphology nor LDH release at any concentration used (n=3; P=NS; data not shown). Similar to paclitaxel, docetaxel enhanced JNK phosphorylation as compared to thrombin alone. The increase in JNK activation after 2 hours of thrombin stimulation was 3.2–fold for paclitaxel and 2.2–fold for docetaxel (n=4; $P=0.0001$ for thrombin plus paclitaxel vs thrombin alone; $P<0.05$ for thrombin plus docetaxel vs thrombin alone; P=NS for thrombin plus paclitaxel vs thrombin plus docetaxel; Figure 7B).

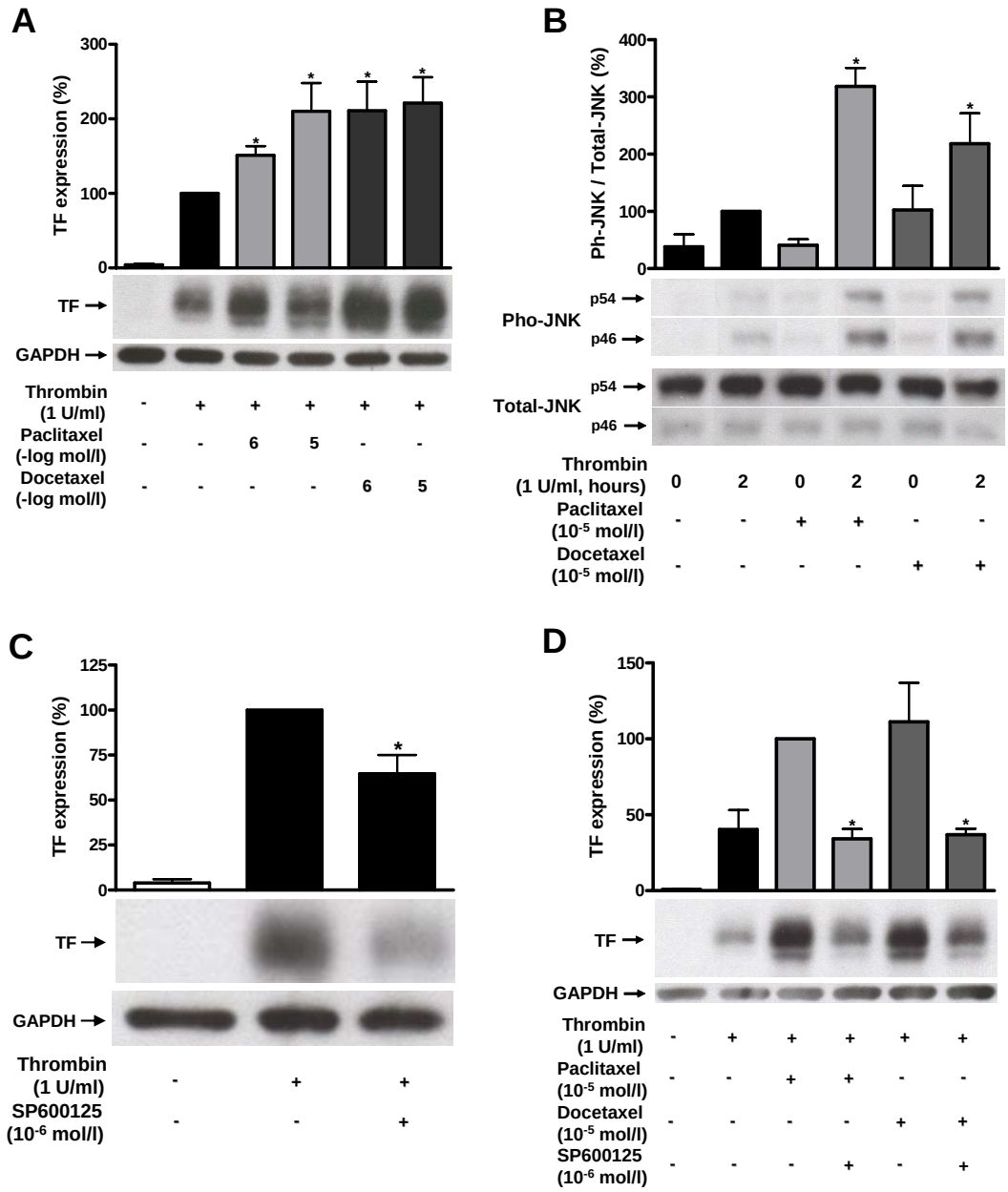


Figure 7. JNK mediates the effect of paclitaxel and docetaxel on TF expression.

A. Similar to paclitaxel, docetaxel (10^{-6} and 10^{-5} mol/L) enhances thrombin-induced TF expression (n=4; **P*<0.05 for thrombin + paclitaxel vs thrombin alone; **P*<0.005 for thrombin + docetaxel vs thrombin alone; *P*=NS for thrombin + paclitaxel vs thrombin + docetaxel). Values are given as percent of TF expression in response to 5 hours thrombin stimulation. Blots are normalized to GAPDH expression. **B.** Paclitaxel and docetaxel enhance JNK phosphorylation as compared to thrombin alone (n=4; **P*=0.0001 for thrombin + paclitaxel vs

thrombin alone; *P<0.05 for thrombin + docetaxel vs thrombin alone; P=NS for thrombin + docetaxel vs thrombin + paclitaxel). Expression of total JNK is not affected. **C.** SP600125 (10^{-6} mol/L) reduces thrombin-induced TF expression to 65% of control (n=3; P<0.01). **D.** In the presence of SP600125 (10^{-6} mol/L), the effect of paclitaxel and docetaxel on thrombin-induced TF expression is blunted (n=4; P<0.0001 for paclitaxel vs paclitaxel + SP600125; P<0.05 for docetaxel vs docetaxel + SP600125; P=NS for paclitaxel + SP600125 vs thrombin alone; P=NS for docetaxel + SP600125 vs thrombin alone).

V: JNK mediates the effect of paclitaxel and docetaxel on TF.

HAECs were pretreated with SP600125, a specific inhibitor of JNK, 90 minutes prior to stimulation with thrombin (1 U/ml). SP600125 (10^{-6} mol/L) reduced thrombin-induced TF expression by 35% (n=3; P<0.01; Figure 7C). Moreover, SP600125 reduced the effect of paclitaxel on thrombin-induced TF expression by 110% and that of docetaxel by 105%, respectively (n=4; P<0.0001 for paclitaxel vs paclitaxel plus SP600125; P<0.05 for docetaxel vs docetaxel plus SP600125; Figure 7D). Hence, inhibition of JNK by SP600125 blunted the effect of paclitaxel and docetaxel on thrombin-induced TF expression (n=4; P=NS for paclitaxel plus SP600125 vs thrombin alone; P=NS for docetaxel plus SP600125 vs thrombin alone; Figure 7D).

Discussion part I

This study reveals that paclitaxel enhances thrombin-induced endothelial TF protein expression and surface activity in a concentration- as well as time-dependent manner via its stabilizing effect on microtubules and selective activation of JNK.

Paclitaxel is a lipophilic diterpenoid that binds to the β-subunit of the tubulin heterodimer; this interaction promotes tubulin polymerization leading to the formation of stable non-functional microtubule bundles and promoting cell cycle arrest in G2/M phase (Rowinsky and Donehower 1995; Crown and O'Leary 2000). Via this mechanism, paclitaxel inhibits proliferation as well as migration of vascular smooth muscle cells and reduces restenosis rates in patients with coronary artery disease (Sollott, Cheng et al. 1995; Babapulle, Joseph et al. 2004; Stone, Ellis et al. 2004). Due to its lipophilic properties, paclitaxel accumulates in the vessel wall reaching particularly high concentrations in the intima (Creel, Lovich et al. 2000; Levin, Vukmirovic et al. 2004); local tissue concentrations are indeed 100-fold higher as compared to perfusate concentrations during ex vivo endovascular perfusion (Creel, Lovich et al. 2000). In a porcine coronary artery stent model, tissue concentrations of paclitaxel reached 3.2 µg/g of arterial tissue after 28 days and drop below detection limit within 3 months only (Vogt, Stein et al. 2004); this tissue concentration of paclitaxel corresponds to 3.7×10^{-6} mol/L at an assumed tissue density of 1 g/cm^3 (Vogt, Stein et al. 2004). Similar tissue

concentrations have been measured in a rabbit iliac artery stent model (Finn, Kolodgie et al. 2005). Thus, the paclitaxel concentrations used in our study are comparable to local tissue concentrations after stent deployment.

In animal models, partial reendothelialization has been observed as early as 4 days after DES deployment, while complete reendothelialization occurs within 3 weeks (Schwartz, Chronos et al. 2004; Vogt, Stein et al. 2004). In humans, partial reendothelialization has been documented 2 weeks after stenting and is usually completed within 12 weeks (Grewe, Deneke et al. 2000; Schwartz, Chronos et al. 2004). Paclitaxel-eluting stents have a biphasic drug release profile in vitro, characterized by an initial burst during the first 48 hours after implantation, followed by a sustained low-level release for at least 2 weeks (Colombo, Drzewiecki et al. 2003; Halkin and Stone 2004; Ranade, Miller et al. 2004). Due to its lipophilic properties, however, very high paclitaxel concentrations have been measured up to 4 weeks after stent implantation in vivo, and the drug remains detectable for up to 12 weeks. Therefore, the time course of reendothelialization coincides with the presence of paclitaxel in the vessel wall after stent deployment. Thus, paclitaxel may indeed alter the biology of the endothelium within the stented area.

In-stent thrombosis has been described in patients treated with paclitaxel-eluting stents, particularly after cessation of antiplatelet therapy (McFadden, Stabile et al. 2004; Iakovou, Schmidt et al. 2005; Ong, McFadden et al. 2005). Our data demonstrate that the effect of paclitaxel is maintained over prolonged time periods and that it becomes effective as soon as a stimulus like thrombin is present; hence, the data are consistent with the clinical observation that cessation of antiplatelet therapy is a risk factor for

thrombosis of drug-eluting stents. Moreover, in view of the coronary paclitaxel concentrations after stent deployment as well as the time-course of reendothelialization, paclitaxel may indeed contribute to the development of subacute or late in-stent thrombosis by enhancing endothelial TF expression. This interpretation is supported by the results of the SCORE trial, which compared the QuaDDS stent (coated with the paclitaxel-derivative 7-hexanoyltaxol) to BMS; the trial had to be terminated prematurely due to very high rates of subacute and late in-stent thrombosis as well as major adverse cardiac events (Grube, Lansky et al. 2004). The increased rates of in-stent thrombosis have been primarily related to the high paclitaxel doses released by these stents, while an unfavourable effect of the stent design may have contributed (Grube, Lansky et al. 2004). Interestingly, the paclitaxel-derivative 7-hexanoyltaxol can be detected up to 10 mm proximal and distal to the stent margins, suggesting that paclitaxel may induce TF expression in the vessel segments proximal and distal to the stent as well (de la Fuente, Miano et al. 2001).

The effect of paclitaxel was due to a specific action on endothelial cell function as it did neither affect cell morphology nor induce any toxicity (Coomber and Gotlieb 1990; Blagosklonny, Darzynkiewicz et al. 2004). This is consistent with previous observations demonstrating that paclitaxel (10^{-5} mol/L) does not induce any cell death in human pulmonary artery endothelial cells or aortic smooth muscle cells after 16 and 36 hours of incubation, respectively (Petrache, Birukova et al. 2003; Blagosklonny, Darzynkiewicz et al. 2004). The increase in TF surface activity was less pronounced than that in protein expression, which may be related to the presence of inactive

encrypted TF on the cell surface or to the distribution of TF in several cellular compartments (Schecter, Giesen et al. 1997).

Thrombin induces TF expression at the transcriptional level via activation of the MAP kinases p38, ERK, and JNK (Liu, Pelekanakis et al. 2004; Steffel, Latini et al. 2005). The increase in TF protein expression by paclitaxel was preceded by an enhanced TF mRNA expression. Consistent with this observation, paclitaxel augmented thrombin-induced JNK phosphorylation. Interestingly, the activation pattern of p38 and ERK was not affected, indicating that paclitaxel selectively activates JNK without affecting other signal transduction molecules in endothelial cells. Consistent with this interpretation, IkB-α degradation was not altered by paclitaxel. Similar observations have been made in different cancer cell lines, demonstrating that the effect of paclitaxel on JNK activation is not restricted to the endothelium (Lee, Li et al. 1998; Wang, Wang et al. 1998; Yujiri, Fanger et al. 1999). To assess whether JNK indeed mediates the induction of TF expression in response to thrombin and, in particular, to paclitaxel, endothelial cells were pretreated with SP600125, a selective inhibitor of JNK catalytic activity. SP600125 decreased thrombin-induced TF expression by about a third, indicating that JNK is not the only signal transduction mediator regulating thrombin-induced TF expression. In contrast, the JNK inhibitor fully prevented the effect of paclitaxel, strongly suggesting that the effect of paclitaxel on TF expression is selectively mediated by the JNK pathway. However, it can not be ruled out completely that other signal transduction pathways may be involved as well.

The microtubule-stabilizing agent docetaxel was used to elucidate

whether the increase in thrombin-induced JNK activation and TF expression by paclitaxel was related to perturbation of microtubule function (Wang, Wang et al. 1998). Both microtubule-stabilizing agents exerted a similar effect on JNK activation and TF expression, and SP600125 blunted the enhancing effect of both docetaxel and paclitaxel on thrombin-induced TF expression. Thus, the action of paclitaxel on JNK activation and TF expression seems to depend on stabilization of microtubule bundles rather than on a substance-specific effect. Consistent with this interpretation, JNK activation in response to changes in the microtubule cytoskeleton has been described in different cancer cell lines (Wang, Wang et al. 1998; Yujiri, Fanger et al. 1999).

Paclitaxel enhanced thrombin-induced endothelial TF expression by 2.1-fold. Rapamycin augmented thrombin-induced TF expression to a similar extent, while the mechanism of action of the two drugs differs completely; binding of rapamycin to its intracellular receptor FKBP-12 abrogates p70S6 kinase phosphorylation leading to enhanced endothelial TF expression, while JNK activation remains unaffected (Steffel, Latini et al. 2005). Large-scale clinical trials have demonstrated that patients receiving paclitaxel-eluting stents have similar rates of in-stent thrombosis as compared to rapamycin-eluting stents (Kastrati, Dibra et al. 2005; Windecker, Remondino et al. 2005). This observation is consistent with the similar degree of TF induction in endothelial cells, suggesting that the latter may indeed be importantly involved in thrombosis of DES.

In conclusion, this study indicates that paclitaxel increases endothelial TF expression via JNK activation due to its microtubule stabilizing effect. The enhanced endothelial TF expression may favor thrombus formation after

paclitaxel-eluting stent deployment, particularly when antithrombotic drugs are withdrawn or thrombin levels are elevated as it occurs in acute coronary syndromes. Therefore, these findings may have interesting implications for drug-eluting stent design.

Results part II

Preface.

Dimethyl sulfoxide (DMSO, Figure 1) is employed for preservation of hematopoietic progenitor cells and therefore infused into patients undergoing bone marrow or stem cell transplantation (Egorin, Rosen et al. 1998).

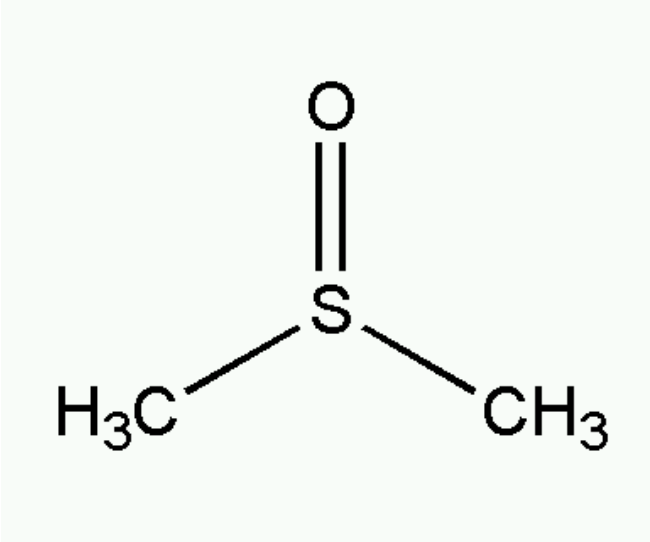


Figure 1: Atomical structure of DMSO, molecular weight 78.

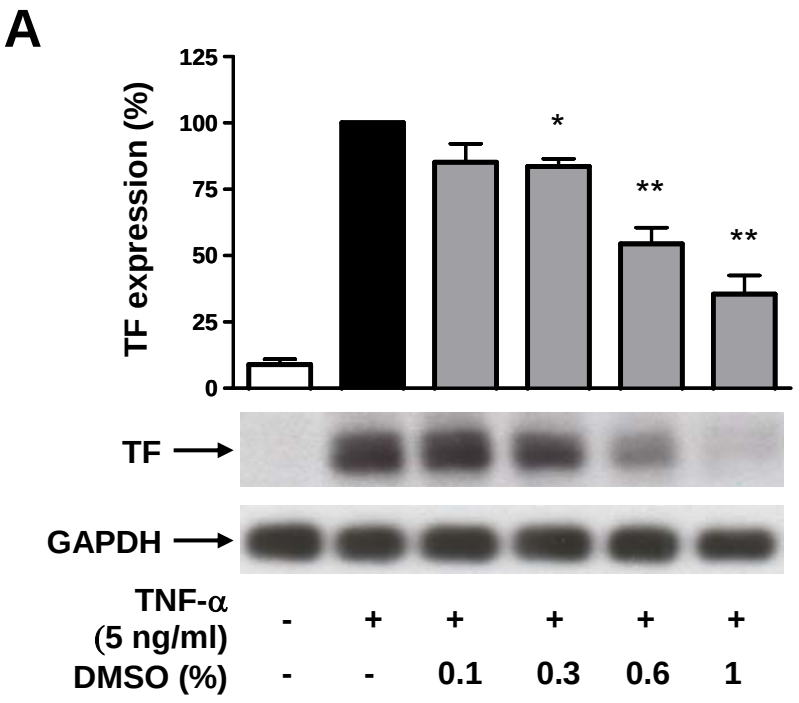
In this context, peak plasma concentrations reach values as high as 20 mmol/L. DMSO is also used as a solvent for chemotherapeutic drugs and, due to its anti-inflammatory properties, has been successfully employed in humans for treating rheumatic (Morassi, Massa et al. 1989), pulmonary

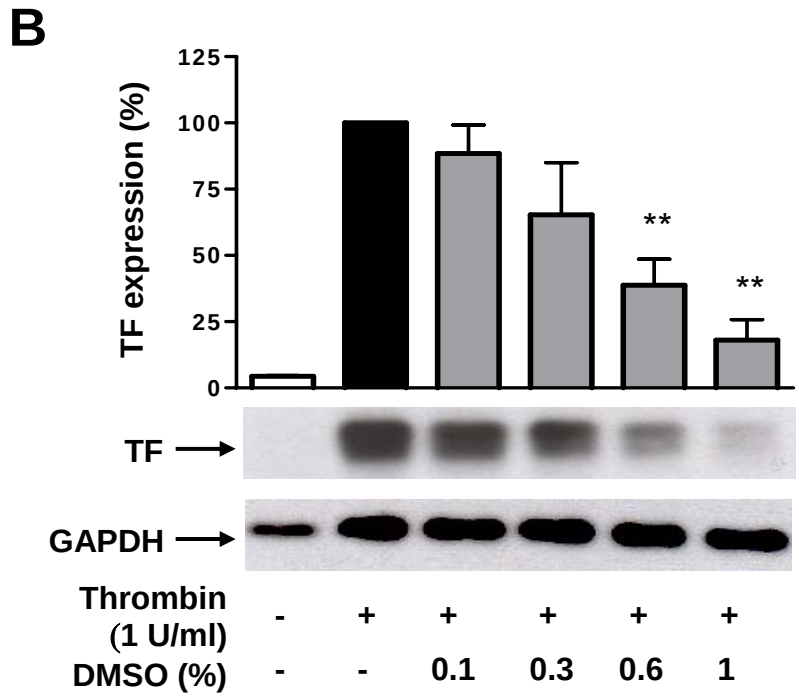
(Iwasaki, Hamano et al. 1994), gastrointestinal (Salim 1992; Salim 1994), neurological (Karaca, Bilgin et al. 1991; Karaca, Kilic et al. 2002), urinary (McCammon, Lentzner et al. 1998), and dermatological (Wong and Lin 1988; Burgess, Hamner et al. 1998) disorders. DMSO further exhibits protective effects in animal models of middle cerebral artery occlusion (Bardutzky, Meng et al. 2005), cerebral hypoperfusion related neuronal death (Farkas, Institoris et al. 2004), mercuric chloride induced kidney injury (Jo, Hu et al. 2004), and chemical liver injury (Lind and Gandolfi 1997). A recent report proposed that DMSO reduces ischemic brain damage through both anti-inflammatory and free radical scavenging properties (Bardutzky, Meng et al. 2005). Despite of all this information, the impact of DMSO on cardiovascular disorders has not been assessed.

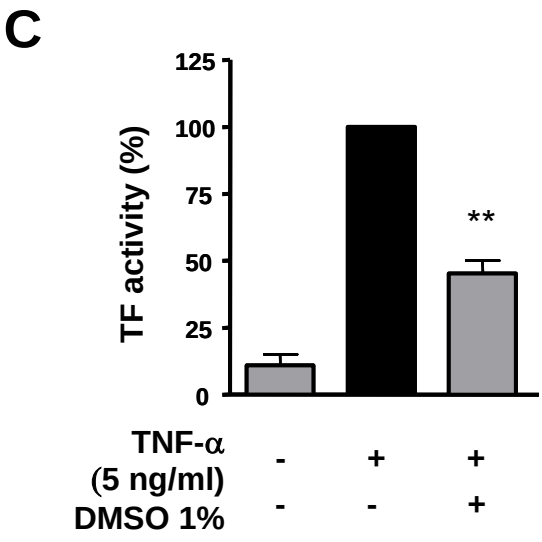
Since intravenous delivery of DMSO has become a recognized practice in clinical oncology due to the increasing use of bone marrow transplants, this study was designed to investigate the impact of DMSO on TF expression and thrombosis as well as on activation of VSMC.

I: Dimethyl Sulfoxide Inhibits Tissue Factor Expression.

Stimulation of HAEC with TNF-α (5 ng/ml) resulted in a 19-fold increase in TF expression (n=4; P<0.0001; 2A); similarly, thrombin (1 U/mL) induced a 24-fold increase of TF (n=4; P<0.0001; 2B). When cells were pretreated with increasing concentrations of DMSO (0.1-1.0%), TF induction by both TNF-α and thrombin was inhibited in a concentration-dependent manner (n=4; P<0.0001 for TNF-α or thrombin alone vs. 1.0% DMSO; 2A, 2B). This effect was paralleled by a similar inhibition of TF surface activity (n=4; P<0.0001 for TNF-α alone vs. 1.0% DMSO; 2C). In THP-1 cells, stimulation with TNF-α (5 ng/ml) induced a 10-fold increase in TF expression (n=4; P<0.0001), which was inhibited by DMSO (n=4; P<0.005 for TNF-α alone vs. 1.0% DMSO; Fig. 2D). In VSMC, the 3.5-fold increase in TNF-α-induced TF expression (n=4; P<0.005) was inhibited as well by DMSO (n=4; P<0.05 for TNF-α alone vs. 1.0% DMSO; Fig. 2D).




B
125
100
75
50
25
0
TF expression (%)
**
**
TF
GAPDH
Thrombin
(1 U/ml)
DMSO (%)
- + + + + +
- - 0.1 0.3 0.6 1


C
125
100
75
50
25
0
TF activity (%)
**
TNF-α
(5 ng/ml)
DMSO 1%
- + +
- - +

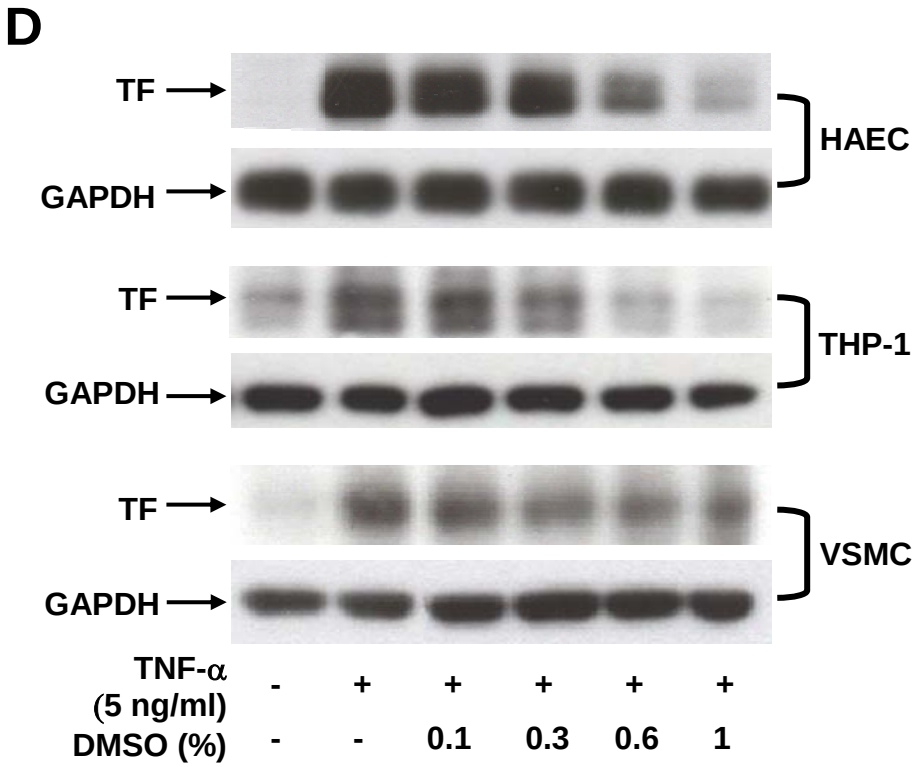


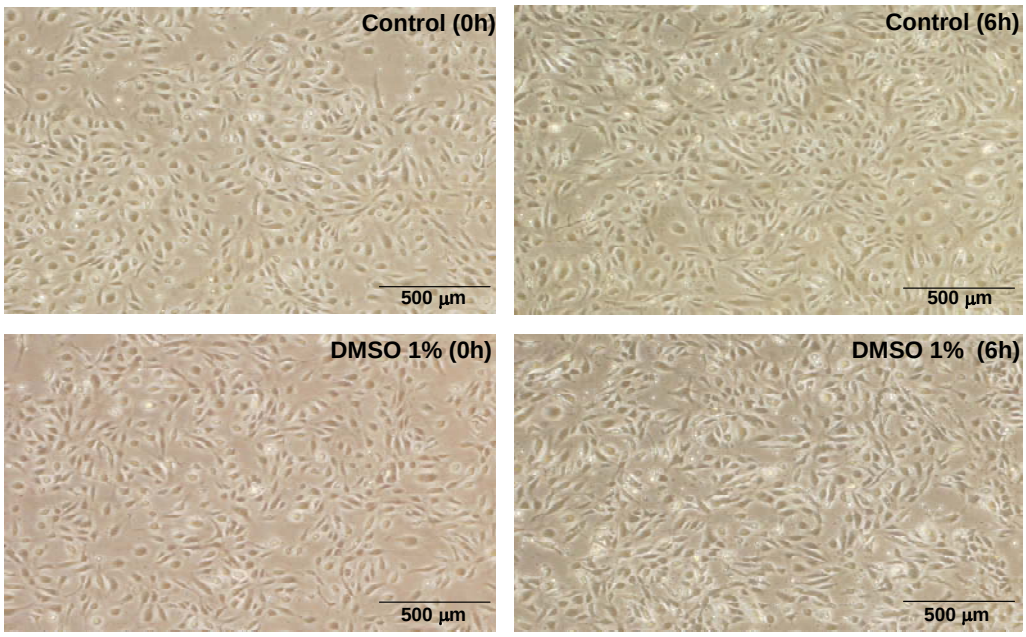
Figure 2. DMSO inhibits TF expression and activity.

A. DMSO inhibits TNF-α induced TF expression of human aortic endothelial cells (HAEC) in a concentration-dependent manner. Values are given as percent of stimulation with TNF-α alone. **P*<0.001, ** *P*<0.0001 vs. TNF-α alone. **B.** DMSO inhibits thrombin-induced TF expression of HAEC in a concentration-dependent manner. Values are given as percent of stimulation with thrombin alone. **P*<0.001, ** *P*<0.0001 vs. thrombin alone. **C.** DMSO inhibits TNF-α induced TF surface activity of HAEC. Values are given as percent of stimulation with TNF-α alone. ** *P*<0.0001 vs. TNF-α alone. **D.** DMSO inhibits TNF-α induced TF expression in a concentration-dependent manner in HAEC, human monocytic cells (THP-1) and in human aortic vascular smooth muscle cells (VSMC). Values are representative of at least 3 different experiments; all blots are normalized to GAPDH expression

II: DMSO does not exert toxicity in human vascular cells.

To assess potential toxic effects of DMSO, HAEC were incubated with 1.0% DMSO for 6 hours, and a morphological examination was performed. No changes in cell morphology were observed when cells with or without treatment were compared (Fig. 3A). In addition, cell death was assessed by both LDH release and trypan blue exclusion in HAEC; again, no signs of toxicity were detected by either method (n=4; P=n.s.; Fig. 3B, 3C).

A



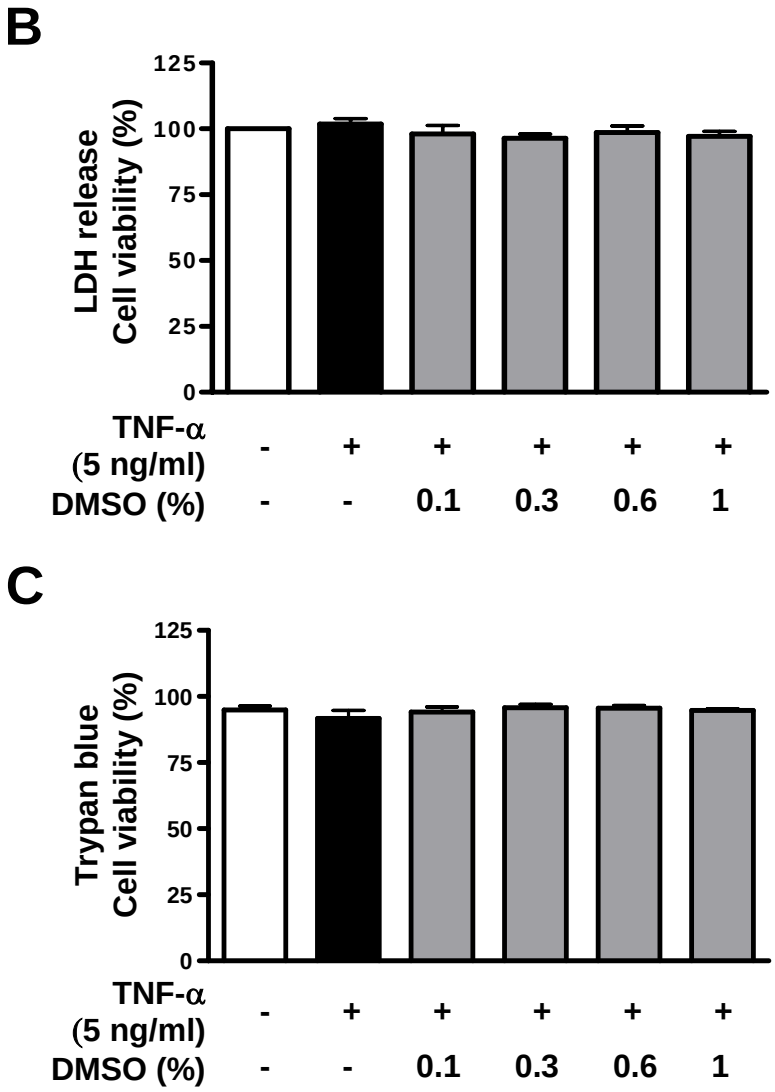
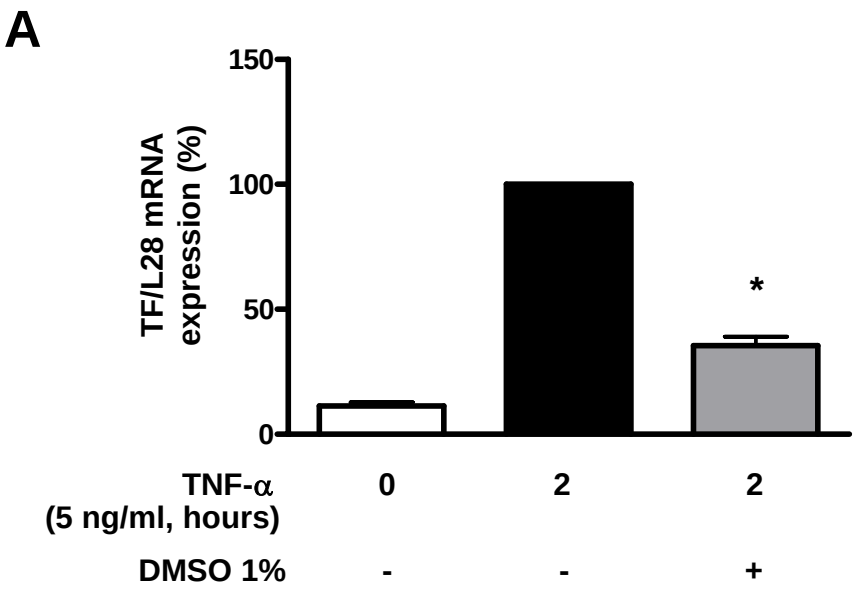


Figure 3. Lack of DMSO-induced toxicity.

A. Incubation with DMSO for 6 h does not affect cell morphology (20 times magnification). LDH release **(B)** and the number of trypane blue positive cells **(C)** remains unaffected by different DMSO concentrations as well. Values are given as percent of stimulation with TNF-α alone

III: DMSO inhibits TF mRNA expression

Peak induction of TF mRNA occurs 2 hours after stimulation with TNF-α (Steffel, Latini et al. 2005). Real-time PCR analysis confirmed a 10-fold induction of TF mRNA expression after 2 h of TNF-α stimulation in HAEC (n=3; P<0.001; Fig. 4A). Treatment with 1.0% DMSO inhibited TF mRNA expression by 65±7% (n=3; P<0.0001 for TNF-α alone vs. 1.0% DMSO; Fig. 4A, 4B).



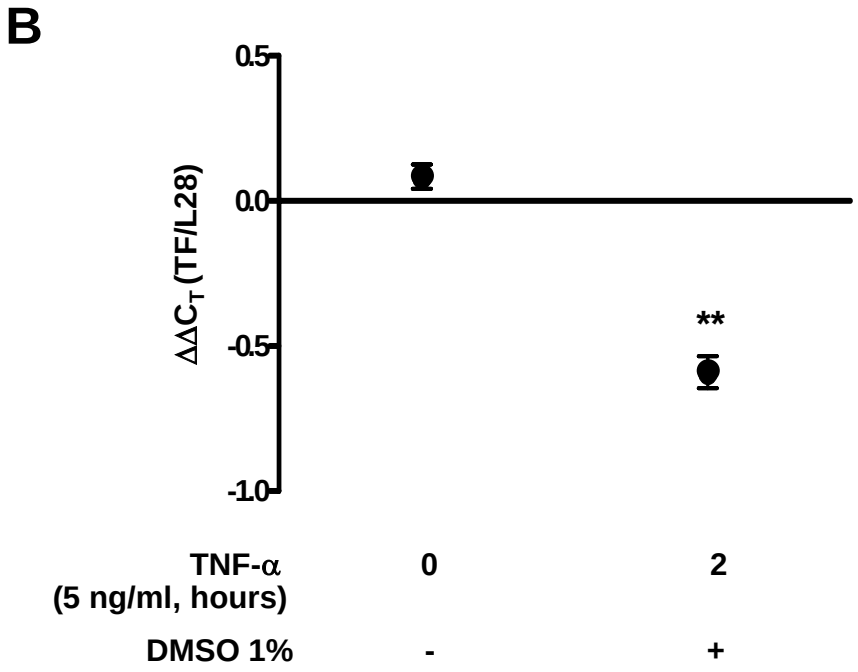


Figure 4. DMSO inhibits TF mRNA expression.

A. Real-time PCR demonstrates increased TF mRNA expression after 2 h of stimulation with TNF-α. DMSO inhibits this effect. Values are given as percent of stimulation with TNF-α alone. **P*<0.001 vs. TNF-α alone. **B.** $\Delta\Delta C_T$ values confirm that DMSO decreases TNF-α induced TF mRNA expression. ** *P*<0.0001 vs. TNF-α alone. All values are representative of 4 different experiments and are normalized to L28 mRNA.

IV: DMSO impairs TF expression via MAPK inhibition

To assess whether DMSO affects MAP kinase activation, HAEC were examined at different time points after stimulation. JNK, p38, and ERK were transiently activated by TNF-α (Fig. 5A). Maximal phosphorylation occurred after 15 minutes and returned to basal levels within 60 minutes (Fig. 5A). DMSO inhibited phosphorylation of JNK and p38 by over 50% while leaving activation of ERK unaltered (Fig. 5A). Indeed, maximal phosphorylation of JNK was decreased by 51±6% (n=4; P=0.0005; Fig. 5B) and that of p38 by 50±3% (n=4; P<0.0001; Fig. 5B); in contrast, maximal activation of ERK was not significantly reduced (n=4; P=n.s.; Fig. 5B). No change in total expression of JNK, p38, or ERK was observed at any time point with or without DMSO. To verify the involvement of MAP kinases in TNF-α-induced TF expression under our experimental conditions, the effect of MAP kinase inhibitors on TF expression was examined in HAEC. SP600125, SB203580, and PD98059, which specifically inhibit JNK, p38, and ERK, respectively, significantly impaired TF induction after TNF-α stimulation (n=4; P<0.001 for TNF-α alone vs. each inhibitor; Fig. 5C).

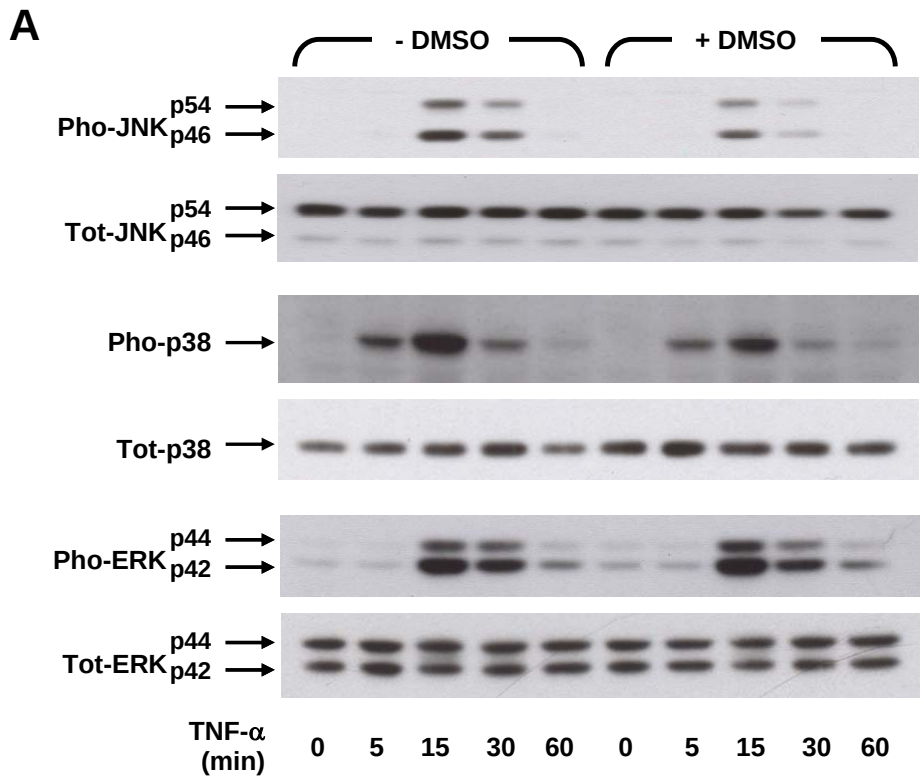


Figure 4. DMSO inhibits MAP kinase activation.

A. Stimulation with TNF-α leads to transient phosphorylation (Pho) of the MAP kinases JNK, p38, and ERK. DMSO inhibits phosphorylation of JNK and p38, but not ERK. Total (Tot) levels of JNK, p38, and ERK remain unchanged.

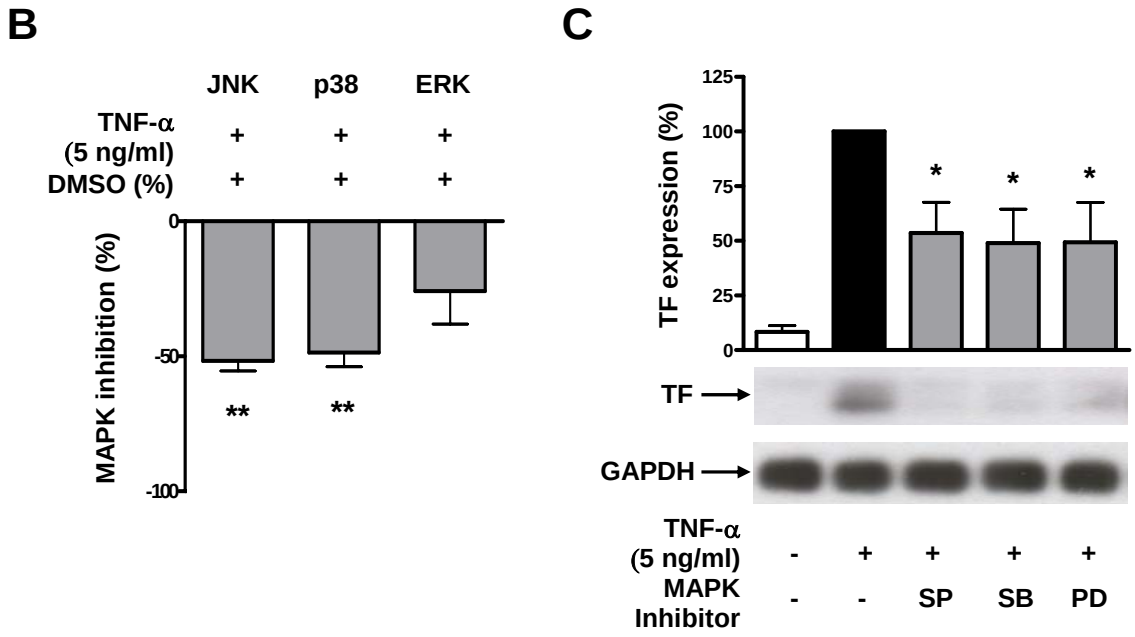


Figure 4. DMSO inhibits MAP kinase activation.

B. DMSO strongly inhibits phosphorylation of JNK and p38, but not ERK. Average values of 4 different experiments. *P<0.001, for JNK and p38 vs. TNF-α alone **C.** SP600125, SB203580, and PD98059, specific inhibitors of JNK, p38, and ERK, respectively, inhibit TNF-α induced TF expression. *P<0.001, vs. TNF-α alone. Blots are representative of at least 3 different experiments.

V: DMSO does not affect TFPI and PAI-1 expression

The effect of DMSO on the physiological inhibitor of TF, TFPI, as well as on the anti-fibrinolytic factor, PAI-1, was assessed. Treatment of TNF-α-stimulated HAEC with 1% DMSO did not affect TFPI expression (n=4; P=n.s.; Fig. 6A). Similarly, treatment of TNF-α-stimulated HAEC with 1.0% DMSO did not alter PAI-1 expression (n=4; P=n.s.; Fig. 6B).

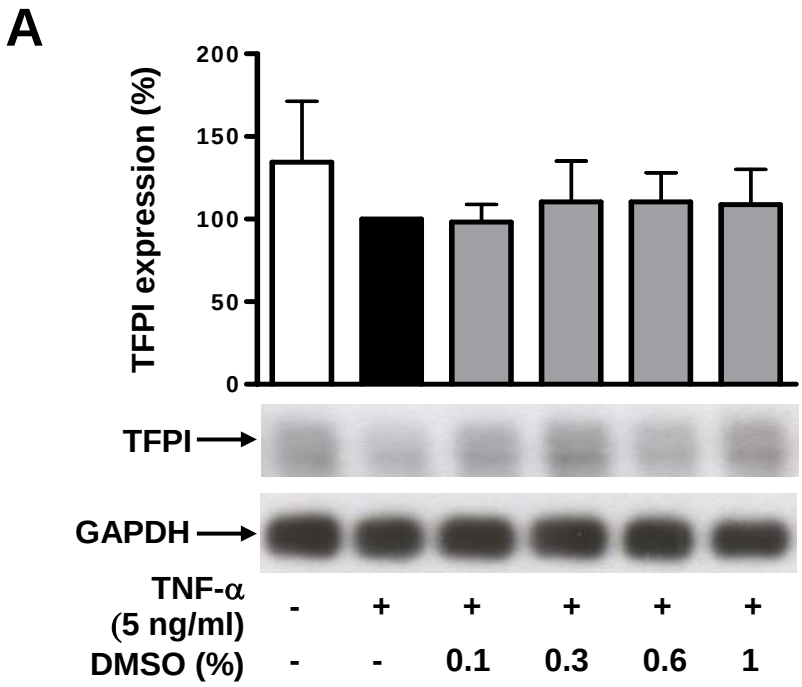


Figure 6. DMSO does not affect TFPI and PAI-1 expression.

A. DMSO does not affect TFPI expression p<0.2.

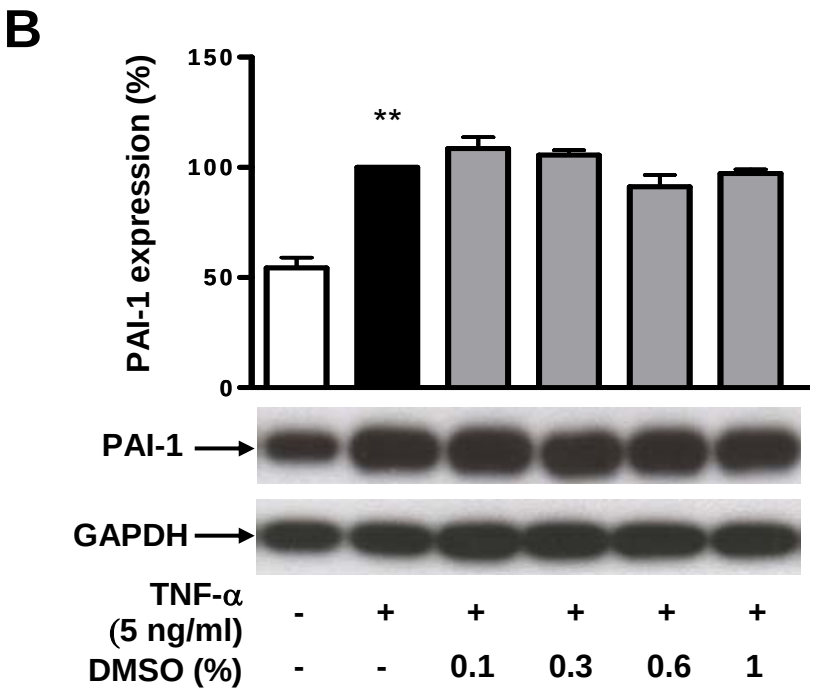


Figure 6. DMSO does not affect TFPI and PAI-1 expression.

B. PAI-1 expression is induced by TNF-α stimulation***P*<0.0001 vs. TNF-α alone. DMSO does not affect TNF-ą induced PAI-1 expression. Values are given as percent of stimulation with TNF-α alone. Blots are representative of at least 3 different experiments.

VI: DMSO suppresses TF activity and prevents arterial thrombosis *in vivo*

Treatment with DMSO (3.8 ml/kg of 40% DMSO in PBS corresponding to 80 mg/kg of DMSO) reduced TF activity in mouse carotid artery *in vivo* (n=3; P<0.002 for vehicle (PBS) vs. DMSO; Fig. 7A).

Photochemically induced arterial injury is dependent on TF and is an established model of arterial thrombosis (Matsuno, Uematsu et al. 1991). Initial blood flow in vehicle (PBS)-treated mice equalled 1.25±0.15 ml/min (n=5); these mice developed thrombotic occlusion within 1 hour after onset of injury (mean occlusion time 63±9 minutes; n=5; P<0.0001 for initial vs. final blood flow; Fig. 7B). During injury, it was evident from transient reductions in flow that a dynamic state of thrombus formation and lysis occurred during the short time interval prior to complete occlusion. Cessation of blood flow coincided with the appearance of a faint white occlusive defect that was visible through the microscope in the lumen of the artery. DMSO (3.8 ml/kg of 40% DMSO in PBS corresponding to 80 mg/kg of DMSO) treated mice exhibited similar initial blood flow (1.14±0.19 ml/min; n=5; P=n.s. for vehicle vs. DMSO), but did not develop thrombotic occlusion. Indeed, 120 min after onset of injury, blood flow in DMSO-treated mice was still very high (0.75±0.15 ml/min; n=5; P<0.01 for vehicle vs. DMSO). However, DMSO-treated mice exhibited a small, but significant decrease in blood flow during the 120 minutes observation period as compared to control mice who did not undergo photochemical injury (n=5; P<0.05). Blood flow in the latter did not change significantly during the 120 minutes observation period (initial flow: 1.12±0.17

ml/min; final flow: 0.94±0.09 ml/min; n=5; P=n.s. for initial vs. final blood flow).

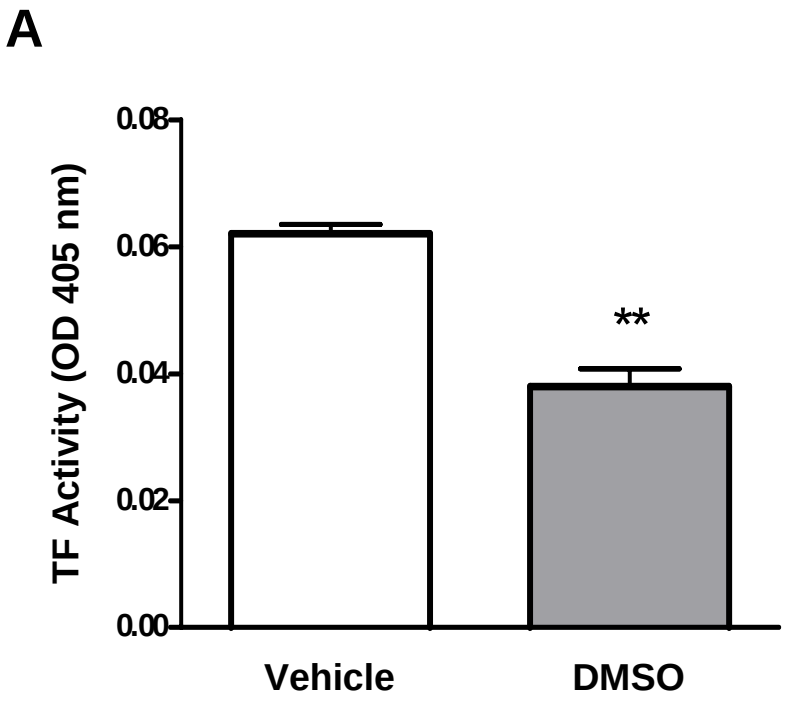


Figure 7. DMSO inhibits carotid artery TF activity and prevents thrombotic occlusion in a mouse model of photochemical injury.

A. DMSO inhibits TF activity in mouse carotid artery. Values are given as absorbance at 405 nm. ** *P*<0.0001 vs. PBS treated (vehicle).

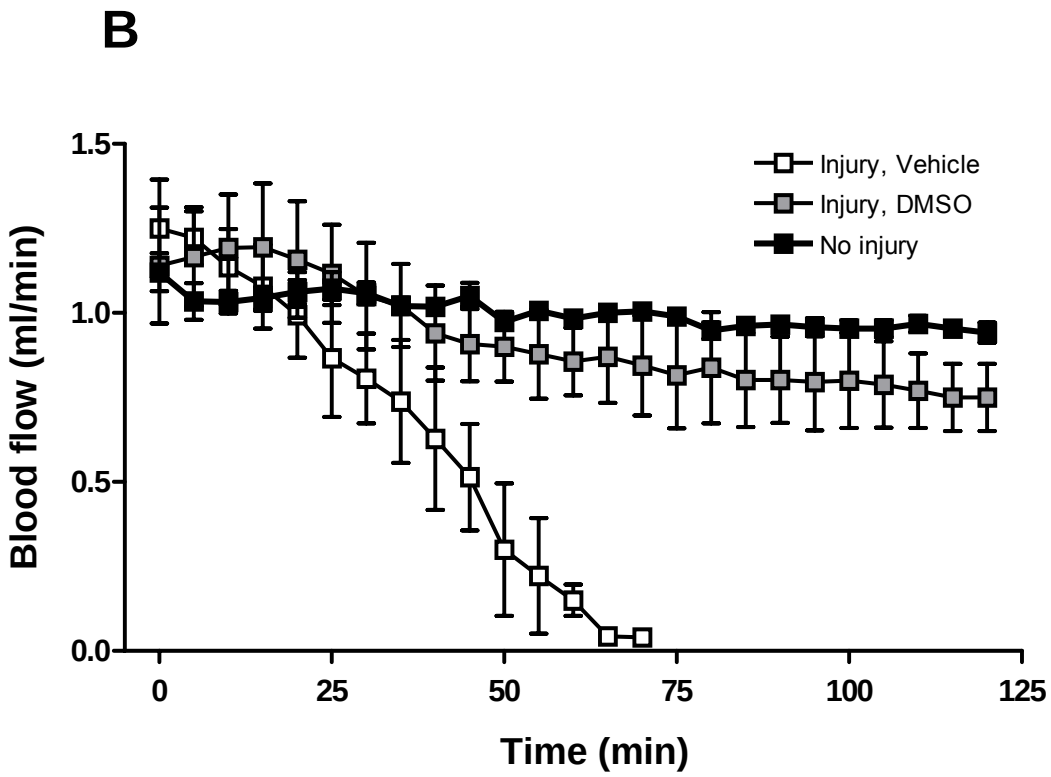
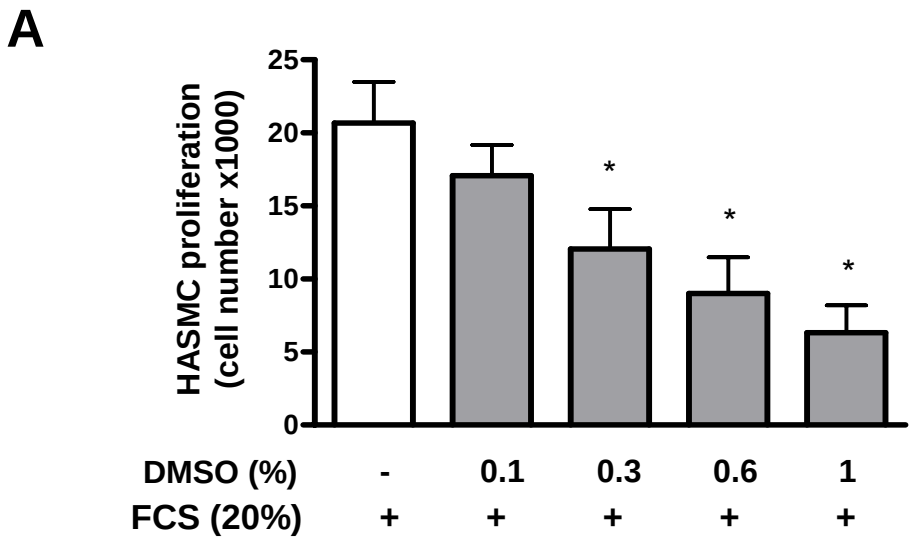


Figure 7. DMSO inhibits carotid artery TF activity and prevents thrombotic occlusion in a mouse model of photochemical injury.

B. Transit-time flow recordings of vehicle treated (PBS) or DMSO treated mouse carotid artery after photochemical injury *in vivo*. Laser injury is initiated at time = 0 min. Uninjured mice are included as an additional negative control. DMSO prevents thrombus formation (P=0.0090 for DMSO treated vs. vehicle treated; P=0.002 for DMSO treated vs. negative control).

VII: DMSO inhibits proliferation and migration of human VSMC

Treatment with DMSO inhibited VSMC proliferation in response to 20% FCS in a concentration-dependent fashion (n=4; $P<0.05$; Fig. 8A). Likewise, DMSO inhibited VSMC migration in response to 10 ng/ml PDGF BB (n=4; $P<0.05$; Fig. 8B). Both rapamycin and paclitaxel are known to enhance thrombin-induced TF expression(Steffel, Latini et al. 2005; Stahli Barbara 2006). To determine whether DMSO can prevent this effect, HAEC were incubated with either drug with or without DMSO (Fig. 8C). Rapamycin (10^{-7} M) and paclitaxel (10^{-5} M) enhanced thrombin-induced TF expression by 241.9±54.61% (n=4; $P<0.05$ vs. thrombin alone; Fig. 8C) and 184±35.3% (n=4; $P<0.05$ vs. thrombin alone; Fig. 8C), respectively. DMSO blunted the effect of rapamycin and paclitaxel on TF expression (n=4; $P=<0.0001$ vs. thrombin plus rapamycin or thrombin plus paclitaxel; Fig. 8C).



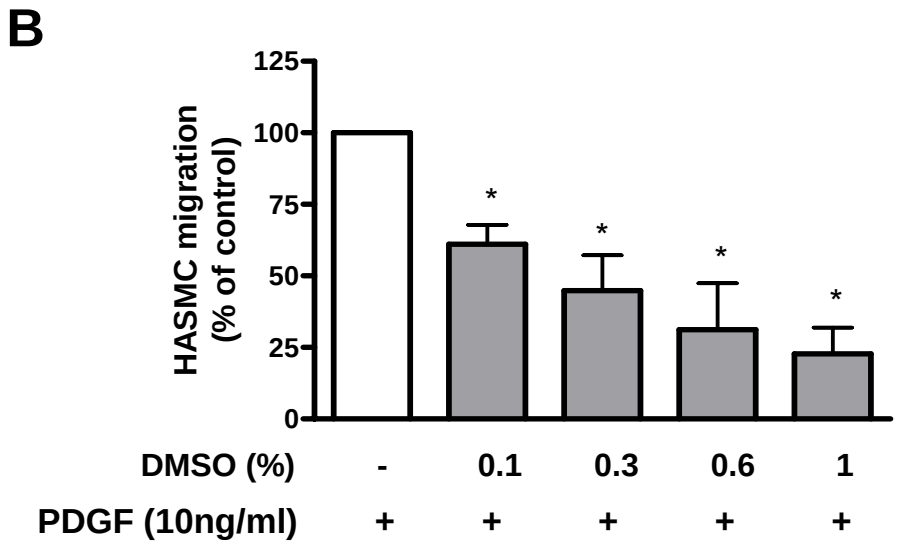


Figure 8. DMSO inhibits proliferation and migration of VSMC

A. DMSO inhibits VSMC proliferation in response to 20% FCS by 70%; n=4 *P<0.05 vs. control. **B**. DMSO inhibits VSMC migration in response to 5 hours 10 ng/ml PDGFstimulation by 75%; n=4*P<0.05 vs. control. Values are given as percent of stimulation with TNF-α alone. ** P<0.0001 vs. TNF-α alone.

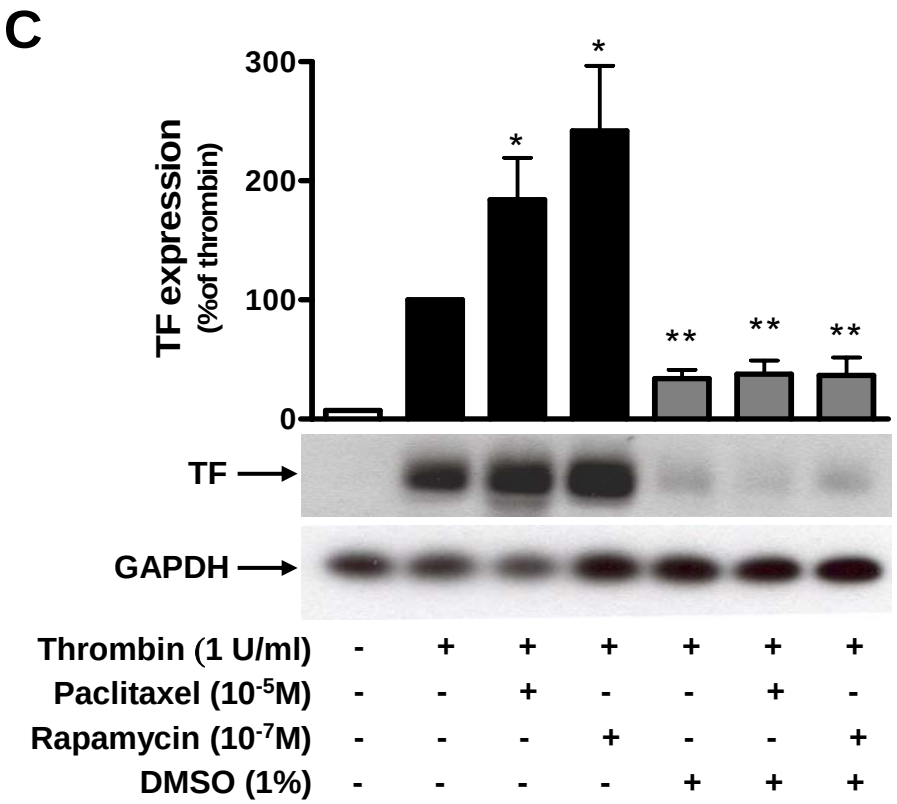


Figure 8. DMSO prevents paclitaxel- and rapamycin-enhanced TF expression

C. Paclitaxel and rapamycin enhance thrombin-induced TF expression in HAEC (n=4; **P*<0.05 for rapamycin or paclitaxel vs. thrombin alone). DMSO blunts thrombin-induced TF expression as well as the effect of rapamycin and paclitaxel on TF expression. ** *P*<0.0001 vs. DMSO, DMSO plus rapamycin, and DMSO plus paclitaxel. Blots are representative of 4 different experiments.

Discussion part II

This study demonstrates that DMSO inhibits TF expression and activity via reduced activation of the MAP kinases JNK and p38. Consistent with this observation, DMSO suppresses TF activity and protects from thrombotic occlusion *in vivo*. The study also demonstrates that DMSO inhibits proliferation and migration of vascular smooth muscle cells; furthermore, it prevents TF induction in response to rapamycin and paclitaxel. This profile renders DMSO ideally suitable for application on drug-eluting stents and for the treatment of acute coronary syndromes.

DMSO treatment did not exert any toxic effect even at the highest concentration used (1.0%; 12.6 mmol/L). An extensive study in human endothelial cells also did not observe any toxic effects of DMSO even at concentrations well above those employed in our study (Bourne, Shearer et al. 1994). Moreover, DMSO has been routinely used to trigger in vitro cellular differentiation at concentrations as high as 1.25% (15.7 mmol/L) (Heidari, Shah et al. 2004). Furthermore, in patients after myeloablative therapy, DMSO is infused intravenously together with hematopoietic progenitor cells; under these conditions, DMSO reaches plasma concentrations of 1.6% (20.0 mmol/L) and only rarely causes adverse effects (Egorin, Rosen et al. 1998). Thus, the DMSO concentrations applied in our study are clearly below those used to induce cellular differentiation or those occurring in treatment of leukaemia patients and hence would be suitable for clinical use.

Thrombin and TNF-α induced TF expression in HAEC with similar

potencies, and incubation with DMSO suppressed this effect irrespective of the stimulus. Furthermore, DMSO reduced TNF-α-induced TF expression not only in HAEC, but also in THP-1 and VSMC, indicating that the effect on TF occurs irrespective of the cell type. Therefore, the protective action of DMSO is neither restricted to a specific stimulus nor to a specific vascular cell type.

Activation of MAP kinases mediates TF expression in response to several stimuli (Mechtcheriakova, Schabbauer et al. 2001; Eto, Kozai et al. 2002; Steffel, Akhmedov et al. 2005; Steffel, Hermann et al. 2005). Indeed, JNK, p38, and ERK were transiently activated in HAEC after stimulation with either TNF-α or thrombin. Treatment with DMSO impaired TF expression by specifically decreasing phosphorylation of JNK and p38, but not ERK; hence, inhibition of these two MAP kinases suppressed TF expression in a potent manner. In line with this observation, pharmacological inhibition of the two MAP kinases induces a more pronounced reduction of TF expression than inhibition of one MAP kinase alone. Consistent with inhibition of MAP kinases, DMSO promoted a strong reduction in TNF-α-induced TF mRNA levels. Thus, the inhibitory action of DMSO is related to a specific inhibition of MAP kinase activation.

TF activity is counterbalanced by its endogenous inhibitor TFPI; the balance of these two factors is essential in determining thrombus formation (Pedersen, Holscher et al. 2005). Treatment with DMSO did not affect TFPI expression indicating that the antithrombotic action of DMSO on TF expression is not compensated by a concomitant reduction in TFPI expression. PAI-1 is a serpin that suppresses fibrinolysis by inhibiting the activity of plasminogen activator. A recent report demonstrated that DMSO is

able to reduce interleukin-1-induced PAI-1 expression in rat microvascular endothelial cells (Okada, Woodcock-Mitchell et al. 1998). In contrast, we did not observe any effect of DMSO on PAI-1 expression in TNF-α-stimulated HAEC; this difference may be related to the different species, the different origin of the cells, or the different stimuli used for induction. Thus, the inhibitory action of DMSO on TF expression does not seem to be modulated by concomitant effects on TFPI or PAI-1.

It is generally accepted that thrombus formation is triggered by TF (Nemerson 1988; Mann, van't Veer et al. 1998; Mackman 2004). Photochemical injury was selected as a model of thrombosis because it is an established protocol for examining TF-dependent thrombosis *in vivo* (Day, Reeve et al. 2005); moreover, this procedure does not require intra-arterial invasion (Matsuno, Uematsu et al. 1991; Day, Reeve et al. 2004). We observed that thrombotic occlusion following injury was prevented in DMSO-treated mice, indicating that treatment with DMSO prevents thrombus formation *in vivo*. Indeed, inhibition of TF activity in the mouse carotid artery within 2 hours of DMSO administration was confirmed and thus is the most likely explanation for the effect of DMSO on thrombus formation after photochemical injury. It can not be excluded, however, that the effects of DMSO *in vivo* may in part be attributable to its ability to impair platelet adherence and aggregation (Dujovny, Rozario et al. 1983).

Involvement of TF in acute coronary syndromes was recently underlined by a study revealing increased levels of TF antigen and activity in atherectomy specimens from patients with unstable angina or myocardial infarction as compared to those with stable angina (Annex, Denning et al.

1995). Moreover, in acute coronary syndromes, plasma concentrations of cytokines such as TNF-α and interleukin-6 are increased at the site of coronary artery occlusion to concentrations high enough to induce TF in vascular cells (Maier, Altwegg et al. 2005). For these reasons, inhibition of TF by DMSO may represent a novel and promising strategy for targeting thrombosis in acute coronary syndromes. In addition, the antiproliferative and antimigratory properties of DMSO as well as its common use in clinical practice underscore its suitability for administration to patients with acute coronary syndromes either intravenously or locally as a coating agent for drug eluting stents.

Although drug-eluting stents are effective in reducing restenosis rates after percutaneous coronary intervention through inhibition of neointima formation and constrictive remodelling, stent thrombosis remains a concern with these devices. Indeed, the rate of stent thrombosis observed with drug-eluting stents is around 2% in controlled clinical trials and may be higher in “real world” patients (Iakovou, Schmidt et al. 2005; Windecker, Remondino et al. 2005). Moreover, if thrombosis of such stents occurs, it is associated with a high morbidity and mortality (Ong, Hoye et al. 2005). Our study demonstrates that DMSO has the potential to inhibit both neointima formation and stent thrombosis if applied on a drug-eluting stent. The known ability of DMSO to inhibit platelet aggration may be very important in this context as well (Dujovny, Rozario et al. 1983). The ability of DMSO to suppress rapamycin and paclitaxel-induced TF expression is of particular relevance, as this effect of rapamycin and paclitaxel represents a possible cause for the occurrence of stent thrombosis in drug-eluting stents (Steffel, Latini et al. 2005; Stahli

Barbara 2006). Indeed, DMSO could be applied on such stents either alone or in combination with rapamycin or paclitaxel; such an application is technically feasible and looks particularly promising.

In summary, this study provides evidence that DMSO specifically suppresses TF expression and activity in addition to preventing TF-dependent thrombosis *in vivo*; furthermore, DMSO inhibits proliferation and migration of VSMC and prevents rapamycin as well as paclitaxel-induced TF expression. As the use of DMSO is established in different areas of modern medicine, we propose this drug as a novel strategy for treating acute coronary syndromes; in particular, DMSO seems to represent an attractive compound for application on drug-eluting stents, either alone or in combination with rapamycin or paclitaxel.

Conclusions

Expression of TF is an important defence mechanism that evolved to prevent excessive bleeding and promote wound repair. Yet, TF expression has been shown to be crucially involved in cardiovascular disease.

Taken together, our work shows that TF expression is importantly regulated by the phosphorylation of the MAP kinases JNK, ERK and p38 (Figure 1). However, TF expression can also take place to a lesser extent when only one or two MAP kinases are activated.

Paclitaxel, an agent universally used to coat stents and target restenosis is indeed very effective at doing so but, as an unwanted effect, it induces TF expression by activation of the MAP kinase JNK (Figure 1). This finding could partly account for the recurrence of in stent thrombosis which, despite the advent of drug eluting stents, remains a central negative outcome in present-day clinical practice. The notion that rapamycin, likewise paclitaxel, induces TF expression, makes the search for a new compound a pressing issue.

DMSO inhibits TF expression in human vascular cells as well as preventing thrombosis in the mouse. In addition, DMSO inhibits the activation of human smooth muscle cells; a key process implicated in restenosis.

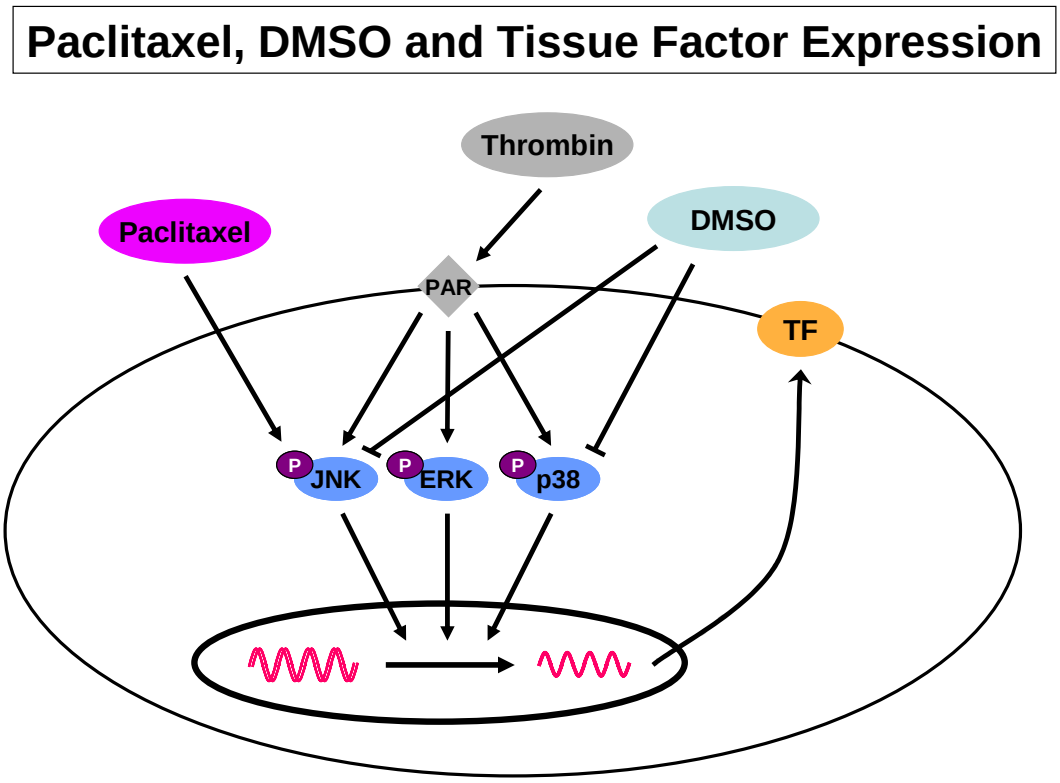


Figure 1: Schematic diagram representing MAP Kinases mediation of TF expression. Paclitaxel enhances TF expression by increasing JNK activation while DMSO prevents TF expression by inhibiting JNK and p38 activation.

As the use of DMSO is established in different areas of modern medicine, we propose this drug as a novel strategy for treating acute coronary syndromes; in particular, DMSO seems to represent an attractive compound for application on drug-eluting stents, either alone or in combination with rapamycin or paclitaxel.

References

Annex, B. H., S. M. Denning, et al. (1995). "Differential expression of tissue factor protein in directional atherectomy specimens from patients with stable and unstable coronary syndromes." Circulation **91**(3): 619-22.

Babapulle, M. N., L. Joseph, et al. (2004). "A hierarchical Bayesian meta-analysis of randomised clinical trials of drug-eluting stents." Lancet **364**(9434): 583-91.

Bardutzky, J., X. Meng, et al. (2005). "Effects of intravenous dimethyl sulfoxide on ischemia evolution in a rat permanent occlusion model." J Cereb Blood Flow Metab **25**(8): 968-77.

Blagosklonny, M. V., Z. Darzynkiewicz, et al. (2004). "Paclitaxel induces primary and postmitotic G1 arrest in human arterial smooth muscle cells." Cell Cycle **3**(8): 1050-6.

Bogdanov, V. Y., V. Balasubramanian, et al. (2003). "Alternatively spliced human tissue factor: a circulating, soluble, thrombogenic protein." Nat Med **9**(4): 458-62.

Bourne, W. M., D. R. Shearer, et al. (1994). "Human corneal endothelial tolerance to glycerol, dimethylsulfoxide, 1,2-propanediol, and 2,3-butanediol." Cryobiology **31**(1): 1-9.

Burgess, J. L., A. P. Hamner, et al. (1998). "Sulfhemoglobinemia after dermal application of DMSO." Vet Hum Toxicol **40**(2): 87-9.

Colombo, A., J. Drzewiecki, et al. (2003). "Randomized study to assess the effectiveness of slow- and moderate-release polymer-based paclitaxel-eluting stents for coronary artery lesions." Circulation **108**(7): 788-94.

Coomber, B. L. and A. I. Gotlieb (1990). "In vitro endothelial wound repair. Interaction of cell migration and proliferation." Arteriosclerosis **10**(2): 215-22.

Creel, C. J., M. A. Lovich, et al. (2000). "Arterial paclitaxel distribution and deposition." Circ Res **86**(8): 879-84.

Crown, J. and M. O'Leary (2000). "The taxanes: an update." Lancet **355**(9210): 1176-8.

Day, S. M., J. L. Reeve, et al. (2004). "Murine thrombosis models." Thromb Haemost **92**(3): 486-94.

Day, S. M., J. L. Reeve, et al. (2005). "Macrovascular thrombosis is driven by tissue factor derived primarily from the blood vessel wall." Blood **105**(1): 192-8.

de la Fuente, L. M., J. Miano, et al. (2001). "Initial results of the Quanam drug eluting stent (QuaDS-QP-2) Registry (BARDDS) in human subjects." Catheter Cardiovasc Interv **53**(4): 480-8.

Dujovny, M., R. Rozario, et al. (1983). "Antiplatelet effect of dimethyl sulfoxide, barbiturates, and methyl prednisolone." Ann N Y Acad Sci **411**: 234-44.

Egorin, M. J., D. M. Rosen, et al. (1998). "Plasma concentrations and pharmacokinetics of dimethylsulfoxide and its metabolites in patients undergoing peripheral-blood stem-cell transplants." J Clin Oncol **16**(2): 610-5.

Eisenberg, M. J. (2004). "Drug-eluting stents: some bare facts." Lancet **364**(9444): 1466-7.

Eitzman, D. T., P. F. Bodary, et al. (2003). "Fabry disease in mice is associated with age-dependent susceptibility to vascular thrombosis." J Am Soc Nephrol **14**(2): 298-302.

Eitzman, D. T., R. J. Westrick, et al. (2000). "Plasminogen activator inhibitor-1 deficiency protects against atherosclerosis progression in the mouse carotid artery." Blood **96**(13): 4212-5.

Eto, M., T. Kozai, et al. (2002). "Statin prevents tissue factor expression in human endothelial cells: role of Rho/Rho-kinase and Akt pathways." Circulation **105**(15): 1756-9.

Farkas, E., A. Institoris, et al. (2004). "Diazoxide and dimethyl sulphoxide prevent cerebral hypoperfusion-related learning dysfunction and brain damage after carotid artery occlusion." Brain Res **1008**(2): 252-60.

Finn, A. V., F. D. Kolodgie, et al. (2005). "Differential response of delayed healing and persistent inflammation at sites of overlapping sirolimus- or paclitaxel-eluting stents." Circulation **112**(2): 270-8.

Furie, B. and B. C. Furie (2005). "Thrombus formation in vivo." J Clin Invest **115**(12): 3355-62.

Fuster, V., P. R. Moreno, et al. (2005). "Atherothrombosis and high-risk plaque: part I: evolving concepts." J Am Coll Cardiol **46**(6): 937-54.

Grewe, P. H., T. Deneke, et al. (2000). "Acute and chronic tissue response to coronary stent implantation: pathologic findings in human specimen." J Am Coll Cardiol **35**(1): 157-63.

Grines, C. L., D. A. Cox, et al. (1999). "Coronary angioplasty with or without stent implantation for acute myocardial infarction. Stent Primary Angioplasty in Myocardial Infarction Study Group." N Engl J Med **341**(26): 1949-56.

Grube, E., A. Lansky, et al. (2004). "High-dose 7-hexanoyltaxol-eluting stent with polymer sleeves for coronary revascularization: one-year results from the SCORE randomized trial." J Am Coll Cardiol **44**(7): 1368-72.

Halkin, A. and G. W. Stone (2004). "Polymer-based paclitaxel-eluting stents in percutaneous coronary intervention: a review of the TAXUS trials." J Interv Cardiol **17**(5): 271-82.

Heidari, Y., A. M. Shah, et al. (2004). "NOX-2S is a new member of the NOX family of NADPH oxidases." Gene **335**: 133-40.

Iakovou, I., T. Schmidt, et al. (2005). "Incidence, predictors, and outcome of thrombosis after successful implantation of drug-eluting stents." Jama **293**(17): 2126-30.

Iwasaki, T., T. Hamano, et al. (1994). "A case of pulmonary amyloidosis associated with multiple myeloma successfully treated with dimethyl sulfoxide." Acta Haematol **91**(2): 91-4.

Jo, S. K., X. Hu, et al. (2004). "Delayed DMSO administration protects the kidney from mercuric chloride-induced injury." J Am Soc Nephrol **15**(10): 2648-54.

Karaca, M., U. Y. Bilgin, et al. (1991). "Dimethly sulphoxide lowers ICP after closed head trauma." Eur J Clin Pharmacol **40**(1): 113-4.

Karaca, M., E. Kilic, et al. (2002). "Ischemic stroke in elderly patients treated with a free radical scavenger-glycolytic intermediate solution: a preliminary pilot trial." Neurol Res **24**(1): 73-80.

Kastrati, A., A. Dibra, et al. (2005). "Sirolimus-eluting stents vs paclitaxel-eluting stents in patients with coronary artery disease: meta-analysis of randomized trials." Jama **294**(7): 819-25.

Lee, L. F., G. Li, et al. (1998). "Paclitaxel (Taxol)-induced gene expression and cell death are both mediated by the activation of c-Jun NH2-terminal kinase (JNK/SAPK)." J Biol Chem **273**(43): 28253-60.

Levin, A. D., N. Vukmirovic, et al. (2004). "Specific binding to intracellular proteins determines arterial transport properties for rapamycin and paclitaxel." Proc Natl Acad Sci U S A **101**(25): 9463-7.

Lincoff, A. M., R. M. Califf, et al. (1999). "Complementary clinical benefits of coronary-artery stenting and blockade of platelet glycoprotein IIb/IIIa receptors. Evaluation of Platelet IIb/IIIa Inhibition in Stenting Investigators." N Engl J Med **341**(5): 319-27.

Lind, R. C. and A. J. Gandolfi (1997). "Late dimethyl sulfoxide administration provides a protective action against chemically induced injury in both the liver and the kidney." Toxicol Appl Pharmacol **142**(1): 201-7.

Liu, Y., K. Pelekanakis, et al. (2004). "Thrombin and tumor necrosis factor alpha synergistically stimulate tissue factor expression in human endothelial cells: regulation through c-Fos and c-Jun." J Biol Chem **279**(34): 36142-7.

Luscher, T. F., F. C. Tanner, et al. (1993). "Endothelial dysfunction in coronary artery disease." Annu Rev Med **44**: 395-418.

Mackman, N. (2004). "Role of tissue factor in hemostasis, thrombosis, and vascular development." Arterioscler Thromb Vasc Biol **24**(6): 1015-22.

Mackman, N., J. H. Morrissey, et al. (1989). "Complete sequence of the human tissue factor gene, a highly regulated cellular receptor that initiates the coagulation protease cascade." Biochemistry **28**(4): 1755-62.

Maier, W., L. A. Altwegg, et al. (2005). "Inflammatory markers at the site of ruptured plaque in acute myocardial infarction: locally increased interleukin-6 and serum amyloid A but decreased C-reactive protein." Circulation **111**(11): 1355-61.

Mann, K. G., C. van't Veer, et al. (1998). "The role of the tissue factor pathway in initiation of coagulation." Blood Coagul Fibrinolysis **9 Suppl 1**: S3-7.

Matsuno, H., T. Uematsu, et al. (1991). "Photochemically induced thrombosis model in rat femoral artery and evaluation of effects of heparin and tissue-type plasminogen activator with use of this model." J Pharmacol Methods **25**(4): 303-17.

McCammon, K. A., A. N. Lentzner, et al. (1998). "Intravesical dimethyl sulfoxide for primary amyloidosis of the bladder." Urology **52**(6): 1136-8.

McFadden, E. P., E. Stabile, et al. (2004). "Late thrombosis in drug-eluting coronary stents after discontinuation of antiplatelet therapy." Lancet **364**(9444): 1519-21.

Mechtcheriakova, D., G. Schabbauer, et al. (2001). "Specificity, diversity, and convergence in VEGF and TNF-alpha signaling events leading to tissue factor up-regulation via EGR-1 in endothelial cells." Faseb J **15**(1): 230-242.

Ming, X. F., C. Barandier, et al. (2004). "Thrombin stimulates human endothelial arginase enzymatic activity via RhoA/ROCK pathway: implications for atherosclerotic endothelial dysfunction." Circulation **110**(24): 3708-14.

Morassi, P., F. Massa, et al. (1989). "[Treatment of amyloidosis with dimethyl sulfoxide (DMSO)]." Minerva Med **80**(1): 65-70.

Morrissey, J. H., S. A. Gregory, et al. (1989). "Tissue factor regulation and gene organization." Oxf Surv Eukaryot Genes **6**: 67-84.

Nemerson, Y. (1988). "Tissue factor and hemostasis." Blood **71**(1): 1-8.

Okada, H., J. Woodcock-Mitchell, et al. (1998). "Induction of plasminogen activator inhibitor type 1 and type 1 collagen expression in rat cardiac microvascular endothelial cells by interleukin-1 and its dependence on oxygen-centered free radicals." Circulation **97**(21): 2175-82.

Ong, A. T., A. Hoye, et al. (2005). "Thirty-day incidence and six-month clinical outcome of thrombotic stent occlusion after bare-metal, sirolimus, or paclitaxel stent implantation." J Am Coll Cardiol **45**(6): 947-53.

Ong, A. T., E. P. McFadden, et al. (2005). "Late angiographic stent thrombosis (LAST) events with drug-eluting stents." J Am Coll Cardiol **45**(12): 2088-92.

Pedersen, B., T. Holscher, et al. (2005). "A balance between tissue factor and tissue factor pathway inhibitor is required for embryonic development and hemostasis in adult mice." Blood **105**(7): 2777-82.

Petrache, I., A. Birukova, et al. (2003). "The role of the microtubules in tumor necrosis factor-alpha-induced endothelial cell permeability." Am J Respir Cell Mol Biol **28**(5): 574-81.

Ranade, S. V., K. M. Miller, et al. (2004). "Physical characterization of controlled release of paclitaxel from the TAXUS Express2 drug-eluting stent." J Biomed Mater Res A **71**(4): 625-34.

Rowinsky, E. K. and R. C. Donehower (1995). "Paclitaxel (taxol)." N Engl J Med **332**(15): 1004-14.

Salim, A. S. (1992). "Role of oxygen-derived free radical scavengers in the management of recurrent attacks of ulcerative colitis: a new approach." J Lab Clin Med **119**(6): 710-7.

Salim, A. S. (1994). "Role of free radical scavengers in the management of refractory duodenal ulceration. A new approach." J Surg Res **56**(1): 45-52.

Schecter, A. D., P. L. Giesen, et al. (1997). "Tissue factor expression in human arterial smooth muscle cells. TF is present in three cellular pools after growth factor stimulation." J Clin Invest **100**(9): 2276-85.

Schwartz, R. S., N. A. Chronos, et al. (2004). "Preclinical restenosis models and drug-eluting stents: still important, still much to learn." J Am Coll Cardiol **44**(7): 1373-85.

Soejima, H., H. Ogawa, et al. (1999). "Heightened tissue factor associated with tissue factor pathway inhibitor and prognosis in patients with unstable angina." Circulation **99**(22): 2908-13.

Sollott, S. J., L. Cheng, et al. (1995). "Taxol inhibits neointimal smooth muscle cell accumulation after angioplasty in the rat." J Clin Invest **95**(4): 1869-76.

Stahli Barbara, C. G. e. a. (2006). "Paclitaxel Enhances Endothelial Tissue Factor Expression via c-Jun Terminal NH2 Kinase Activation." Circ. Res. Revisions submitted).

Steffel, J., A. Akhmedov, et al. (2005). "Histamine induces tissue factor expression: implications for acute coronary syndromes." Circulation **112**(3): 341-9.

Steffel, J., M. Hermann, et al. (2005). "Celecoxib decreases endothelial tissue factor expression through inhibition of c-Jun terminal NH2 kinase phosphorylation." Circulation **111**(13): 1685-9.

Steffel, J., R. A. Latini, et al. (2005). "Rapamycin, but not FK-506, increases endothelial tissue factor expression: implications for drug-eluting stent design." Circulation **112**(13): 2002-11.

Steffel, J., T. F. Luscher, et al. (2006). "Tissue factor in cardiovascular diseases: molecular mechanisms and clinical implications." Circulation **113**(5): 722-31.

Stone, G. W., S. G. Ellis, et al. (2004). "One-year clinical results with the slow-release, polymer-based, paclitaxel-eluting TAXUS stent: the TAXUS-IV trial." Circulation **109**(16): 1942-7.

Stone, G. W., S. G. Ellis, et al. (2004). "A polymer-based, paclitaxel-eluting stent in patients with coronary artery disease." N Engl J Med **350**(3): 221-31.

Tanner, F. C., T. Largiader, et al. (2004). "Nitric oxide synthase gene transfer inhibits biological features of bypass graft disease in the human saphenous vein." J Thorac Cardiovasc Surg **127**(1): 20-6.

Viswambharan, H., X. F. Ming, et al. (2004). "Reconstituted high-density lipoprotein inhibits thrombin-induced endothelial tissue factor expression through inhibition of RhoA and stimulation of phosphatidylinositol 3-kinase but not Akt/endothelial nitric oxide synthase." Circ Res **94**(7): 918-25.

Vogt, F., A. Stein, et al. (2004). "Long-term assessment of a novel biodegradable paclitaxel-eluting coronary polylactide stent." Eur Heart J **25**(15): 1330-40.

Wang, T. H., H. S. Wang, et al. (1998). "Microtubule-interfering agents activate c-Jun N-terminal kinase/stress-activated protein kinase through both Ras and apoptosis signal-regulating kinase pathways." J Biol Chem **273**(9): 4928-36.

Windecker, S., A. Remondino, et al. (2005). "Sirolimus-eluting and paclitaxel-eluting stents for coronary revascularization." N Engl J Med **353**(7): 653-62.

Wong, C. K. and C. S. Lin (1988). "Remarkable response of lipoid proteinosis to oral dimethyl sulphoxide." Br J Dermatol **119**(4): 541-4.

Yang, Z., U. Arnet, et al. (1994). "Thrombin-induced endothelium-dependent inhibition and direct activation of platelet-vessel wall interaction. Role of prostacyclin, nitric oxide, and thromboxane A2." Circulation **89**(5): 2266-72.

Yujiri, T., G. R. Fanger, et al. (1999). "MEK kinase 1 (MEKK1) transduces c-Jun NH2-terminal kinase activation in response to changes in the microtubule cytoskeleton." J Biol Chem **274**(18): 12605-10.

Zhu, S., H. Viswambharan, et al. (2005). "Sirolimus increases tissue factor expression but not activity in cultured human vascular smooth muscle cells." BMC Cardiovasc Disord **5**: 22.

CURRICULUM VITAE

Giovanni G. Camici

Wehntalerstrasse 8 8057 Zurich,
Switzerland

Private: +41 76 5867776
Work : +41 44 6355081
Fax : +41 44 6356827

E-mail giovannicamici@hotmail.com

Personal Information

Marital status: Single
Nationality: Italian
Place & Date of birth: Pisa, 7th July 1976

Work experience

PhD Student 10.2003/present

Cardiovascular Research Institute of Physiology,
Zurich University, Zurich, & Institute of Physiology
University of Friburg, Switzerland.
"Tissue Factor and cardiovascular disease "

Research assistant 06.2001/10.2003

Cardiovascular Research Institute of Physiology,
Zurich-Irchel University, Zurich, Switzerland.

Research assistant (RA-1B) 09.1999/04.2001

Neuromuscular Unit, Imperial College of Science, Technology,
and Medicine, Hammersmith Campus, London, UK.
"Mutations of Lamin a/c gene in Emery Dreifuss Muscular
Dystrophy (EDMD) and isolated cardiomyopathy."

University thesis project 06.1998/09.1998

Neuromuscular Unit, Imperial College of Science, Technology,
and Medicine, Hammersmith Campus, London, UK.
"Oligonucleotide transfection into C2C12 muscle cells: a

comparison of viral and non-viral transfection techniques".
(Grade: A)

Education

PhD admission examinations 09.2003
University of Friburg, Switzerland
Examined in:
Human Physiology (4.5/6)
Developmental Biology (5.5/6)
Biochemistry (3.5/6)

University degree 1995/1999
Queen Mary University of London, UK
BSc in Biology
2nd class Honours Degree.

High school 1990/1995
European school, Oxford, UK
European Baccalaureate

Skills

Skilled in a wide spectrum of laboratory techniques; qualified to carry out radioactive work and animal experimentations (Swiss licence LTK module 1E).

Good knowledge of PC's Windows, sequencing software (ABI), densitometry software Scion Image, statistical software Graphpad prism, e-mail and Internet. Typing skill 50 words/min.

Languages

Fluent in English and Italian. Intermediate level French.

Publications

Dimethyl Sulfoxide Inhibits Tissue Factor Expression, Thrombus Formation, and Vascular Smooth Muscle Cell Activation: A Novel Treatment Strategy for Drug-Eluting Stents.
Giovanni G Camici, Jan Steffel, Alexander Akhmedov, Nicola Schafer, Jeannette Baldinger, Urs Schulz, Kushiar Shojaati, Christian Matter, Zhihong Yang, Thomas F. Lüscher, and Felix C. Tanner
Circulation Aug 2006, in press

Paclitaxel Enhances Thrombin-Induced Endothelial Tissue Factor Expression via c-Jun Terminal NH_2 Kinase Activation.
Barbara E. Stähli*; Giovanni G Camici*; Jan Steffel; Alexander Akhmedov,; Kushiar Shoojati; Michelle Graber; Thomas F. Lüscher and Felix C. Tanner.
Circ Res. 2006 Jul 21;99(2):149-55. Epub 2006 Jun 22

Genetic Deletion of $p66^{Shc}$ Adaptor Protein Prevents Hyperglycemia-Induced Endothelial Dysfunction and Oxidative Stress.
Giovanni G Camici*, Marzia Schiavoni*, Markus Bachschmid, Pietro Francia, Ines Martin-Padura, Martin Hersberger, Felix C Tanner, Pier Giuseppe Pelicci, Massimo Volpe, Piero Anversa, Thomas F Lüscher, Francesco Cosentino
Manuscript under revision *PNAS* 2006

Protective Effects of Erythropoietin in Myocardial Infarction.
Camici G, Hermann M, Stallmach t, Gassmann M, Luscher TF and Ruschitzka F
Manuscript under revision *J Vas Res* 2006.

Selective COX-2 Inhibitors and Renal Injury in Salt sensitive Hypertension
Matthias Hermann, MD; Sidney Shaw, PhD; Giovanni Camici, BSc; Eva Kiss; MD Nico Bühler; Remy Chenevard, MD; Thomas F. Lüscher, MD; Hermann J. Gröne, MD; Frank Ruschitzka, MD
Hypertension. 2005 Feb;45(2):193-7. Epub 2005 Jan 3..

Differential effects of selective cyclooxygenase-2 inhibitors on endothelial function in salt-induced hypertension.
Hermann M, Camici G, Fratton A, Hurlimann D, Tanner FC, Hellermann JP, Fiedler M, Thiery J, Neidhart M, Gay RE, Gay S, Luscher TF, Ruschitzka
Circulation. 2003 Nov 11; 108(19): 2308-11. Epub 2003 Nov

Skeletal muscle pathology in Autosomal Dominant Emery Dreifuss Muscular Dystrophy with Lamin A/C mutations.
Sewry CA, Brown SC, Mercuri E, Bonne G, Feng L, Camici G, Morris GE, Muntoni F.
Neuropathol. Appl Neurobiol. 2001 Aug; 27 (4) 281-90.

International Meetings

G. Camici, M Schiavoni, , P. Francia, M. Volpe, T.F. Lüscher, F. Cosentino "Deletion of p66shc Gene Protects Against Oxidative Stress-Induced Endothelial Dysfunction in Type I Diabetes Mellitus"
Poster at the Swiss Society of Cardiology Congress Lausanne (CH), 2005

G. Camici M Schiavoni, , P. Francia, M. Volpe, T.F. Lüscher, F. Cosentino "Deletion of p66shc Gene Protects Against Oxidative Stress-Induced Endothelial Dysfunction in Type I Diabetes Mellitus"
Oral presentation at the American Heart Association
New Orleans, (USA) 2004

M Schiavoni G. Camici, , P. Francia, M. Volpe, T.F. Lüscher, F. Cosentino "Deletion of p66shc Gene Protects Against Oxidative Stress-Induced Endothelial Dysfunction in Type I Diabetes Mellitus"
Poster presentation at the European Society of Cardiology
Monaco (Germania), 2004

Matthias Hermann, MD; Giovanni Camici, BSc; Aisha Fratton; Felix C. Tanner, MD; Jens Hellermann, MD; Joachim Thiery, MD; Michel Neidhart, MD; Renate Gay, MD; Steffen Gay, MD; Thomas F. Lüscher, MD; Frank Ruschitzka, MD
"Differential Effects of Selective COX-2 Inhibitors on Endothelial Function in Salt-induced Hypertension"
Poster at the European Society of Cardiology
Monaco (Germania), 2004

Camici G, Hermann M, Ruschitzka
"Eyhtropoietin in myocardial infarction and acute coronary artery disease"
Oral presentation at the Swiss Society of Cardiology Congress
Basilea (CH), 2004.

Camici G, Hermann M, Ruschitzka
"Effect of Rosuvastatin on Mineralcorticoid Induced Hypertension."
Poster presentation at the Cardiology Update Meeting
Davos (CH), 2003

Laminopathies: a common cause of muscular dystrophy and isolated cardiomyopathy.
Poster at the Association of Physicians annual meeting, Chelsea and Westminster Hospital, Imperial College
Londra (UK), 2000.

Teaching activities

Supervisor, Biology degree thesis of Nicola Schaffer
03/2005 – 06/2005
University of Zurich, Switzerland

Supervisor, MD degree thesis of Janette Baldinger
04/2005 – 10/2005
University of Zurich, Switzerland

Supervisor, MD degree thesis of Urs Schulz
05/2005 – 02/2006
University of Zurich, Switzerland

Lecturer and experiments supervisor,
MSc course "Human Biology"
Semester 2005/2006
University of Zurich, Switzerland

Grants

CHIP (Center Integrative Physiology, Zurich)
10/2005, sum funded : CHF 20,810

"Wissenschaftliche" Foundation for Scientific Research,
University of Zurich
2006, sum funded : CHF 29,883

References

Prof Thomas F. Luscher:
University Hospital Zurich, Switzerland
Telephone no. +41-1-2552121,
e-mail: cardiotfl@gmx.ch

Prof Felix C. Tanner:
Cardiovascular Research Institute of Physiology,
Zurich University, Zurich, Switzerland
Telephone no.+41-1-6356469
e-mail: felix.tanner@access.unizh.ch

Prof. Francesco Muntoni:
Imperial College, London, UK
Telephone no.+44 208-3832125
e-mail: f.muntoni@ic.ac.uk

Dr Matthew Dunckley:
Imperial College, London, UK
e-mail: m.dunckley@ic.ac.uk

Appendix I

Manuscripts included:

Paclitaxel Enhances Thrombin-Induced Endothelial Tissue Factor Expression via c-Jun Terminal NH_2 Kinase Activation.

Barbara E. Stähli*; Giovanni G. Camici*; Jan Steffel; Alexander Akhmedov,; Kushiar Shoojati; Michelle Graber; Thomas F. Lüscher and Felix C. Tanner.

*= both authors contributed equally to this manuscript

Circ Res. 2006 Jul 21;99(2):149-55. Epub 2006 Jun 22

Dimethyl Sulfoxide Inhibits Tissue Factor Expression, Thrombus Formation, and Vascular Smooth Muscle Cell Activation: A Novel Treatment Strategy for Drug-Eluting Stents.

Giovanni G Camici, Jan Steffel, Alexander Akhmedov, Nicola Schafer, Jeannette Baldinger, Urs Schulz, Kushiar Shojaati, Christian Matter, Zhihong Yang, Thomas F. Lüscher, and Felix C. Tanner

Circulation Aug. 2006, in press.

Appendix II

Certification of originality

I, herewith declare that all data and concepts presented in this thesis result from no other sources other than my own work, unless stated otherwise.

Giovanni G Camici, Fribourg 2006.