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Signaling through NADPH oxidases: Role in vascular remodeling processes

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Erklärung

Diese Dissertation wurde im Sinne von § 13 Abs. 3 bzw. 4 der Promotionsordnung vom 29. Januar 1998 von Frau PD Dr. Agnes Görlach betreut und von Frau Prof. Dr. Angelika M. Vollmar vor der Fakultät für Chemie und Pharmazie vertreten.

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Johann Wolfgang von Goethe

.....

Natur und Kunst Sie scheinen sich zu fliehen,
Und haben sich, eh' man es denkt, gefunden;
Der Widerwille ist auch mir verschwunden,
Und beide scheinen gleich mich anzuziehen.

Es gilt wohl nur ein redliches Bemühen!
Und wenn wir erst in abgemess'nen Stunden
Mit Geist und Fleiss uns an die Kunst gebunden,
Mag frei Natur im Herzen wieder glühen.

So ist's mit aller Bildung auch beschaffen:
Vergebens werden ungebundne Geister
Nach der Vollendung reiner Höhe streben.

Wer Grosses will muss sich zusammenraffen;
In der Beschränkung zeigt sich erst der Meister,
Und das Gesetz nur kann uns Freiheit geben.

.....

Nur allein der Mensch
Vermag das Unmögliche:
Er unterscheidet,
Wählet und richtet;
Er kann den Augenblick
Dauer verleihen.

With all my love dedicated to
my mother Ljiljana and my grandmother Živana

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1. Introduction

1.1 Vascular remodeling

The integrity of the vessel wall is a prerequisite for vascular function. The vessel wall normally consists of three well-defined concentric layers that surround the lumen[1].

The intima is the innermost monolayer of endothelial cells (EC) that lines the inside of the vessel wall being in direct contact with the flowing blood. The endothelium primarily functions as a selective barrier between blood and underlying vascular tissues and as the regulator of blood vessel diameter. It releases different growth factors and vasoactive mediators, which regulate the physical and biochemical properties of the vessels and affect vascular contractility and cell growth. Endothelial cells are in contact with the underlying middle layer (or media) composed of vascular smooth muscle cells (VSMC).

The medial layer as the 'muscle' of the blood vessel maintains the vascular tone and regulates contraction, blood vessel tone and diameter, blood pressure, and blood flow distribution. VSMC within blood vessels proliferate at an extremely low rate and exhibit very low synthetic activity.

The outer-most layer, also termed adventitia, mainly contains fibroblasts which form a fibrous connective tissue. This tissue is very strong, and helps to prevent the rupture or bursting of larger arteries that carry blood under high pressure.

In addition to hemodynamic factors, such as flow and pressure, the vascular wall is constantly exposed to a variety of humoral factors, including coagulation factors, vasoactive peptides, cytokines, growth factors and certain hormones. Changes in the homeostatic balance between these factors can induce adaptive alterations of the vessel structure, architecture and function. These processes are known as vascular remodeling.

The main features of remodeling are hypertrophy (cell growth) and/or hyperplasia (proliferation) of the predominant cell type within each of these layers, as well as increased deposition of extracellular matrix components and fibrin. During vascular remodeling

"activated" EC may express adhesion molecules recruiting leucocytes which promote inflammatory responses and aggregation of platelets. In addition, increased permeability of EC and endothelial injury expose the underlying medial layer to blood-borne factors. Altered production of various vasoactive factors affects the contractile responses, gene expression and growth of the vascular smooth muscle cells and facilitate their proliferation as well as migration from the media to the subendothelial layer[2]. Moreover, VSMC change their phenotype from quiescent, contractile cells into a synthetic form and start to excessively secrete growth factors and the components of the extracellular matrix. These changes are summarized in the Fig. 1.

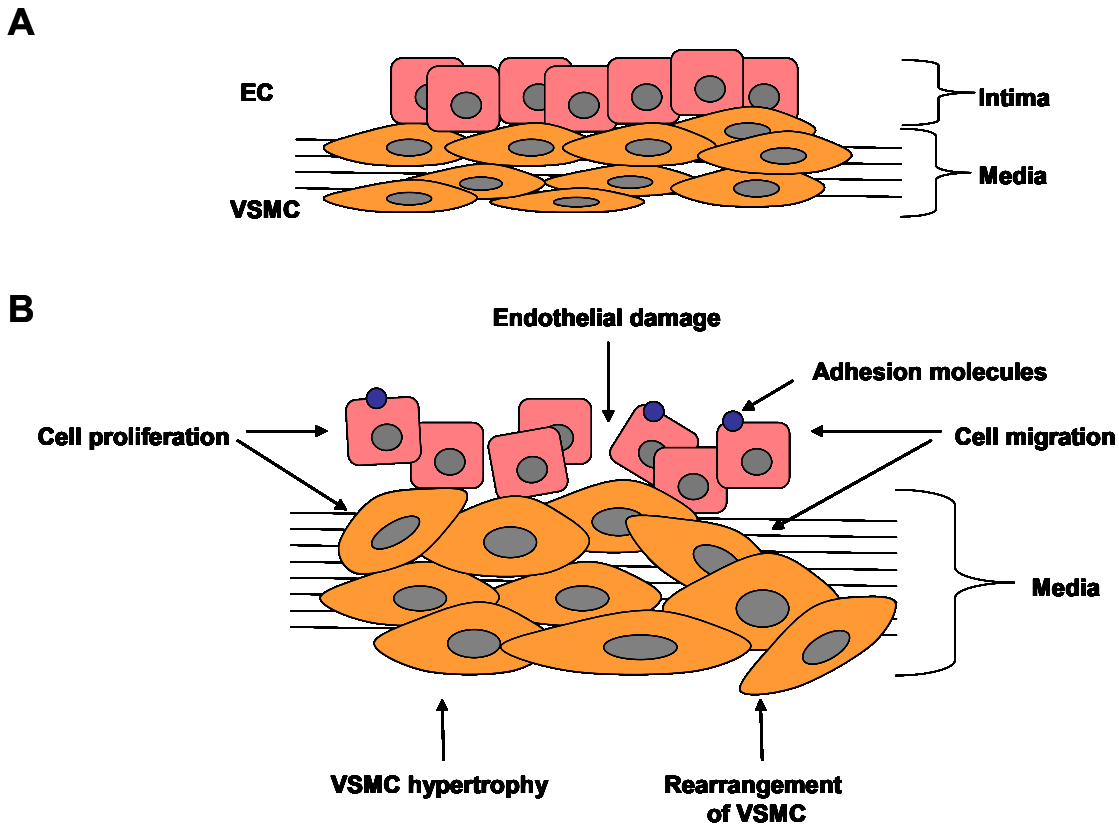


Figure 1. Cellular changes during vascular remodeling

A) Endothelial (EC) and vascular smooth muscle cells (VSMC) in the normal, non-diseased vessel. B) Cellular changes resulting in vascular remodeling.

Various cardiovascular diseases such as atherosclerosis, diabetes mellitus and hypertension are associated with vascular remodeling. Vascular remodeling is also a main pathogenic determinant of pulmonary hypertension (PH). This disease is characterized by elevations in pulmonary artery pressure and pulmonary vascular resistance which are primarily due to extensive vascular remodeling leading to thickening and muscularization of the vessel wall with subsequent stiffening and vasoconstriction of the vessels[3, 4]. In addition, disordered endothelial cell proliferation and migration, along with neoangiogenesis as well as proliferation of VSMC and their transformation into myofibroblasts can lead to the formation of so-called plexiform lesions resulting in luminal narrowing[5, 6]. Plexiform lesions are present in about 30% of lung-biopsy specimens, and are mostly located near the origin of a small pulmonary artery[7, 8].

1.1.1 Humoral factors in the pathogenesis of vascular remodeling

Altered production of different humoral factors influences cellular physiology by affecting growth, proliferation and migration of EC and VSMC leading to structural remodeling of the vasculature. Among them, vasoactive peptides such as endothelin (ET-1), angiotensin II (AngII), atrial natriuretic peptide (ANP) and urotensin-II (U-II) as well as activation of the coagulation cascade play a major role in mediating functional and structural changes in the vasculature[9-14].

1.1.1.1 Vasoactive peptides in vascular remodeling

In addition to AngII which has been shown to affect vascular tone and remodeling processes in the systemic and pulmonary vasculature[11, 13], ET-1 has been suggested to play a prominent role in pulmonary vascular remodeling[11, 14].

ET-1 is composed of 21 amino acids and is secreted from endothelial cells[15]. It binds to two distinct G protein-coupled receptors (GPR), endothelin receptor A (ET_A) and endothelin receptor B (ET_B)[16,17, 18]. When activated by binding of endothelin-1, the ET_A receptors located

in VSMC mediate a potent vasoconstrictive response[17], while ET_B receptors on EC mediate vasodilation via increased production of nitric oxide and prostacyclin[19-21]. However, ET-1 acts not only as a vasoactive peptide, but it is also a potent growth-promoting agent leading to proliferation of VSMC and EC, therefore being associated with vascular remodeling[14, 22, 23].

In recent years, the structurally related peptide urotensin-II has been found to also act as a vasoconstrictor and it was declared as the "new endothelin", as initial studies suggested similarities between the two peptides in structure, receptor molecule and contractile actions within the cardiovascular system[24, 25]. Urotensin-II is a phylogenetically ancient peptide that was initially isolated from *Gillichthys mirabilis,* the fish also known as goby, where it is involved in the control of vascular tone and osmoregulation[26,27]. This peptide is highly conserved across the species, from goby till humans. It is composed of 11 amino acid residues with a cyclic hexapeptide region important for binding to its receptor molecule (Fig. 2)[25].

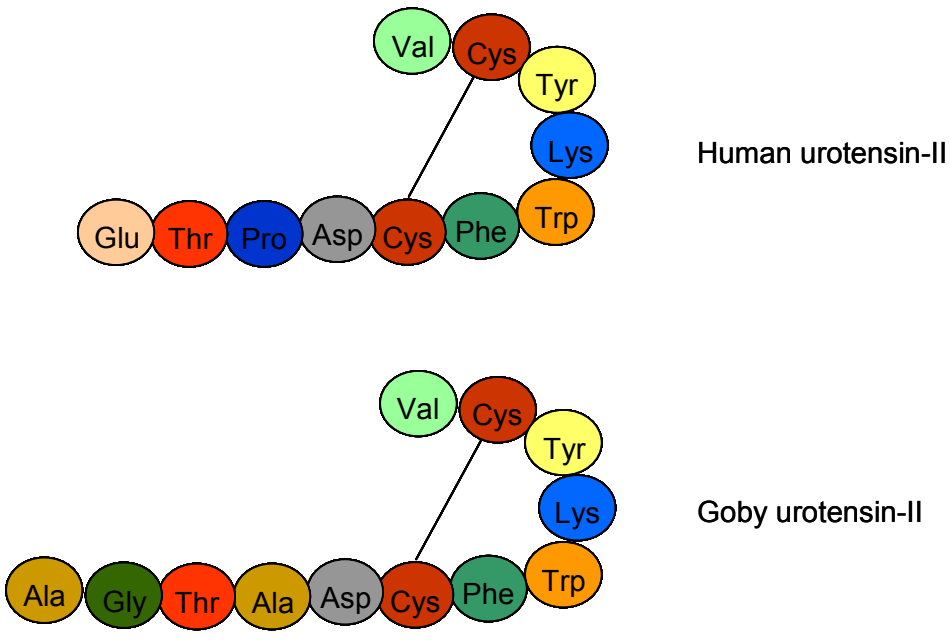


Figure 2. The structure of the human and goby urotensin-II

Explanations are given in the text.

Human U-II (hU-II) has been identified as endogenous ligand for the G-protein coupled receptor GPR14[28], recently renamed as urotensin-II receptor. Both, hU-II and its receptor are expressed in different cardiovascular tissues including smooth muscle and endothelium[29, 30]. Several studies compared the contractile potency of hU-II relative to ET-1. hU-II was shown to be more potent than ET-1, with the strongest effect within the pulmonary vasculature (Table 1)[28]. In the pulmonary artery, the potency of hU-II was 28 times higher than the one of ET-1 (Table 1).

Human U-II has been suggested to contribute to several cardiovascular diseases associated with vascular remodeling, such as pulmonary hypertension. Elevated hU-II levels have been found in rats with pulmonary hypertension and have been associated with pulmonary artery hypertrophy[31]. In addition, pulmonary arteries from animals with pulmonary hypertension showed significantly increased maximal contractive responses to hU-II compared with controls[32]. However, the signaling pathways and functional consequences of hU-II action in the vasculature are not defined.

Vessel	Human urotensin-II -log (EC)	Endothelin-1 -log (EC)	Relative potency
Left anterior descending coronary artery	9.39+0.40	8.20+0.32	15
Mesenteric artery	9.35+0.26	8.24+0.28	13
Pulmonary artery	9.29+0.16	7.84+0.06	28
Renal artery	9.59+0.36	8.83+0.24	6

Table 1. Relative contractile potency of human hU-II compared to ET-1 in non-human primates
Table was modified from Ames et al[28]. Relative potency shows how many times hU-II is more potent than ET-1.

1.1.1.2 Prothrombotic state in vascular remodeling

Endothelial injury allows the leakage of various blood-born factors and their contact with the underlying smooth muscle cells resulting in increased coagulability and thrombogenicity within the vessel wall[33].

The primary link between vascular cells and the hemostatic system is provided by the cell surface protein TF[34-36]. Human TF is a 47-kDa transmembrane glycoprotein. It consists of 263 amino acids, 219 of which form the large extracellular domain, 23 amino acids are integrated membraneously and the cytoplasmic tail is only 21 amino acid residues in length[35, 37]. TF is differentially expressed in various vascular cell types[35, 37, 38]. In adult blood vessels, TF is abundant in adventitial fibroblasts whereas little or no TF is constitutively expressed in the intima or media[36]. Thus, cellular initiation of TF-dependent blood coagulation seems to require induction of TF expression on the surface of cells, which are normally or pathophysiologically in contact with plasmatic factors of hemostasis. The major role of TF is the initiation of blood coagulation by binding factor VII/VIIa with high affinity. This complex promotes the activation of factor X with subsequent thrombin generation (Fig. 3)[39-42].

Thrombin is produced predominantly on the surface of circulating platelets as a result of proteolytic activation of the 72-kDa zymogen prothrombin, which is constitutively synthesized in the liver and released into the circulation[40, 41, 43]. Thrombin exerts most of its actions on vascular target cells via an irreversible proteolytic activation of protease-activated receptors (PAR) that belong to the family of G-protein coupled receptors[44, 45]. Three of the four currently identified PAR family members (PAR-1, PAR-3, and PAR-4) have been shown to be cleaved and activated by thrombin[46].

Thrombin is a multifunctional serine protease that regulates the blood coagulation cascade by affecting a series of factors, cofactors and proteins inside the plasmatic hemostatic system leading to conversion of fibrinogen to fibrin[40, 41]. Fibrin deposition within the lumen of the vessel wall may serve as a provisional matrix for the invading VSMC, which proliferate within the arterial media and migrate towards the vascular lumen. Moreover, fibrin may directly stimulate proliferation of VSCM leading to vascular remodeling [47, 48].

Under normal conditions, fibrin deposition is counterbalanced by fibrinolysis and fibrin clearance. This process is mediated by urokinase or tissue type plasminogen activator (uPA or tPA) which catalyze the conversion of plasminogen to plasmin that degradates fibrin. Fibrinolytic processes are antagonized by the serine protease plasminogen activator inhibitor-1 (PAI-1)[48]. PAI-1 is a 47-kDa protein and is the primary inhibitor of uPA or tPA. Inhibition of uPA or tPA by PAI-1 is facilitated by vitronectin, a 75-kDa glycoprotein that binds the inhibitor with high affinity, stabilizes the inhibitor in its active conformation, and mediates the binding of the inhibitor to fibrin clots[49]. Therefore, by blocking uPA or tPA activity, PAI-1 is inhibiting fibrinolytic processes and increasing fibrin deposition (Fig. 2).

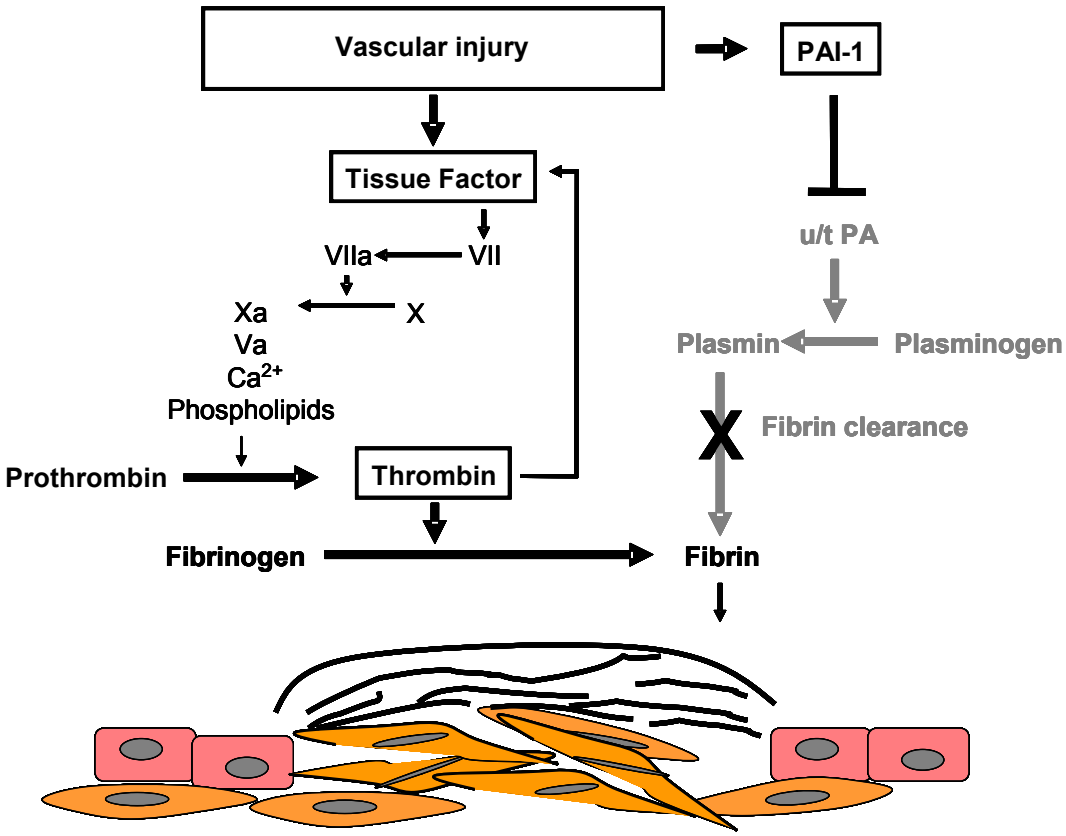


Figure 3. Vascular injury induces TF release and PAI-1 expression

Explanations are given in the text.

1.2 Vascular signaling cascades

1.2.1 Reactive oxygen species in the vasculature

Reactive oxygen species (ROS), which result from the incomplete reduction of oxygen and include superoxide anion radicals ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$) have been initially considered as toxic byproducts of the aerobic metabolism that lead to tissue damage[50-54] .

One electron reduction of oxygen results in the formation of $O_2^{-\cdot}$ which is membrane impermeable but can cross cell membranes via anion channels. Superoxide anion radicals are converted to H_2O_2 spontaneously or by the superoxide dismutase (SOD) (Fig. 4). Hydrogen peroxide is an easily diffusible molecule that has a longer half life than $O_2^{-\cdot}$. Hydrogen peroxide is enzymaticaly metabolized by catalase or glutathione-peroxidase (GPx). Additionally, in a Fenton reaction with transition metals like ferrous ions (Fe^{2+}), H_2O_2 produces highly reactive hydroxyl radicals ($OH^{-\cdot}$). On the other hand, $O_2^{-\cdot}$ can react with $NO^{\cdot}$ to form peroxynitrite ($ONOO^-$) (Fig. 4).

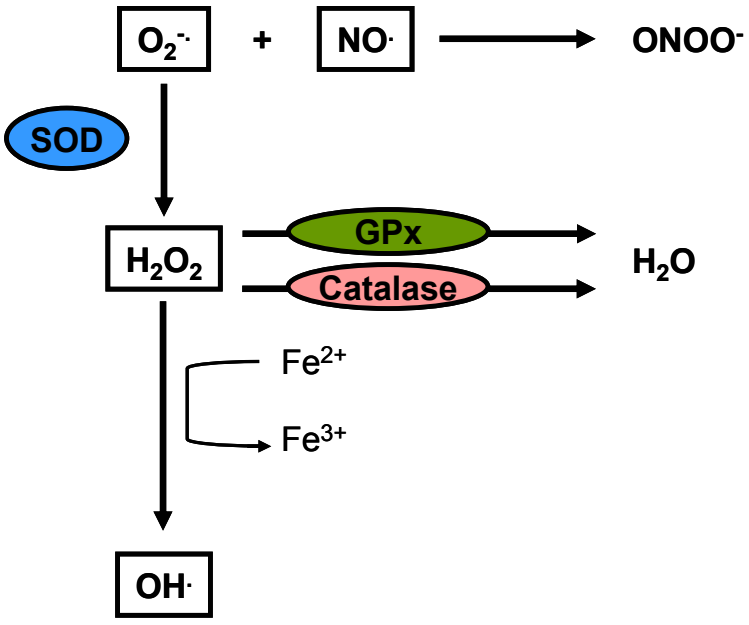


Figure 4. Reactive oxygen and reactive nitrogen species in vascular cells

The explanations are given in the text. SOD- superoxide dismutase; GPx- glutathione-peroxidase.

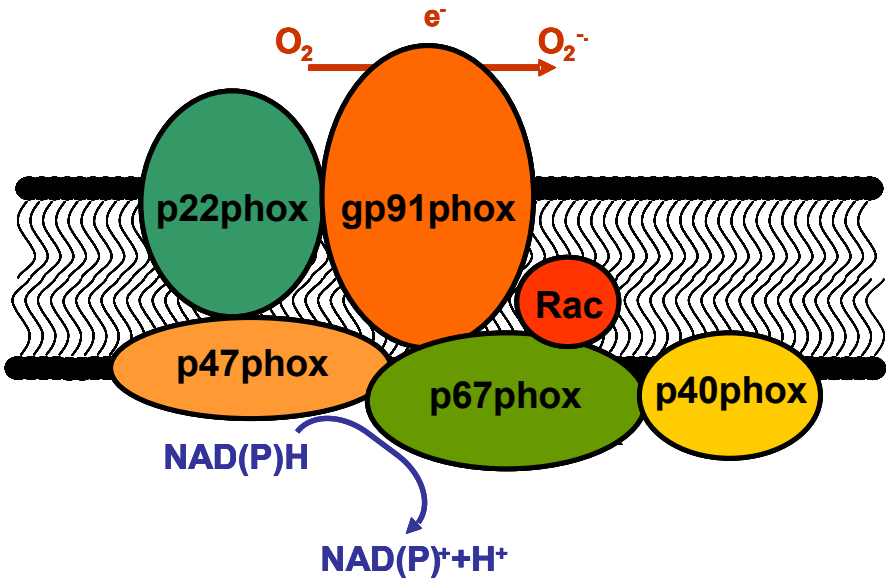
However, more recently, it has been appreciated that these molecules have many non-toxic functions, and evidence has been accumulated that they exert a prominent role as signaling molecules and second messengers in the vascular wall connecting receptor-mediated agonist stimulation and modulation of gene expression and proliferation of VSMC and EC[55-57]. Indeed, ROS may be classified as second messengers, since they are generated at the time of receptor activation; they are short lived species and can transiently alter the activity of certain effectors. On the other hand, the specificity of ROS may be determined by the site of their production. In the vasculature, ROS production has been reported in endothelial, smooth muscle and adventitial cells[56, 58, 59].

ROS regulate vascular function by modulating cell growth, proliferation, apoptosis, migration, inflammation, secretion, and production of different growth factors and proteins of the extracellular matrix[58, 60-62]. Under normal physiological conditions, the oxidant formation is balanced by the rate of oxidant elimination. Thus, any change in this balance, where pro-oxidants overwhelm cellular antioxidant capacity, results in oxidative stress. Oxidative stress and subsequent oxidative damage are mediators of vascular injury and associated with the development of many cardiovascular diseases[12, 57, 63]. Characteristically, increased ROS production is observed in disorders associated with a prothrombotic state[58, 64-70] and enhanced generation of ROS has been linked to a procoagulant state[71-74]. Moreover, increased ROS levels have been associated with deterioration of endothelial function[75, 76] which is characterized by decreased bioavailability of $NO^{\cdot}$. $NO^{\cdot}$ is synthesized by the enzyme endothelial nitric oxide synthase (eNOS), and released from EC in the direction of VSMC where it triggers vasodilatory responses. However, $NO^{\cdot}$ can be scavenged by $O_2^{-\cdot}$ resulting in vasoconstriction. Different growth factors, mechanical stress, vasoconstrictors and vasoactive peptides, including ET-1 and AngII, have been reported to increase the levels of ROS in VSMC[77, 78], further potentiating vasoconstrictive responses.

1.2.2 Vascular NADPH oxidases

Vascular ROS production has been associated with a number of systems including the mitochondrial electron transport chain, lipoxygenases, cyclooxygenase, xanthine and glucose oxidase, NADPH oxidases, nitric oxide synthases, the cytochrome P450s, peroxidases and various hemoproteins[79]. However, NADPH oxidases which catalyze the 1-electron reduction of oxygen using NADH or NADPH as electron donor resulting in the formation of superoxide anion radical (Fig. 5) have now been recognized as the most prominent sources of ROS generation in vascular cells[55, 56, 58, 80, 81].

The NADPH oxidase has been initially identified in phagocytes, where this multicomponent enzyme produces high amounts of microbicidal oxygen radicals (Fig. 5)[82].



$$NAD(P)H + 2O_2 \rightarrow NAD(P)^+ + H^+ + 2O_2^{-\cdot}$$

Figure 5. The structure of the activated neutrophil NADPH oxidase

The explanations are given in the text.

The leukocyte oxidase is composed of two membrane proteins, p22phox and gp91phox, that comprise the catalytical core of this enzyme, named flavocytochrome b558, as a result of its absorption maximum at 558 nm. Flavocytochrome b558 contains all redox components necessary for sequential transmembrane electron transport: two non-identical hemes and FAD- and NADPH-binding moieties. Regulatory components of the enzyme include the cytoplasmic subunits p40phox, p47phox, p67phox and the small GTP-binding protein Rac[52-54].

The enzyme is dormant in resting cells, and p47phox, p67phox and p40phox form a heterotrimeric complex in the cytoplasm. However, upon stimulation with pathogens, activation of the enzyme is initiated by phosphorylation of the cytosolic subunits that change their conformation and translocate to the cytochrome b558 at the membrane. In addition, loading of Rac with GTP triggers its translocation to the membrane, where it interacts with p67phox, further positioning flavocytochrome b558 in the right direction for electron transfer. Subsequent activation of the oxidase complex leads to electron flow through the membrane and release of large amounts of O_2^- in the well-characterized respiratory burst (Fig. 5).

The physiological significance of the phagocytic NADPH oxidase is illustrated by chronic granulomatous disease (CGD) caused by mutations in any of the genes encoding gp91phox, p22phox, p47phox or p67phox subunits of the NADPH oxidase[83]. Phagocytes of patients suffering from CGD are unable to produce $O_2^{-\cdot}$, and patients are therefore highly susceptible to bacterial and fungal infections.

Recently, NADPH oxidases have been found to be expressed in various non-phagocytic cells, including vascular cells[55, 58, 77, 81, 84]. In contrast to the neutrophil oxidase, basal ROS levels have been observed in EC and VSMC. Stimulation of vascular cells with different agonists including ET-1, AngII, tumor necrosis factor α (TNFα), platelet-derived growth factor (PDGF), insulin growth factor-1 (IGF-1), interleukin-1 alpha (IL1-α), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), low density lipoproteins (LDL) or their oxidized form (oxLDL), resulted in ROS production. However, ROS levels achieved in

vascular cells are substantially lower than in leukocytes, suggesting crucial differences between neutrophil and vascular NADPH oxidases[80, 82].

Recent studies demonstrated the presence of multiple NADPH oxidase forms in vascular cells. Based on homologies with each other and their apparent evolution from an ancestral NOX, these homologues are named NOX1, NOX2 (the former gp91phox), NOX3, NOX4 and NOX5 (Fig. 6)[80, 85-88]. NOX1, NOX3 and NOX4 share around 50-60% homology with NOX2, while NOX5 is distinct in its structure from NOX1-4, since it has an N-terminal extension with calcium binding moieties, known as EF hands[82]. NOX2 is mainly expressed in phagocytes and EC, whereas NOX1 and NOX4 are expressed in EC and VSMC[80, 81, 86, 89]. NOX3 protein has been identified in embryonic tissue, while NOX5 expression has been reported in tumor tissues[85]. In addition, two related proteins, called DUOX1 and DUOX2, mainly expressed in thyroid tissue, have NOX-homologous regions as well as regions with peroxidase activity (Fig. 6)[90, 91].

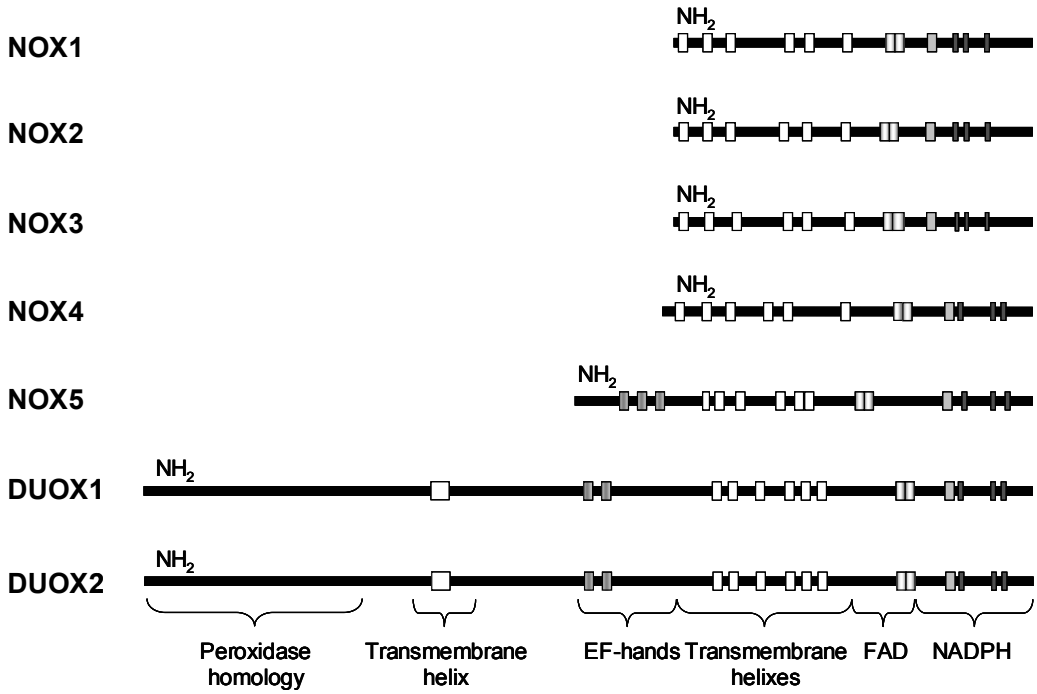


Figure 6. Homologues of the neutrophil gp91phox (NOX2) catalytic subunit

NOX1, NOX2, NOX3 and NOX4 contain 6 transmembrane helixes, as well as FAD- and NADPH-binding sites. In addition to these domains, NOX5 contains EF-hands which are calcium binding regions. DUOX1/2 homologues contain a peroxidase homology domain at the N-terminus.

In addition, two proteins with homology to the cytosolic components p47phox and p67phox, p41phox termed NOX organizer 1 (NOXO1) and p51phox named as NOX activator 1 (NOXA1), respectively, have been discovered and shown to regulate NOX1 and NOX3 activity[92-95]. These newly identified proteins might also modulate enzyme activity in either a tissue- and/or stimulus-specific fashion.

1.2.2.1 p22phox

p22phox is a 22-kDa membrane protein that contains 195 amino acids. p22phox together with gp91phox comprises the catalytical core of NADPH oxidase[96, 97]. Moreover, upon agonist stimulation, p22phox binds to p47phox to assemble the active oxidase[53].

In contrast to the NOX proteins, expression of p22phox is ubiquitous and has been shown in all vascular cell types underlining the essential function of this component for the assembly and activation of NADPH oxidases. The importance of p22phox is further supported by the identification of several polymorphisms in the p22phox gene. A polymorphism in the p22phox promoter revealed higher promoter activity and has been associated with hypertension[98]. Moreover, increased levels of p22phox and other NADPH oxidase subunits have been identified in the vascular wall after balloon angioplasty, as well as in atherosclerosis, hypertension, diabetes and other disorders and have been associated with elevated ROS levels in these disorders[66-68, 75, 76, 99].

1.2.2.2 Rac-1

Rac-1 is a small GTPase composed of 192 amino acids with a molecular weight of 20-kDa. There are two isoforms of Rac identified, Rac-1 and Rac-2. Rac-2 expression is restricted to hematopoetic cells and it is the predominantly active isoform in human neutrophils[100, 101], while Rac-1 is ubiquitously expressed and present in all vascular cell types.

Rac belongs to the family of Rho GTPases. Rho proteins cycle between an inactive GDP-bound, and active GTP-bound conformation. In the cytosol Rac is trapped in an inactive, GDP-bound state, by binding of guanine nucleotide exchange inhibitors (GDIs). Upon

stimulation, conversion of Rac in the active state is tightly regulated by guanine nucleotide exchange factors (GEFs) which allow the release of GDP and binding of GTP (Fig. 7). Subsequent Rac inactivation is regulated by GTPase activating proteins (GAPs) that increase the rate of GTP hydrolysis and downregulate GTPase signaling that acts as molecular switch and participates in a large variety of signaling events regulating processes such as cytoskeletal remodeling, mitogenesis, cell adhesion, cell-cycle progression and cell survival[102-104]. In addition, active Rac is necessary for the activation of the NADPH oxidase complex. Upon activation, Rac-1 translocates to the plasma membrane where it associates with p67phox to assemble with the oxidase complex. Furthermore, Rac-1 is geranyl-geranylated at the C-terminus which enables its interaction with the plasma membrane[82, 105].

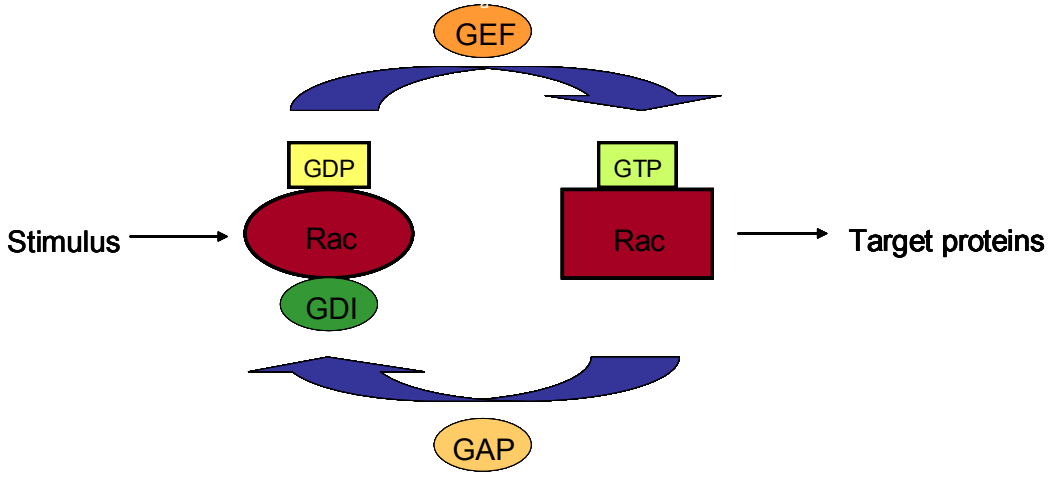


Figure 7. Rac activation

Rac-1/2 is trapped in an inactive, GDP-bound conformation, by binding of guanine nucleotide exchange inhibitors (GDIs). Activation of Rac-1/2 in response to stimuli is catalyzed by guanine nucleotide exchange factors (GEFs), and Rac-1/2 inactivation through hydrolysis of GTP is catalysed by GTPase activating proteins (GAPs).

1.2.3 Protein kinases and reactive oxygen species

Protein kinases are the common participants in signal transduction pathways that transfer the extracellular signals from activated membrane-associated receptors to downstream effectors by catalyzing the phosphorylation of their substrates.

Mitogen activated protein kinases (MAPKs) are serine/threonine kinases that are activated by a number of signaling pathways and are directly phosphorylated by another kinase, MAP-kinase-kinase (MAPKK). The MAPKK is activated by a MAP-kinase-kinase-kinase (MAPKKK), which is thought to be activated by the binding of activated Ras[106].

MAP kinases play a crucial role in vascular signaling, since they influence gene expression and proliferation of vascular cells. There are three families of MAPKs, p41/42 extracellular signal regulated kinases (ERK), c-jun N-terminal kinases (JNK) and p38 MAP kinase (p38MAPK). ERK is mainly associated with proliferation and differentiation, while JNK and p38MAPK regulate responses to cellular stresses. Moreover, enhanced activation of MAPKs has been observed in different cardiovascular diseases associated with vascular remodeling[62, 107].

Another group of protein kinases important for signal transduction is the serine/threonine protein kinase B (PKB, also called Akt) and its upstream kinase phosphatidyl-inositol-(3) kinase (PI3K). Subsequent to the activation of PI3K, Akt is recruited to the membrane where it is activated via the phosphorylation by phosphoinositide-dependent kinase 1 (PDK1). Akt is an important relay to control metabolic activity, growth and proliferation of vascular cells[108].

Another important class of protein kinases is p21-activated kinase (PAK). The PAK family consists of six isoforms, PAK1, PAK2 and PAK3 and the recently discovered PAK4, PAK5 and PAK6. PAK1-3 (so called PAK group A) are similar in the catalytic domain to PAK4-6 (PAK group B), but have significantly different structural organization[109]. PAK is a serine/threonine protein kinase which was one of the first Cdc42/Rac effectors identified. As an important mediator of Rac activity, PAK plays a significant role in modulating a range of

biological activities, including cytoskeleton reorganization, cell survival and cell proliferation[110].

It has been suggested that redox homeostasis plays an important role in the proper functioning of protein kinases. Several studies showed that ROS can activate protein kinases including MAPK and PI3K/Akt[107]. The mechanisms proposed for the ROS-induced activation of protein kinases include receptor clustering and protein tyrosine phosphatase inhibition[111]. The inactivation of tyrosine phosphatases is due to the reversible oxidation of cysteine residues that participate in dephosphorylation of protein kinases[112].

1.2.4 Redox-sensitive transcription factors

The proliferative activity of vascular cells and other remodeling processes are mediated by modulation of gene expression, thus requiring the activation of specific transcription factors.

1.2.4.1. NFκB transcription factor

Nuclear factor kappa B (NFκB) is known to mediate gene expression in response to many stimuli, including oxidative stress [111, 113]. It is a basic-leucine-zipper transcription factor and a member of the *Rel*-related transcription factor family. The predominant form of NFκB is a dimer composed of p50 and p65 subunits. In resting cells, the NFκB complex is trapped in the cytosol by binding to the inhibitory κB proteins (IκBα or IκBβ) (Fig. 8). Upon stimulation, phosphorylation of the IκB proteins by kinases (IκBKα or IκBKβ) marks them for destruction in the proteasome. The NFκB complex dissociates, translocates into the nucleus and binds DNA at specific motifs (Fig. 8).

NFκB is involved in the control of inflammatory responses, cellular growth or apoptosis as well as in the pathogenesis of cardiovascular diseases[111, 114, 115]. ROS have been initially implicated in stimulation of NFκB transcriptional activity, although the mechanisms of activation are controversial and appear to be cell-type specific[115, 116].

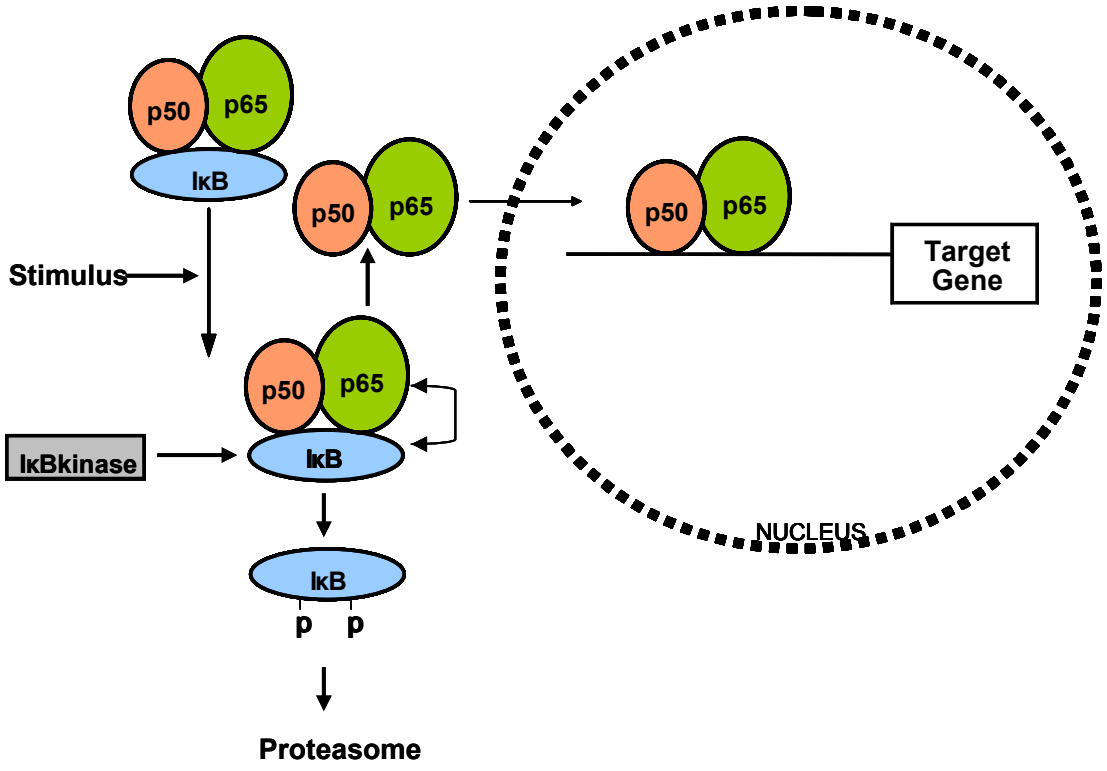


Figure 8. The regulation of NFκB transcriptional activity

The explanations are given in the text.

1.2.4.2. HIF-1 transcription factor

Another redox-sensitive transcription factor is the hypoxia-inducible factor (HIF), which mediates adaptive changes including proliferation in response to reduced O_2 bioavailability[117].

HIF is a heterodimer composed of two subunits, α and β, which are helix-loop-helix PAS proteins (Fig. 9)[111]. There are 3 different α-isoforms described: HIF-1α which appears to be ubiquitously expressed, HIF-2α which has been described to be predominantly expressed in vascular cells and HIF-3α whose role has not been clarified to date. While the HIF-1β subunit (also termed ARNT for arylhydrocarbon receptor nuclear translocator) is constitutively expressed, the level of the HIFα subunits is strongly regulated by the cellular O_2 concentration. In normoxia, HIF-1α is hydroxylated by prolyl hydroxylases (PHD), allowing the physical interaction with the tumor suppressor von Hippel-Lindau protein (pVHL). pVHL is

a recognition component for the E3 ubiquitin-protein ligase that ubiquitinylates HIF-1α and targets its degradation by the 26S proteasome[118-120]. In addition, hydroxylation of an asparagine in the C-terminal transactivation domain by hydroxylase termed factor inhibiting HIF-1 (FIH-1), prevents binding of the coactivator p300/CBP[121]. Under hypoxic conditions, hydroxylases are inactive, and non-hydroxylated HIF-1α escapes the proteasomal degradation, accumulates in the cells, and translocates to the nucleus. In the nucleus, HIF-1α subunit binds p300/CBP coactivators and dimerizes with its partner proteins from the ARNT family, which allows binding to specific response elements named hypoxia-responsive elements (HRE) in the promoters of target genes such as PAI-1 or VEGF (Fig. 9).

Very recently, it has been recognized that HIF-1α can be induced and activated also in response to certain stimuli in non-hypoxic conditions[113 122-125].

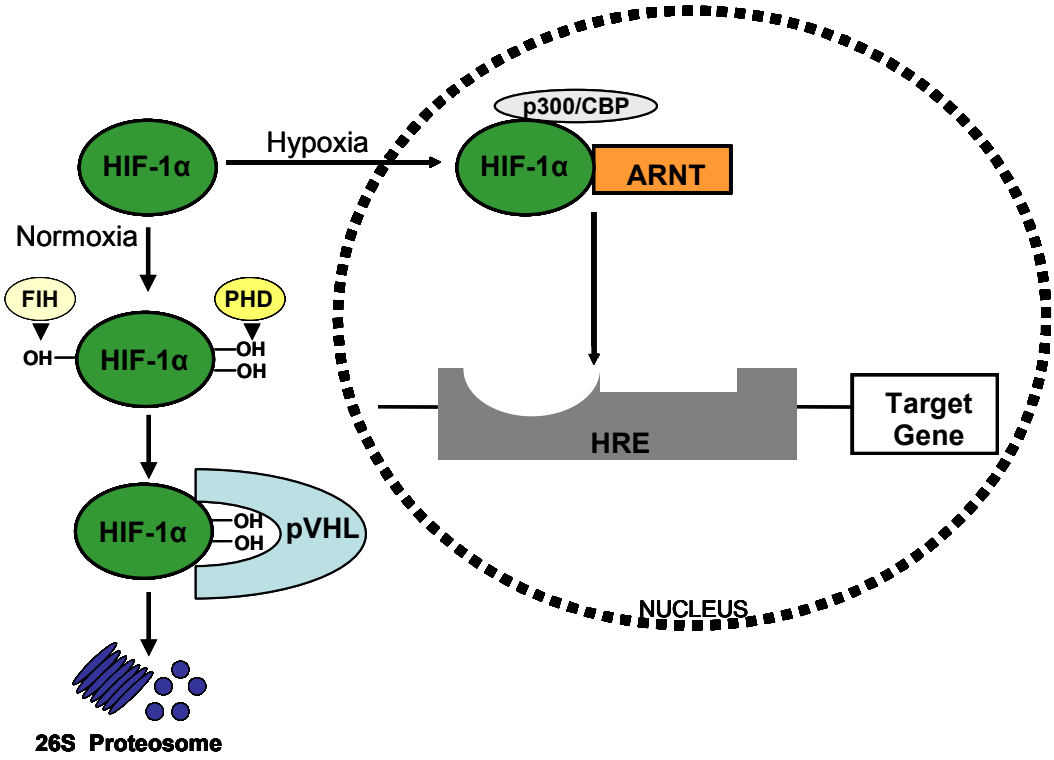


Figure 9. The regulation of HIF-1 transcriptional activity

The explanations are given in the text. PHD- prolyl hydroxylase; pVHL- von Hippel-Lindau protein; FIH-1-factor inhibiting HIF-1; p300/CBP-HIF-1 coactivators.

1.3 Aim of the study

Thrombotic events and proliferation play an important role in vascular remodeling in the systemic as well as in the pulmonary vasculature. Coagulation factors and vasoactive peptides have been shown to activate endothelial and smooth muscle cells.

ROS have been suggested to play an important role in vascular signaling, and NADPH oxidases have been identified as vascular sources of ROS.

It was therefore hypothesized that ROS derived from NADPH oxidases could initiate common signaling pathways in response to coagulation factors and vasoactive peptides, thereby activating proliferation and procoagulant activity.

The aim of the study was therefore to investigate:

A) how thrombin induces NADPH oxidase-derived ROS production and proliferation of vascular cells.

B) how NADPH oxidase-derived ROS and the GTPase Rac-1 influence the expression of the prothrombotic genes tissue factor and PAI-1 in pulmonary artery smooth muscle cells, the major cell type involved in pulmonary vascular remodeling.

C) whether the vasoactive peptide urotensin-II could activate NADPH oxidases and ROS-derived signaling cascades and proliferation of pulmonary artery smooth muscle cells.

2. Materials and methods

2.1 Materials

2.1.1 Chemicals

Chemicals	Company
Acetic acid	Roth
Actinomycin D	Sigma
Agar Agar	Roth
Agarose NEEO	Roth
Alamar Blue	Biosource
Ammoniumpersulfat	Roth
Ampicilin	Calbiochem
Antihuman PAI antiobody	Loxo
BAPTA-AM	Sigma
Bovine serum albumin	Sigma
Calcium chloride	Merck
Chloroform	Merck
Coumaric acid	Sigma
Cycloheximide	Sigma
Diaminofluorescein –2 Diacetate	Alexis
5-(and-6)-chloromethyl-2‘,7‘-dichlorodihydrofluorescein diacetate	MoBiTec
DEPC	Roth
Dihydroethidium	MoBiTec
Dihydrorhodamine 123	MoBiTec
Diphenyleneiodonium chloride	Alexis
Dimethylsulfoxide	Roth
Dithiothreitol	Roth
EDTA	Roth
EGTA	Roth
Ethanol	Merck
Ethidium bromide	Roth
Formaldehyd	Merck
Glucose	Merck
Glycerol	Roth
Glycin	Roth
Guanidinethiocyanate	Roth
Hank’s balanced salt solution	Gibco
Hydrochloric acid	JT Baker
HEPES buffer	Gibco
Hydrogen peroxide	Merck
Isoamilalcohol	Merck
Isopropanol	JT Baker

Kanamycin	Calbiochem
Luminol	Sigma
LY294002	Calbiochem
Magnesium chloride	Roth
Maleic Acid	Sigma
MOPS	Roth
N-Acetyl-Cysteine	Sigma
N-Lauryl-Sarcosine	Sigma
N-Nitro-L-Arginine	Calbiochem
Non-fet dry milk powder	Merck
PAK-1 PBD	Biomol
PBS Tablets	Gibco
PD98059	Calbiochem
Phorbol 12-Myristate 13 Acetate	Sigma
PIM	Calbiochem
PMSF	Calbiochem
Ponceau S	Sigma
Potassium chloride	Merck
Roti-Aqua-Phenol	Roth
Rotiphorese Gel	Roth
S-2238	Haemochrom Dignostik
SB 220025	Calbiochem
SDS ultra pure	Roth
SH-5	Alexis
Shingosine1 Phosphate	Alexis
Sodium acetate	Merck
Sodium chloride	Roth
Sodium citrate	Roth
Sodium dihydrogen phosphate	Sigma
Sodium fluorid	Merck
Sodium ortovanadat	Merck
SP600125	Biomol
Sulfuric acid	Roth
TEMED	Roth
Thrombin	Haemochrom Dignostik
Triton®X-100	Sigma
Tris	Roth
Tryptone	Roth
Tween 20	Sigma
Ovalbumin	Sigma
Urotensin-II	PolyPeptide
Vitamin C	Sigma
Wortmannin	Calbiochem
Xylencyanol	Roth
Yeast extract	Roth
β-Mercaptoethanol	Roth

Cell culture media and additives	Company
Dulbecco's Modified Eagl's Med.(glucose 1000mg)	Gibco
Fetal calf serum	Pan Biotech
HAT Supplement	Gibco
MCDB 131-Medium	Gibco
Penicillin /Streptomycin	Gibco
SmBm-2 medium	Cambrex
SmGm-2 Single Q	Cambrex
Trypsin EDTA	Gibco

Kits	Company
Dual-Luciferase Reporter Assay System	Promega
BrdU proliferation assay	Roche
QIAprep Spin Miniprep Kit	Quiagen
Quiagen Plasmid Maxi Kit	Quiagen
Rac activity pull down assay	Biomol
Quick Change mutagenesis kit	Stratagene
Nucleo Seq sequencing clean-up kit	Macherey-Nagel

Restriction Enzymes	Company
*Eco*RI	New England Biolabs
*Eco*RV	New England Biolabs
*Bsr*GI	New England Biolabs
*Ava*I	Roche
SmaI	New England Biolabs
Mung bean nuclease	New England Biolabs
Klenow fragment	New England Biolabs

Plastic ware	Company
T75 flasks	Greiner
10 and 6 cm dishes	Sarstedt
6-, 24- and 96-well plates	Greiner
2, 5, 10 and 25 ml pippets	Sarstedt
1.5 and 2 ml tubes	Sarstedt
15 ml and 50 ml tubes	Sarstedt
Cryovials	Greiner

2.1.2 Plasmids

2.1.2.1 Expression plasmids

In the experiments, the following plasmids were used:

Expression plasmids
pcDNA3.1
pcDNA3.1p22phoxS
pcDNA3.1p22phoxAS
psDNA6.1ECFP
pcDNA6.1hp22phoxECFP
pcDNA3.1NOX4S
pcDNA3.1NOX4AS
pcDNA3.1RacV12-myc
pcDNA3.1RacN17-myc
pRSV-NFκB-1
pRSV-RelA
pCMV-IκBαdn
p-FlagCMV2-IκK-2
PCMV-PAK-R295
PCMV-PAK-T423

To obtain pcDNA3.1p22phoxS and pcDNA3.1p22phoxAS (p22S and p2AS) the full length cDNA fragment encoding human p22phox derived from pBShp22phox (provided by Dr. M. Dinauer, Indianopolis, USA) was digested with *Eco*RI, blunt-ended with Mung bean nuclease and the fragment was ligated into pcDNA3.1 in sense and antisense direction resulting in p22S and p22AS. This fragment was also cloned into pcDNA6.1ECFP (provided by Dr. T. Kietzmann, Kaiserslautern, Germany), digested with *Bsr*GI and filled in with Klenow fragment and additional nucleotides encoding a polyalanine linker were inserted using the Quick Change mutagenesis kit resulting in pcDNA6.1hp22phoxECFP.

The human NOX4 full length cDNA was cut out from pCMV4-SPORT-NOX4 (RZPD, Accession number NM-016931) by *Ava*I, and blunted using Klenow fragment. It was then inserted into pcDNA3.1 linearised with *Eco*RV resulting in pcDNA3.1NOX4 sense and antisense constructs (NOX4S and NOX4AS).

Plasmids encoding myc-tagged constitutively active mutant of Rac1, pcDNA3.1RacV12-myc (RacG12V), and myc-tagged dominant-negative mutant of Rac1, pcDNA3.1RacN17-myc (RacT17N) were present in the laboratory and have already been described[71, 126].

The expression vectors p50 (pRSV-NFκB-1) and p65 (pRSV-RelA) encoding full length p50 and p65/rel, respectively, were provided by Dr. T. Kietzmann and have been described previously[127]. The expression vector pCMV-IκBαdn (IκBdn) was purchased from Becton Dickinson. The expression vector p-FlagCMV2-IκK-2 (IκKdn) encoding full length IκK-2 mutated at S177A/S181A was provided by Dr. T. Kietzmann and has been described previously[128].

PCMV-PAK-R295 (PAK-R295) and PCMV-PAK-T423E (PAK-T295) were kindly provided by Dr. T. Kietzmann.

All constructs were confirmed by DNA sequencing using T7 and BGH primers:

Sequencing primers	Sequence
T7	5'd[TAATACGACTCACTATAGGG]3
BGH	5'd[TAGAAGGCACAGTCGAGG]3'

To sequence the constructs Polymerase Chain Reaction (PCR) was performed using the following sequencing mix:

Sequencing mix:

DNA template (plasmid)	100 ng
BigDye Terminator	3 µl
Sequencing primers	100 µM
Water	up to 10 µl

The PCR products were cleaned from unincorporated dye using the Nucleo Seq sequencing clean-up kit and run on an ABI 3100 Genetic Analyzer.

2.1.2.2 Reporter constructs

To determine promoter activity, the promoter sequence of the gene of interest is coupled to the luciferase gene:

In the experiments, the following plasmids were used:

Promoter plasmids
pTF111
pTF636
pGI3 hPAI-1

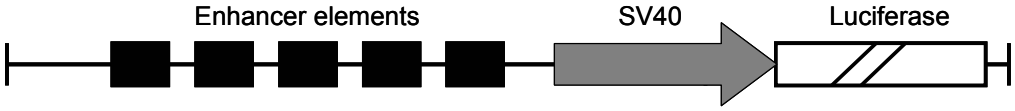
pTF111 contains the minimal human TF promoter (-111 to +121 bp) linked to the luciferase gene of the pgl2 basic vector. It was present in the lab and has been described previously[129].

pTF636 was obtained by PCR from the full length human TF promoter construct p19Luc (kindly provided by Dr. Nigel Mackman, La Jolla, USA). The fragment encompassing the sequence from –636 to -111 bp was inserted into the *Sma*I site from pTF111.

The pGI3 hPAI-1 promoter construct containing the human PAI-1 promoter from -796 to +13 was provided by Dr. T. Kietzmann.

2.1.2.3 Enhancer plasmids

To study the transactivation potential of transcription factors, copies of transcription factor binding sites were inserted as enhancer elements in front of a non-specific promoter and the luciferase gene:



In the experiments, the following plasmids were used:

Enhancer plasmids
pNFκB-Luc
pGL3-EPO-HRE

pNFκB-Luc containing 5 NFκB sites in front of the SV40 promoter linked to the luciferase gene was from Clontech.

pGL3-EPO-HRE vector containing 3 hypoxia responsive elements (HRE) in front of the SV40 promoter was present in the laboratory and has already been described[122].

2.1.2.4 Transformation and purification of plasmids

Bacterial Transformation: To transform and enrich the plasmids, the *E. coli* strains DH5α and XL-10 Gold were used:

Competent cells	Company
XL-10 Gold	Invitrogen
DH5α	Stratagen

An aliquot of 100 µl of competent cells was gently mixed with 1 µg of plasmid DNA, and incubated on ice for 30 min. Cells were subjected to 42°C for 30 seconds (sec),1 ml of SOC medium was added and the cell suspension was gently rocked for 1 hour (h) at 37°C. Thereafter, 100 and 50 µl of the bacterial suspension was spread on LB plates containing either 1% ampicilin or 1% kanamycin.

SOC medium	Final (%) in H_2O
Yeast extract	0.5%
Tryptone	2%
NaCl	0.05%

After dissolving in 950 ml of water, 10 ml of 250 mM KCl solution (final concentration 2.5 mM) were added and the pH was adjusted to 7.0. The solution was then autoclaved and thereafter 20 ml of sterile 1 M solution of glucose (final concentration 5 mM) and 5 ml of sterile 2 M $MgCl_2$ (final concentration 0.5 mM) were added.

LB medium	Final (%) in H_2O
Yeast Extract	0.5%
Tryptone	1%
NaCl	1%

The pH was adjusted to 7.2 and the solution was autoclaved.

LB plates	Final concentration in prepared LB medium
Agar	1.5%
Antibiotic (Ampicilin or Kanamycin)	1%

Minipreps and Maxipreps: Plasmids were purified by miniprep or maxiprep using QIAprep Spin Miniprep Kit and Qiagen Plasmid Maxi Kit.

Transfection of the plasmids: To deliver plasmid DNA to cells, the following transfection reagents were used:

Transfection reagents	**Company**
Effectene	Qiagen
FuGENE 6	Roche
SuperFect	Qiagen

2.1.3 Antibodies

Primary antibodies were prepared in 5% non-fat dry milk powder or 5% bovine serum albumin (BSA) diluted in Tris buffered salt solution containing 0.3% Tween 20 (TBS-T).

TBS-T	Final concentration
Tris	50 mM
NaCl	150 mM
“Smoky” HCl	0.3%
Tween	0.3%

The pH was adjusted to 7.5.

The monoclonal antibody against p22phox (mAb 44.9) was kindly provided by Dr. D. Roos, Amsterdam, and was used in a concentration of 1:250 in 5% milk in TBS-T.

The antibody against NOX4 was generated by immunizing rabbits with the peptides CAYLRGSQKVOSRRT and CSYGTKFEYNKESFS representing amino acids 81-95 and 566-578, respectively, of the rat NOX4 protein, and was kindly provided by Dr. Pfeilschifter (Frankfurt am Main). The NOX4 antibody was used in a concentration of 1:500 in 5% milk in TBS-T.

All other antibodies were commercially available.

Antibodies	Company	Concentation
Actin	Santa Cruz	1:1000 in 5% milk
c-myc	Santa Cruz	1:1000 in 5% milk
HIF-1 alpha	BD Transduction laboratories	1:1000 in 5% milk
PAI-1	American Diagnostica	1:200 in 5% milk
p-PAK(Thr)	Cell Signaling	1:500 in 5% BSA
p-p38MAPK	Cell Signaling	1:1000 in 5% BSA
p-JNK	Cell Signaling	1:1000 in 5% BSA
p-ERK	Cell Signaling	1:1000 in 5% BSA
p-Akt	Cell Signaling	1:1000 in 5% BSA
Rac-1	BD Transduction laboratories	1:1000 in 5% milk
TF	American Diagnostica	1:200 in 5% milk

Anti-mouse or anti–rabbit horseradish peroxidase-conjugated secondary antibodies were purchased from Calbiochem, and used in a concentration of 1:10000 in 5% milk or BSA.

2.1.4. Solutions

2.1.4.1 Solutions for Northern blot

DEPC Water

500 µl DEPC was added to 900 ml dH_2O and left overnight with slightly opened lit at room temperature. The solution was then autoclaved.

GT	Final concentration
Guanidine thiocyanate	4 M
Na-citrate	25 mM
N-Lauryl Sarcosine	0.3%

The solution was prepared in DEPC-H_20 and heated at 65°C.

The lysis buffer GT-DTT was prepared by adding 7 µl of 375 mM DTT to 1 ml of GT solution (final concentration 2.62 mM) just before use.

MOPS buffer (10X)	Final concentration
MOPS	0.2 M
Sodium acetate	80 mM
EDTA	10 mM

The pH was adjusted to 7.0.

500 µl DEPC was added to 900 ml 10X MOPS water and left overnight with slightly opened lit. The solution was then autoclaved.

SSC (20X)	Final concentration
NaCl	3 M
Sodium citrate	0.3 M

500 µl DEPC was added to 900 ml 20X SSC and left overnight with slightly opened lit. The solution was then autoclaved.

Premix for RNA probes

Formamid	10 ml
Formaldehyd 37%	3.5 ml
10 X MOPS	2.0 ml
Ethidium Bromide	0.03 µg

The solution was aliquoted and stored at - 20°C.

Blue marker for RNA probes (10 X)

Glycerin	1 ml
Bromphenolblue	1.6 mg
Xylenecyanol	1.6 mg
EDTA	1 mM
DEPC H_20	2 ml

The solution was stored at 4 °C.

Hybridisation solution (for 250 ml)	Final concentration
20 X SSC	25%
Formamid	50%
10 X Blocking reagent	5%
N-Lauryl Sarcosinat	1%
SDS	0.02%

Washing solution	Final concentration
Maleic acid	0.1 M
NaCl	0.15 M

The pH was adjusted to 7.5.

Blocking solution

10X Blocking solution (Roche) was diluted 1:9 in washing solution.

Detection solution	Final concentration
Tris	0.1 M
NaCl	5 M

The pH was adjusted to 9-9.5.

2.1.4.2 Solutions for Western blot

Running gel

Percentage	8%	10%	12%
H_20 (ml)	3.4	2.8	2.1
30% acrylamid (Rotiphorese gel) (ml)	2.7	3.3	4
1M trisHCl pH 8.8 (ml)	3.7	3.7	3.7
10% SDS (µl)	100	100	100

To initiate polymerization, 80 µl ammoniumpersulfate and 10 µl of TEMED were added.

Stacking gel

Percentage	5%
H_20 (ml)	2.14
30% acrylamid (ml)	0.488
1M trisHCl pH 6.8 (ml)	0.375
10% SDS	30

To initiate polymerization, 15 µl ammoniumpersulfate and 3 µl of TEMED were added.

Laemmli buffer (3X)	Final concentration
Tris (pH 6.8)	187 mM
SDS	6%
Glycerol	30%
Bromphenol blue	0.06%
DTT	15 mM
EDTA	60 mM

Running buffer	Final concentration
Tris	25 mM
Glycin	200 mM
SDS	0.5%

Transfer buffer	Final concentration
Tris	25 mM
Glycin	200 mM
Methanol	20%

Stripping buffer	Final concentration
SDS	2%
Tris pH 6.8	62.5 mM
β-Mercaptoethanol	0.07%

Enhanced chemiluminescent reagent 1

ECL 1	Final concentration
Tris pH 8.8	100 mM
Luminol	2.5 mM
Coumaric acid	0.4 mM

Enhanced chemiluminescent reagent 2

ECL 2	Final concentration
Tris pH 8.8	100 mM
H_2O_2	0.15%

Before use, ECL1 and ECL2 reagents were mixed in a 1:1 ratio.

2.2 Cell culture

2.2.1 Endothelial cells

The human endothelial cell line Ea.hy 926 (kindly provided by Dr. U. Foerstermann, Mainz, Germany) is a permanent cell line established by hybridization of human umbilical vein endothelial cells with the permanent human cell line A549[130]. This cell line is well characterized for its endothelial phenotype and biology, and expresses markers of highly differentiated vascular endothelium, including factor VIII-related antigen, which is maintained for more than 100 cumulative populations[130]. Cells were grown in DMEM medium with 1 g/l glucose, containing 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Since Ea.hy 926 cells need to be cultured in selective medium to maintain the endothelial phenotype, medium was supplemented with 1.5 X Hypoxanthine, Aminopterin, Thymidine (HAT) and cells were maintained at 37°C under the humidified atmosphere of 5% CO_2. Cells were cultured in T75 flasks and passaged twice a week by trypsinisation. First, cells were briefly rinsed and then incubated with 2 ml of 0.05% trypsin containing 0.53 mM EDTA•4Na for 1-2 minutes (min) at 37°C. The digestion was stopped by adding complete medium and cells were subcultured in the ratio 1:3 in T75 flasks or in 10 cm dishes and plates. Prior to stimulation cells were starved from serum in DMEM medium containing 0.1% serum albumin, 1.5x HAT, 100 U/ml penicillin and 100 µg/ml streptomycin for 24 h.

2.2.2 Smooth muscle cells

Pulmonary artery smooth muscle cells (PASMC) were purchased from Cambrex and grown in the provided SmBm medium. PASMC were maintained at 37°C under the humidified atmosphere of 5% CO_2. Cells were passaged twice per week by trypsinisation, as already described for endothelial cells. PASMC were used only up to passage 12 in order to maintain the phenotypic characteristics of PASMC *in vivo*, as confirmed by monitoring the expression

of smooth muscle α-actin in all experiments. Cells were deprived from serum for 16 to 24 h before stimulation.

Rat smooth muscle cells (rSMC) derived from embryonic rat aorta, (kindly provided by Dr. H.H.H. Schmidt, Giessen, Germany) were cultured in DMEM medium supplemented with 10% fetal calf serum, 1 g/l glucose, 100 U/ml penicillin and 100 µg/ml streptomycin. For subculturing, cells were trypsinised twice per week, and grown to confluency. Before experiments, cells were serum-starved for 16 h.

2.2.3 Other cell types

HepG2 cells (ATCC, Cat. No. HB-8065) were cultured in DMEM medium supplemented with 1 g/l glucose, 10% heat inactivated fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin and grown at 37°C under an atmosphere of 5% CO_2. Cells were passaged twice per week in a subcultivation ratio of 1:4.

2.2.4 Storage of the cells

To store the cells in liquid nitrogen, confluent cells were detached by trypsinisation and centrifuged at 1000 rpm for 5 min. Cells were then resuspended in precooled culture medium containing 10% DMSO and transferred to cryovials. To allow gradual freezing, vials were then placed in a cold isopropanol freezing box and kept for one day at -70°C. Thereafter, cells were frozen at -70°C and kept for at least 24 h to acclimate to extreme cold. Finally, cells were transferred to and stored in liquid nitrogen (-196°C).

2.3 Transfection experiments

2.3.1 Transfection of endothelial cells

Ea.hy 926 cells were transfected using the Effectene transfection reagent. The Effectene reagent is a non-liposomal lipid formation that spontaneously forms micelle structures that show no variations in size or batch. In the first step of transfection, DNA is highly condensed by interaction with DNA-condensation buffer (Buffer EC). Thereafter, Effectene reagent is added in order to coat condensed DNA molecules and produce Effectene-DNA complexes, thus allowing transfer of DNA into eukaryotic cells. When performing transfections, Ea.hy 926 cells were seeded one day before the experiments in 96-well plates or 10 cm dishes to achieve 50-70% confluency on the next day. Per one well of 96 well plates 35 ng of plasmid DNA was diluted in DNA-condensation buffer EC to a total volume of 5 µl, and 0.5 µl of Effectene reagent was added. For a 10 cm dish 3.5 µg of DNA, 500 µl of EC buffer and 50 µl of Effectene reagent were used. The mixture was incubated 10 min at room temperature to allow the formation of the transfection complex. Thereafter, the growth medium from the plate was gently aspirated, 100 µl of serum containing media per one well of a 96 well plate or 10 ml per 10 cm dish was added to the transfection complex, mixed by pipetting and immediately added to the cells. The transfection efficiency was about 60%.

2.3.2 Transfection of smooth muscle cells

To transfect PASMC, the FuGENE 6 transfection reagent was used. FuGENE 6 is a non-liposomal transfection reagent composed of a blend of lipids supplied in 80% ethanol. When transfecting PASMC, cells were cultured for 24 h to a density of 70% and transfections were performed using 6 µg of plasmid DNA and 36 µl FuGENE 6 reagent per 10 cm dish. FuGENE 6 was diluted in serum-free MCDB medium, mixed with plasmid DNA and the transfection complex was incubated for 15 min at room temperature. The growth medium of

the cells was changed and the transfection complex was then added to the cells. The day after, medium was changed and cells were serum-deprived for 16 to 24 h prior to stimulation. rSMC were transfected using the SuperFect reagent. SuperFect transfection reagent is an activated dendrimer with a defined spherical architecture, with branches radiating from a central core and terminating at positively charged amino groups. Net positive charge allows them to bind to negatively charged receptors on the surface of eukaryotic cells (e.g. sialylated glycoproteins). Once inside the cell, SuperFect reagent buffers the lysosome after it has fused with the endosome, leading to pH inhibition of lysosomal nucleases and stability of SuperFect–DNA complexes. For transfection experiments, cells were plated in 24-well plates (25000 cells/ well) one day before transfection. A ratio of a total of 1 µg of DNA to 5 µl Superfect per well was used in each transfection. After 2 h of incubation with the transfection complex, the medium was changed, and the cells were further cultured for 5 h. Cells were then washed and serum-starved for 16 h.

2.3.3 Transfection of other cell types

HepG2 cells were transfected using FuGENE 6 reagent as described for PASMC. HepG2 cells were plated onto 3.5 cm glass bottom dishes with a glass thickness of 0.17 mm (Willco Wells) the day before the transfection to achieve 40-60% of confluence on the next day. For transfection, 18 µl of FuGENE 6 reagent was mixed with 3 µg plasmid DNA. Transfection efficiency was about 70%.

2.4 Luciferase reporter gene assay

2.4.1 Principle of the assay

The Dual-Luciferase Reporter Assay System was used to determine reporter gene activity. Plasmids containing promoter or enhancer elements in front of the luciferase gene have been described (*2.1.2.2 and 2.1.2.3*). The expression of the luciferase is driven by promoter or enhancer sequences of genes of interest (Fig. 10).

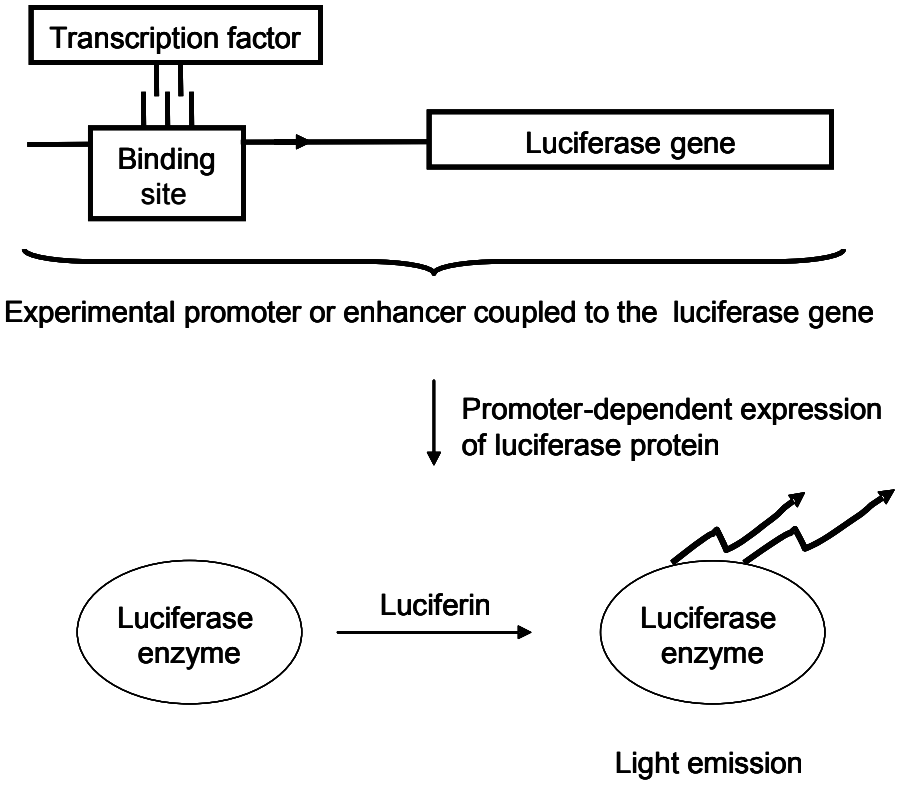


Figure 10. Scheme presenting the principle of the Luciferase reporter gene assay
Explanations are given in the text.

Firefly luciferase is a monomeric, 61kDa-enzyme that catalyzes luciferin oxidation using ATP Mg^{2+} as a cosubstrate. Light is produced by converting chemical energy of luciferin oxidation through an electron transition, forming oxyluciferin.

Transfection efficiency was controlled by cotransfection of 0.25 µg Renilla Luciferase expression vector (pRLSV40, Promega). Activity of the co-transfected reporter enzyme Renilla provides an internal control that serves as the baseline response. Thus, the activity of the experimental reporter after substracting background was normalized to the activity of the Renilla internal control or standardized to the protein content.

2.4.2 Luciferase assay

When performing luciferase assays, rSMC were used since these cells are transfectable with high efficiency. Cells were transfected with different reporter or enhancer constructs that are coupled to the luciferase gene as described (*2.3.2*). Transfected and serum-starved rSMC were treated as described in respective legends. At the end of the experiment, cells were washed 2 times with cold PBS and lysed with 150 µl of passive lysis buffer (PLB, Promega). PLB is specifically formulated to promote rapid lysis of cultured mammalian cells, to provide optimum performance and stability of the firefly and Renilla luciferase reporter enzymes, and to minimize autoluminescence. After shaking the cells for 10 min in PLB, 75 µl of total cell lysate was transferred to tubes. The chemiluminescence was measured in a Berthold Luminometer (Berthold Technologies) by automatically injecting 100 µl of the substrate luciferin. Finally, protein content of the total lysate was determined by performing Bradford assay.

2.5 Measurements of ROS production

2.5.1 DCF fluorescence in the plate reader

The generation of reactive oxygen species (ROS) was measured using the fluoroprobe 5-(and-6)-chloromethyl-2‘,7‘-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA (DCF)). Fluorescent dyes, like fluorescein, are chemically reduced to colorless, nonfluorescent products that are in the presence of ROS easily oxidized back to fluorescent compounds. Dichlorodihydrofluorescein diacetate passively diffuses into the cells where its diacetate groups are cleaved by cellular esterases, giving a more polar compound that stays trapped in the cells. The carboxylated analogue of H_2DCFDA passes even easier through the membranes and has better retention in the cells, since it has two negative charges at physiological pH. Moreover, thiol-reactive chloromethyl groups react with gluthatione and other thiols, further preventing the leakage of the dye from the cells (Fig. 11).

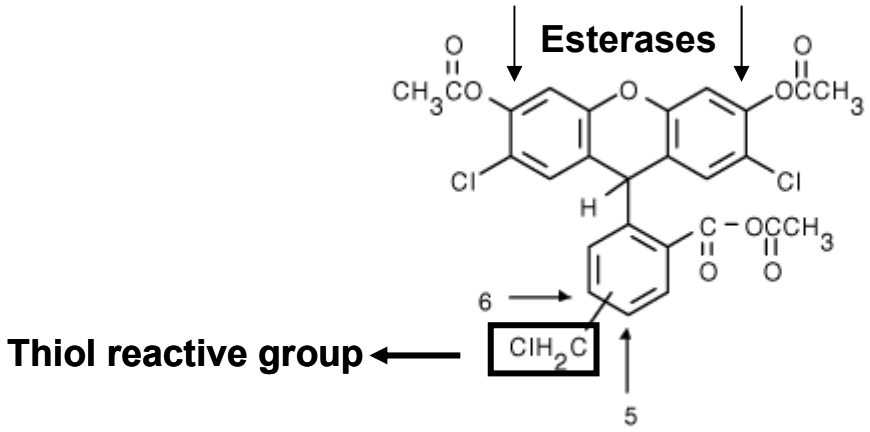


Figure 11. Structure of the 5-(and-6)- chloromethyl-2',7'- dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA)

Explanations are given in the text.

Subsequent oxidation of DCF gives a fluorescent product that stays trapped in the cells and indicates ROS production. DCF is sensitive to H_2O_2, but it also detects peroxynitrite ($ONOO^-$) that is formed in a reaction between $NO^\cdot$ and $O_2^{\cdot -}$. Endothelial cell express eNOS and produce high amounts of $NO^\cdot$. Therefore, when measuring ROS in endothelial cells, the contribution of $ONOO^-$-dependent DCF oxidation was excluded by preincubating the cells for 30 min with the eNOS inhibitor N-ω-nitro-L-arginine methyl ester (L-NAME,10 µM), and by adding L-NAME to HBSS.

To determine DCF fluorescence in a microplate reader (Tecan), EC or PASMC were grown in 96-well plates to 80% confluence, or transfected as described (2.3), and made quiescent in serum free medium for 24 h. Cells were then treated, washed with HBSS and incubated in the dark with DCF (8.5 µM) dissolved in HBSS for 10 min at 37°C. Cells were then washed with HBSS to remove excess dye, and fluorescence was monitored using 480 nm excitation and 540 nm emission wavelength. In some experiments, cells were preincubated with various inhibitors or antioxidants for 30 min and then exposed to stimuli.

To evaluate ROS levels, background fluorescence of unloaded cells was subtracted from DCF fluorescence. Moreover, DCF fluorescence was standardized to the number of viable cells using the alamarBlue test. Continued cellular growth maintains a reduced environment in the cells, and therefore causes the redox indicator from alamarBlue to change from the oxidized, nonfluorescent, blue form to the reduced, fluorescent, red form. Therefore, to determine the number of viable cells, cells were incubated with alamarBlue in phosphate-buffered saline (PBS), pH 7.4 at 37°C to allow the indicator to change from blue to the fully reduced red form. The absorbance was then measured at the wavelength of 580 nm.

2.5.2 Flow cytometric analyses using DCF fluorescence

Alternatively, ROS production was assessed by flow cytometric analysis of DCF stained cells. Flow cytometry is a method where a fluidic system carries the suspended cells from the sample tube to the laser intercept point allowing differentiation and counting of the cells. When the cells are loaded with the fluorescent dye, fluorescence analysis makes it possible to quantify fluorescence from single cells.

To confirm the validity of the method, detection of ROS production was tested by using freshly isolated leukocytes ($1x10^6$ cells/ml) loaded with 8.5 µM DCF and treated with phorbol 12-myristate 13 acetate (PMA, 100 nM) for 5 min. PMA is known to induce respiratory burst in phagocytes. After performing measurements, the fluorescence peak of PMA-stimulated leukocytes was significantly shifted to higher fluorescent signal than in unstimulated leukocytes (Fig. 12). The quantification was done using the arithmetic mean of the peak.

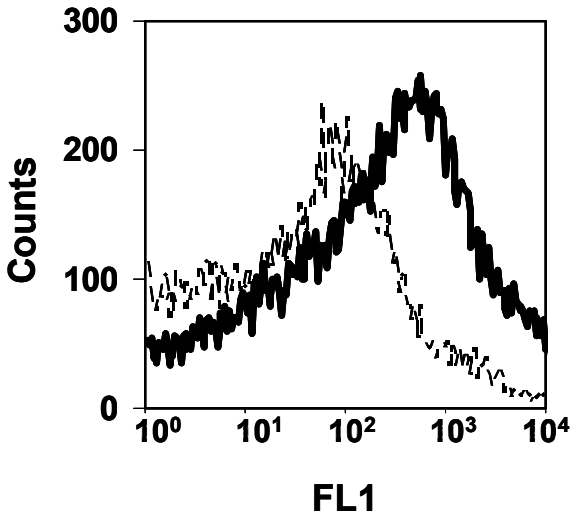


Figure 12. ROS generation from freshly isolated leukocytes

Leukocytes were loaded with 8.5 µM DCF and left untreated (-----), or stimulated with 100 nM PMA for 5 min (──). ROS levels were detected by performing flow cytometric analyses. Counts on the y-axis represent relative number of cells investigated and FL1 on the x-axis relative intensity of fluorescence.

EC or PASMC were grown to 80% confluence and made quiescent in serum free media for 24 h. Thereafter, cells were treated as described in respective figure legends. To evaluate ROS production, cells were detached and harvested by trypsinisation, collected by centrifugation at 1000 rpm for 5 min and resuspended in HBSS at a concentration of $1x10^6$ cells/ml.

When performing experiments with endothelial cells, L-NAME (10 µM) was added to the buffers. Cells were then loaded with 8.5 µM DCF for 15 min in the dark at 37°C before stimulation. The DCF fluorescence was monitored by analyzing 10 000 cells using 480 nm excitation and 540 nm emission wavelength in a flow cytometer (Partec).

2.5.3 Dihydroethidium staining

Generation of ROS was visualized by the fluoroprobe dihydroethidium (DHE), which is also called hydroethidine. DHE passively diffuses into the cells exhibiting blue fluorescence. After oxidation, predominantly with $O_2^{\cdot -}$, DHE is oxidized to ethidium, giving a more polar compound that intercalates within DNA and stains the nucleus in fluorescent red (Fig. 13).

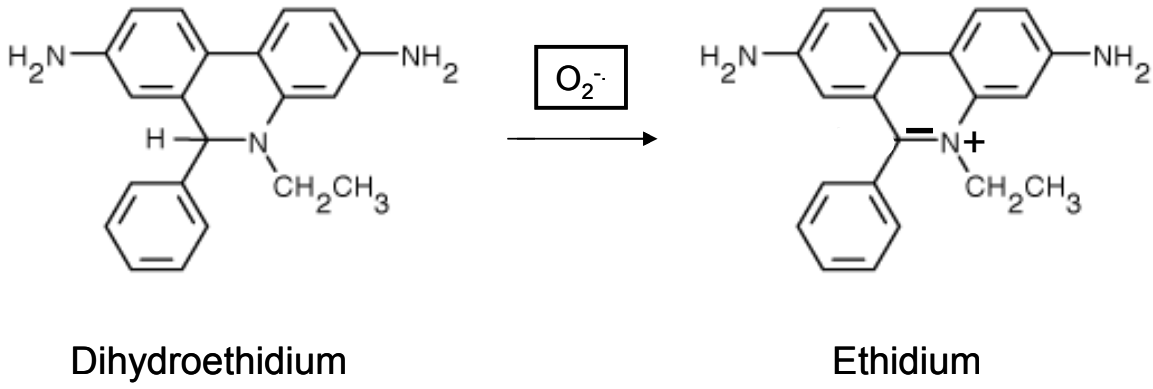


Figure 13. Structure of the dihydroethidium

After oxidation with $O_2^{\cdot -}$, DHE is converted to the polar compound ethidium that intercalates into the DNA.

To evaluate the levels of ROS, EC or PASMC were grown in 96-well plates to 80% confluence, and growth-arrested in serum free media for 24 h. Cells were then stimulated, washed with HBSS and incubated in the dark with DHE (50 μM) for 10 min at 37°C. Cells were washed with HBSS again to remove excess dye. Fluorescence was monitored in a fluorescence microscope (Olympus) and images were obtained using the Openlab Modular Software for Scientific Imaging (Improvision).

2.6. Single cell two photon confocal laser microscopy

Two photon confocal microscopy is a technique invented by Denk et al in 1990[131]. In conventional confocal laser scanning fluorescence microscopy, absorption of a single photon delivers energy for the fluorophore to reach the excited state from which it returns to the ground state by emitting a photon of fluorescence (Fig. 14A). Interestingly, the excited state can also be reached by the near simultaneous absorption of two longer wavelength photons (Fig. 14B). This absorption results in the squared dependence on laser light intensity in contrast to the linear light dependence of conventional, single-photon fluorescence imaging. This gives this technique the possibility of high contrast, three-dimensional resolution of fluorescence within living cells, reduced specimen photodamage and enhanced penetration depth of the light.

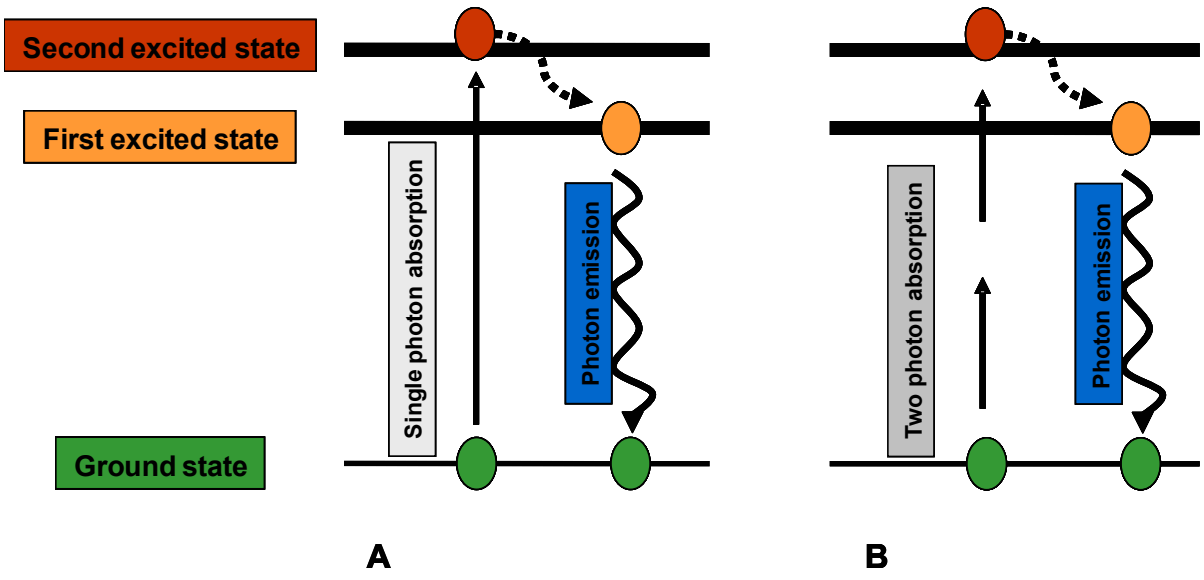


Figure 14. Principle of two photon confocal microscopy

A. Single photon absorption and fluorescence emission B. Simultaneous absorption of two photons and fluorescence emission.

The possibility of two photon confocal microscopy to simultaneously excite different fluorophores allows the detection of two different fluorescent colors without any cross-talk.

When performing experiments, HepG2 cells were used, since Ea.hy 926 cells did not adhere to the experimental glass dishes. Moreover, transfection efficiency of HepG2 cells is much higher, further increasing the sensitivity of the method. Cells were plated onto 3.5 cm glass bottom dishes with a glass thickness of 0.17 mm (Willco Wells) and transfected with 3 µg of either pcDNA6.1ECFP or pcDNA6.1hp22phoxECFP. After 24 h, the transfected cells were transferred to a culture chamber equipped with a thermostat, enabling observation at 37°C at constant pO_2. Cells were loaded with DHR using 30 µM DHR solution in DMEM for 5 min and washed with DMEM. Then RH123 fluorescence in single cells was recorded using 2-photon confocal laser scanning microscopy (2P-CLSM) and an excitation wavelength of 850 nm was applied to colocalize RH123 (emission 530 nm) and ECFP fluorescence (emission 580 nm) as described[132]. Fluorescence was registered by a photomultiplier using a 60-fold water immersion objective (Plan Achromat, Nikon), digitized and visualized by the EZ2000 software (Version 2.1.4 Coord Automatisering). The signal-to-noise ratio was determined and images were deconvoluted with the Huygens System software (Version 2.2.1, Scientific Volume Imaging) using the Maximum Likelihood Estimation (MLE) method. The data were reconstructed and isosurfaces were calculated with the Application Visualisation System (AVS Waltham) as described[133].

2.7 Northern blot analysis

2.7.1 Preparation of total RNA

Total RNA from EC or PASMC was purified using guanidine thiocyanate-acid phenol extraction. For RNA extraction, cells were plated in 10 cm dishes. At the end of the experiment, cells were washed with ice cold serum free medium and plates were frozen on liquid nitrogen. Thereafter, cells were lysed in 800 µl GT-DTT buffer, which denatures cellular and nucleoprotein complexes and releases RNA. The lysate was incubated for 30 min on ice with 800 µl phenol and 200 µl chloroform-isoamyl alcohol (24:1) to remove contaminating chromosomal DNA from the RNA preparation. Moreover, 80 µl of 2 M sodium-acetate buffer (pH 4) was added to maintain the pH of the denatured cell lysate during acid extraction and to provide the salt necessary for RNA precipitation with isopropanol. After centrifugation for 30 min at 13000 rpm, DNA and proteins remained in the lower organic phase, while RNA was present in the upper aqueous phase. After separation of phases, RNA was purified by two isopropanol precipitations at -20 °C for 2 h. Finally, the pellet of RNA was diluted in DEPC treated H_2O, heated at 65 °C for 10 min and RNA concentration was measured in a Hitachi spectrophotometer model U-2000 (Colora).

2.7.2 Electrophoresis and detection

10 µg of total RNA was mixed with RNA Premix in a 1:1 ratio and 10X RNA blue marker was added to the mix. The samples were then heated at 65°C for 10 min and loaded on prepared 1.3% agarose gels. The gel was run in 1X MOPS buffer on 40 V for 4 h. After separation, RNA was transferred from the gel to a nylon membrane (Porablot) for 24 h using capillary transfer in 20X SSC buffer. After the transfer was checked by UV detection of incorporated ethidium bromide, transferred RNA was cross-linked by UV irradiation in Stratalinker 1800 (Stratagen). Northern membranes were then incubated in hybridization solution for 2 h at

65°C. Northern hybridizations were carried out with digoxigenin-labeled antisense RNA for human p22phox and TF (that were present in the laboratory) or for human PAI-1 (provided by Dr. T. Kietzmann) at 65°C for 16 h. Thereafter, membranes were washed 2 times in 2X SSC solution containing 0.1% SDS and then 2 times in 0.1 SSC solution containing 0.1% SDS at 65°C. Membranes were blocked for 30 min in 1X blocking solution and then incubated for 1 h with anti-DIG antibody conjugated with alkaline phosphatase (Roche) diluted 1:10000 in blocking solution. After washing the membranes in washing solution, detection was performed by using the chemiluminescent substrate CDP-Star (Roche) diluted 1:100 in detection buffer. Blots were scanned and analysed using GelDoc software (BioRad). Loading of equal amounts of RNA was confirmed by ethidium bromide staining of 18S.

2.8 Western blot analysis

2.8.1 Preparation of protein samples

Cells were washed with ice cold serum free medium and plates were frozen on liquid nitrogen. Cells were then lysed in GT-DTT buffer and total proteins from EC or PASMC were purified using guanidine thiocyanate-acid phenol extraction, as described for purification of total RNA (*2.7.1*). The proteins which remained in the lower organic phase were precipitated overnight at 4°C by addition of 3 ml isopropanol and 600 µl of ethanol. Thereafter, the protein pellet was washed in 70% ethanol and dissolved in PBS containing 1% SDS and 1 mM NaF.
Alternatively, cells were lysed in freshly prepared buffer containing 50 mM tris(hydroxymethyl)aminomethane-HCl (Tris-HCl) pH 7.5, 150 mM NaCl, 25 mM NaF, 1% Triton, 2 mM EDTA, 2 mM EGTA, 10 mM Na_2PO_7, 2 mM activated Na_3VO_4 and supplemented with the mix of protease inhibitors PIM (2 µg/ml) and PMSF (44 µg/ml). After scraping of the cells, samples were incubated for 30 min on ice, and then centrifuged 10 min at 13000 rpm to remove cellular debris. This protocol was used to detect phosphorylated proteins.
Before performing electrophoresis, samples were mixed with 3X Laemmli loading buffer and boiled at 95°C for 5 min.

2.8.2 Electrophoresis and immunodetection

50 µg of isolated proteins were separated by 8-12% SDS polyacrylamide gel electrophoresis (PAGE) in running buffer in a Mini-Protean 3 System (Biorad) and transferred to nitrocellulose Protran membranes (Schleicher & Schuell) using the Mini Trans-blot System (Biorad) in cold transfer buffer. After transfer, blots were rinsed in water and incubated in ponceau S solution for several minutes. Thereafter, blots were washed in water until the background was reduced. The membranes were placed on a transparency sheet and

photocopied for documentation. Thereafter, blots were rinsed for several times in TBS-T to remove the ponceau S. Membranes were then blocked for 2 h in TBS-T containing 5% non-dry milk or 5% BSA and then overnight at 4°C with specific antibodies. Thereafter, membranes were washed 3 times for 5 min with TBS-T washing solution and incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h. After washing the membranes 5 times for 5 min with TBS-T, protein bands were visualized by performing luminol-enhanced chemiluminescence. Blots were scanned and analysed using GelDoc software (BioRad).

2.8.3 Stripping and reprobing

Equal loading of proteins was confirmed by reprobing the membranes with an antibody against actin. To remove bound antibodies, membranes were incubated in stripping buffer for 30 min at 50°C. Thereafter, membranes were washed 2 times for 30 min in TBS-T, blocked for 2 h in TBS-T containing 5% non-dry milk and then incubated with respective antibody.

2.9 Rac-1 pull down assay

2.9.1 Principle of the assay

Rac-1 activity was evaluated by using Rac-1 pull down activity assay. This assay uses a affinity precipitation method to detect active Rac-1. The assay reagent is a fusion-protein of the Rac-1 substrate, PAK-1, where the p21-binding domain of PAK (PBD, residues 67-150) is fused to glutathione S-transferase and then bound to glutathione agarose beads. Therefore, the beads interact only with active Rac-1 (Rac-1-GTP), and precipitate the whole complex. To confirm the validity of the method, lysates were loaded with either GTPγS or GDP before precipitation with PAK-PDB agarose. The signal from these cells confirmed that beads precipitate only Rac-1-GTP, which is the active form of Rac-1 (Fig. 15).

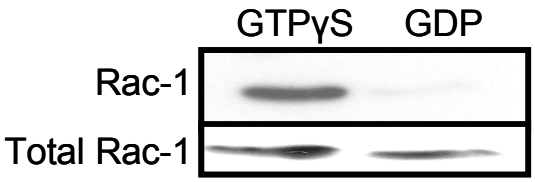


Figure 15. Validation of Rac-1 activity assay

Cell lysates were incubated with 100 µM GTPγS or 1mM GDP in the presence of 10 mM EDTA for 15 min at 30°C. The reaction was terminated by placing lysates on ice and adding $MgCl_2$ to a final concentration of 60 mM. Thereafter, lysates were incubated with the beads and the assay was performed as described. Western blot analysis was performed by using an antibody against Rac-1. Only Rac-1 from lysates loaded with GTP (active form of Rac-1: Rac-1-GTP) was detected.

2.9.2 Rac-1 pull-down assay

Serum-starved PASMC were stimulated with thrombin (3 U/ml) for the indicated time. Cells were lysed in buffer containing 25 mM HEPES, 150 mM NaCl, 1% Igepal CA-630, 10% glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM sodium orthovanadate, 10 µg/ml leupeptin, 10 µg/ml aprotinin and incubated for 30 min on ice. Thereafter, the lysate was centrifuged at 13 000 rpm for 15 min to remove cellular debris. The supernatant was then transferred to another tube and 5 µg of PAK-PBD-bound agarose beads was added. The mixture was rocked for 60 min at 4°C. Thereafter, beads were collected by pulsing, washed 3 times with lyses buffer, resuspended in 2X Laemmli buffer and boiled for 5 min. Levels of active Rac-1 were evaluated by SDS-PAGE and subsequent immunoblot analysis using a Rac-1 antibody. The supernatant after removal of the beads was also analysed by Western blot in order to determine levels of total Rac-1 protein.

2.10 Procoagulant activity

PASMC were plated in 24 well plates and starved from serum for 16 h. Cells were then stimulated with thrombin (1 U/ml) for 6 h. Thereafter, the culture medium was removed and cells were washed 3 times with 1 ml prewarmed (37°C) HEPES buffer. Platelet poor plasma (PPP, 480 µl) was then added to the cells. PPP was isolated from freshly taken blood which was mixed 1:10 with sodium citrate buffer (3.8%), and centrifuged at 2100 U/min for 30 min. The upper phase was then separated and added to the cells together with 120 µl Buffer A. The formation of thrombin was initiated by the addition of 120 µl 0.1 M $CaCl_2$ (pH 7.35; final concentration 16.7 mM). Every second minute from the beginning of the reaction, 20 µl aliquots were taken and mixed with prewarmed (37°C) Buffer B (480 µl) that contained 0.2 mM chromogenic substrate S-2238. S-2238 is cleaved by thrombin thereby changing color into yellow whose optical densities are a quantitative measure of thrombin formation. After 3 min, the reaction was stopped by adding 300 µl of concentrated acetic acid and the absorbance at 405 nm was measured in a spectrophotometer.

Buffer A	Final concentration
Tris	0.05 M
NaCl	0.1 M

The pH was adjusted to 7.35.

Buffer B	Final concentration
Tris	0.05 M
NaCl	0.1 M
EDTA	20 mM
Ovalbumin	0.05 mg/ml

2.11 Proliferation assay

2.11.1 Principle of the BrdU incorporation assay

Cellular proliferation requires DNA replication. Therefore, monitoring of DNA synthesis is an indirect marker of the rate of proliferation in response to different stimuli.

To asses proliferative activity of EC and PASMC we used 5-bromo-2‘deoxyuridine (BrdU) labeling. BrdU is an analogue of the DNA nucleoside thymidine, and is incorporated in newly synthesized DNA of proliferating cells. The incorporated BrdU is detected by an immunoassay, using a peroxidase-conjugated antibody that specifically recognizes BrdU bound to denaturated DNA. The bound antibody is detected by a peroxidase-catalysed colorimetric reaction, using tetramethyl-benzidine (TMB) as a substrate.

2.11.2 Proliferation assay

Cells were seeded in 96 well plates to achieve 50% of confluency. In the experiments where DNA synthesis of transfected cells was investigated, cells were transfected on the following day with corresponding vectors, and starved from serum overnight. On the next day, cells were exposed to stimuli as described in the respective legends. Alternatively, when analyzing PAI-1-dependent proliferation of PASMC, cells were stimulated with thrombin (3 U/ml) for 8 h. The culture medium was then removed and another set of serum-starved PASMC seeded in 96 well plates was incubated with the supernatant in the presence of an inhibitory antibody that binds PAI-1 (Loxo) or mouse IgG (Santa Cruz) as a control.

To asses proliferation, cells were then incubated with BrdU (10 µM) for 16 h. Thereafter, cells were fixed for 30 min and then incubated for 1 h with the peroxidase-conjugated antibody against BrdU. Immunodetection was performed by adding the colorimetric substrate TMB. When the blue color developed, the reaction was stopped with 1 M H_2SO_4. Absorbance was

measured in an ELISA reader (Tecan Safire) at 450 nm with a reference wavelength at 690 nm.

2.12 Statistical analysis

Values presented are means ± standard deviation. All experiments are performed at least 3 times (n>3). To calculate significance, Student-Newman-Keuls *t*-test was performed. $P<0.05$ was considered statistically significant.

3. Results

3.1 p22phox regulates ROS production and proliferation of endothelial cells in response to thrombin

The mechanisms linking thrombosis to ROS production in the endothelium are not well understood. Endothelial cells are described to generate ROS, but the underlying mechanisms are still unclear. Therefore the role of thrombin in regulating NADPH oxidase-dependent ROS production and expression of its subunit p22phox, as well as p22phox-dependent proliferation of the endothelial cell line Ea.hy 926 were investigated.

3.1.1 Thrombin activates ROS production and increases p22phox expression

To evaluate the effects of thrombin on ROS production in endothelial cells, Ea.hy 926 cells were stimulated with thrombin and ROS production was measured by DCF fluorescence in a flow cytometer. Exposure to thrombin resulted in significantly increased ROS production peaking after 15 min and thereafter declining again (Fig. 16A). Furthermore, prolonged exposure to thrombin for more than 1 h also increased ROS production peaking at 3 h of stimulation (Fig. 16A). Since NADPH oxidases have been shown to contribute to endothelial ROS production, it was determined whether elevated ROS production in response to prolonged thrombin stimulation would be associated with increased mRNA and protein levels of the NADPH oxidase subunit p22phox. Ea.hy 926 cells were stimulated with thrombin (3 U/ml) for increasing time periods, and p22phox mRNA and protein levels were measured by Northern blot and Western blot analyses, respectively (Fig.16B,C). Thrombin stimulation resulted in an upregulation of p22phox mRNA by 3-fold after 1 h followed by a 3.8 fold-increase in p22phox protein levels at 3 h (Fig.16B,C) thus closely accompanying the kinetics of thrombin-stimulated ROS production (Fig.16A) suggesting that increased ROS production in response to prolonged thrombin stimulation may be allowed by upregulation of p22phox.

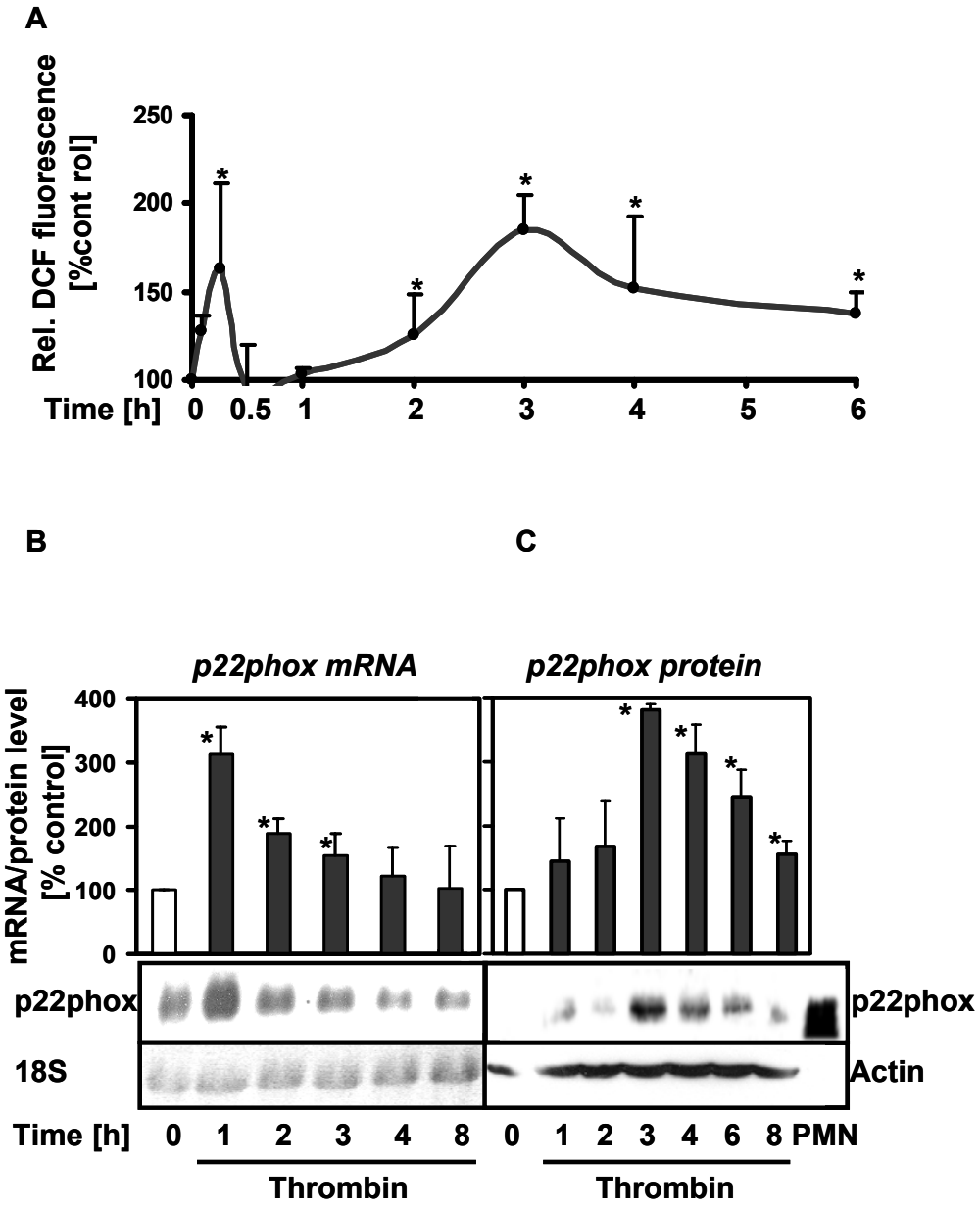


Figure 16. Thrombin stimulates ROS production and p22phox expression

A) Ea.hy 926 cells were treated with 3 U/ml thrombin for 5, 15 and 30 min. DCF fluorescence was monitored by flow cytometry. Additionally, Ea.hy 926 cells were stimulated with thrombin from 1 to 6 h and DCF fluorescence was monitored in a microplate reader. Data are presented as relative increase compared to control at time 0 (100%) (n=3, *p<0.05 versus non-stimulated cells (Ctr)). B) Ea.hy 926 cells were exposed to 3 U/ml thrombin for the indicated time points (from 1 to 8 h) and Northern blot and C) Western blot analyses were performed. Lysates of polymorphonuclear leukocytes (PMN) were used as a positive control. In each experiment the p22phox mRNA or protein levels at time 0 were set equal to 100% (n=3, *p<0.05 versus non-stimulated cells (Ctr)). Representative Northern and Western blots are displayed.

3.1.2 Expression of p22phox colocalizes with intracellular ROS production

To determine whether enhanced expression of the NADPH oxidase subunit p22phox is linked to increased ROS production, human p22phox fused to the enhanced cyan fluorescent protein ECFP was overexpressed and intracellular ROS production was measured using DHR fluorescence by 2- photon confocal laser scanning microscopy (2P-CLSM) (Fig. 17A). 2P-CLSM allows the simultaneous detection of the blue fluorescence from transfected ECFP vectors and of the red fluorescence, from the conversion of non-fluorescent DHR to fluorescent rhodamine123 (RH123) by ROS, without any cross-talk due to the emission filter settings. Overlap of ECFP fluorescence with RH123 fluorescence after deconvolution and reconstruction indicates the sites of ROS generation and p22phox expression. Since transfected Ea.hy 926 cells did not attach to the specific wells required for the optical analysis, HepG2 cells were used in this experiment. Transfection of ECFP control plasmid resulted in disseminated blue fluorescence but almost no red RH123 fluorescence (Fig. 17A). In contrast, in pcDNA6.1hp22phoxECFP transfected cells, blue fluorescence was clearly localized to intracellular structures surrounding the nucleus and colocalized with elevated RH123 fluorescence, indicating that expression of this NADPH oxidase subunit is associated with intracellular ROS production (Fig. 17A). Furthermore, overexpression of p22phox increased ROS production in Ea.hy 926 cells as was quantified by DCF fluorescence in a microplate reader (Fig. 17B), indicating that increased levels of p22phox are associated with enhanced ROS production. Whereas stimulation with thrombin did not significantly enhance ROS production in p22phox overexpressing cells compared to control cells, depletion of p22phox by transfecting an antisense vector completely abrogated thrombin-stimulated ROS production (Fig. 17B,C). These findings confirm that p22phox is required for ROS production and that thrombin stimulation is mimicking the situation with enhanced p22phox expression.

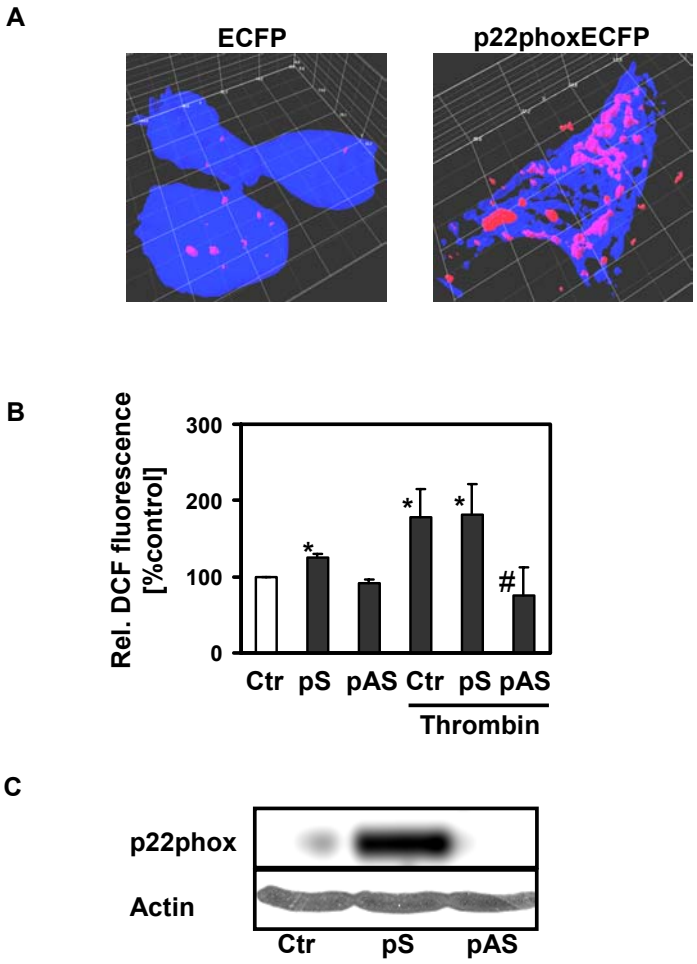


Figure 17. Expression of p22phox is linked to intracellular ROS production

A) HepG2 cells were transfected with pcDNA6.1hp22phoxECFP (p22phoxECFP) or control vector (ECFP) and loaded with 30 µM DHR for 5 min. RH123 fluorescence showing ROS generation and ECFP fluorescence showing the expression of the p22phox fusion protein were recorded simultaneously by 2-photon confocal laser scanning microscopy. Overlapping of ECFP fluorescence (blue fluorescence) with RH123 fluorescence (red fluorescence) after deconvolution indicates the sites of p22phox expression and ROS production, respectively. B) Ea.hy 926 cells were transfected with control vector (Ctr) or a p22phox sense (pS) or antisense vector (pAS) and stimulated with thrombin (3 U/ml) for 3 h. ROS levels were evaluated by performing DCF measurements. Data are presented as relative increase to control (100%) (n=3, *p<0.05 versus cells transfected with control vector (Ctr); #p<0.05 versus thrombin-stimulated cells transfected with control vector (Ctr)). C) Ea.hy 926 cells were transfected with control vector (Ctr) or a p22phox sense (pS) or antisense vector (pAS) and the expression was confirmed by performing Western blot analyses using an antibody against p22phox. The loading of equal amounts of proteins was confirmed by reprobing the membranes with a β-actin antibody. A representative Western blot is shown (n=3).

3.1.3 Thrombin regulates p22phox expression in a redox-sensitive manner

Since thrombin induced a biphasic elevation of ROS whereby the delayed phase of ROS production correlated with an upregulation of p22phox, and overexpression of p22phox significantly increased intracellular levels of ROS, the hypothesis was investigated whether thrombin stimulation of p22phox protein expression is redox-sensitive. Ea.hy 926 cells were exposed to the antioxidant vitamin C (VitC, 100 µM) or the flavin inhibitor diphenyleneiodonium (DPI, 10 µM) for 30 min prior to stimulation with thrombin for 3 h, and generation of ROS as well as expression levels of p22phox mRNA and protein were determined. Treatment with VitC as well as DPI did not only inhibit thrombin-stimulated ROS production (Fig. 18A) but also prevented upregulation of p22phox mRNA and protein levels (Fig. 18B) further suggesting that thrombin regulates p22phox expression via a redox-sensitive pathway.

To further support redox-sensitivity of p22phox expression, Ea.hy 926 cells were exposed to 50 µM H_2O_2 for increasing time periods and p22phox mRNA levels were determined (Fig. 19A). H_2O_2 transiently increased p22phox mRNA levels by 2.9-fold at 2 h of stimulation, corresponding to elevated ROS levels at this time as determined by dihydroethidium (DHE) fluorescence (Fig. 19B). Quantitative analysis of ROS production by DCF fluorescence in response to treatment with H_2O_2 for 3 h revealed a dose-dependent increase in DCF fluorescence peaking at 10 to 50 µM (Fig. 19C) which was accompanied by a significant increase in p22phox protein levels (Fig. 19C) further supporting that p22phox expression is redox-sensitive.

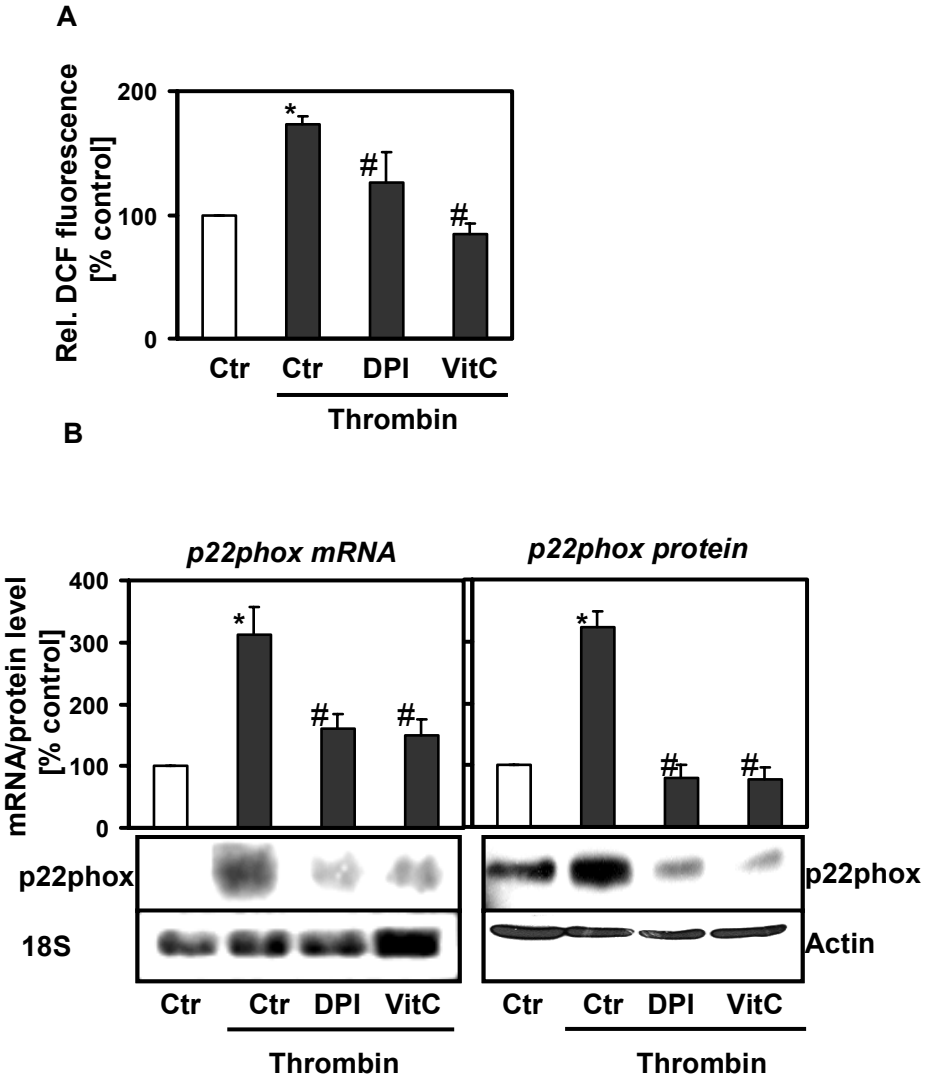


Figure 18. Thrombin-induced p22phox expression is redox-sensitive

Ea.hy 926 cells were exposed to the antioxidant vitamin C (VitC, 100 µM) or the flavin inhibitor diphenyleneiodonium (DPI, 10 µM) for 30 min prior to stimulation with 3 U/ml thrombin for 3 h. A) ROS levels were assessed by performing DCF measurements in a microplate reader. Data are presented as relative increase to control (100%) (n=3, *p<0.05 versus non stimulated cells (Ctr), #p<0.05 versus thrombin-stimulated cells (Ctr)). B) p22phox mRNA and protein levels were evaluated by performing Northern blot or Western blot analyses, respectively. Equal loading was controlled by 18S staining or probing with an actin antibody, respectively. In each experiment the p22phox mRNA or protein levels under non-stimulated, untreated conditions were set equal to 100% (n=3, *p<0.05 versus non-stimulated cells (Ctr), #p<0.05 versus thrombin-stimulated cells (Ctr)). Representative Northern and Western blots are displayed.

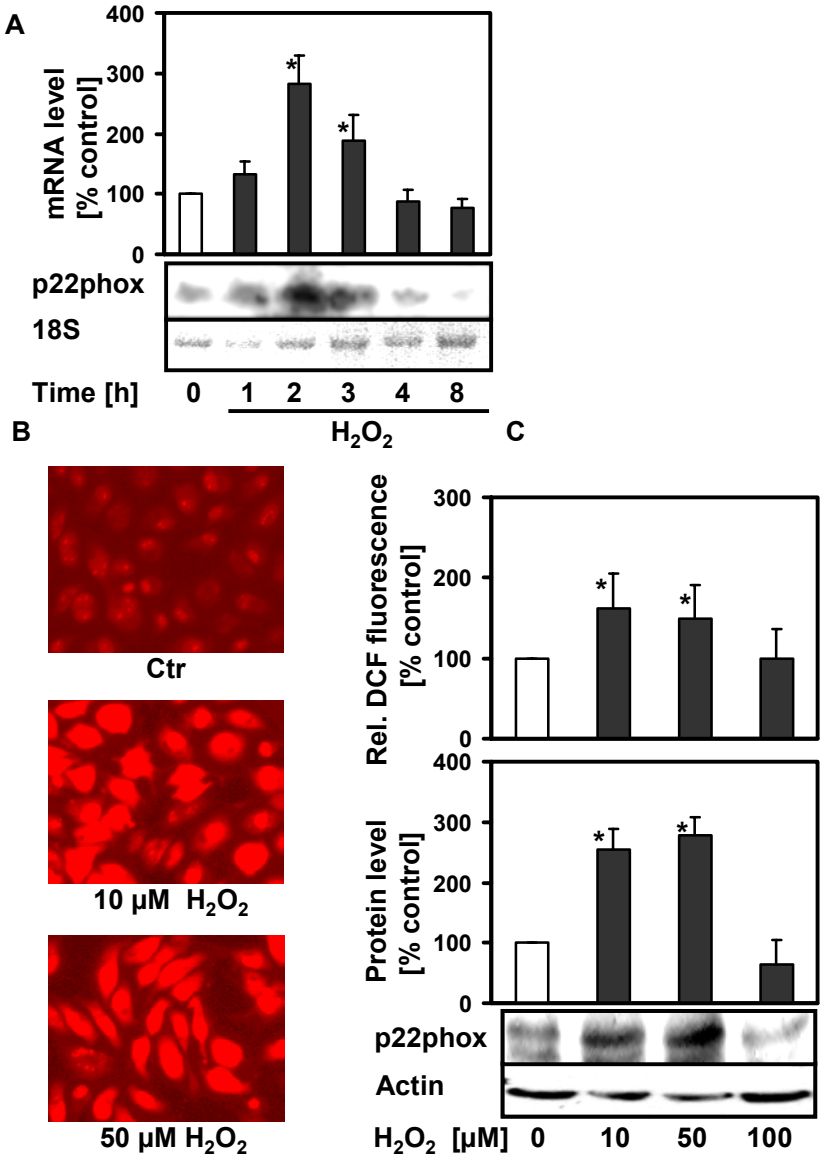


Figure 19. H_2O_2 increases ROS production and p22phox expression

A) Ea.hy 926 cells were stimulated with 50 µM H_2O_2 for increasing time points (from 1 to 8 h) and p22phox mRNA levels were determined by Northern blot analyses. In each experiment the p22phox mRNA levels under non-stimulated conditions were set equal to 100% (n=3, *p<0.05 versus non-stimulated cells (Ctr)). B) Ea.hy 926 cells were treated with H_2O_2 (10 or 50 µM) for 3 h, washed with HBSS and incubated with DHE (50 µM) for 10 min in the dark at 37°C. Representative images from a total of 3 experiments are shown. C) Ea.hy 926 cells were stimulated with increasing concentrations of H_2O_2 (10, 50 or 100 µM) for 3 h. DCF fluorescence was monitored in a microplate reader. Data are presented as relative increase to control (100%) (n=3, *p<0.05 versus non-stimulated cells). Concomitantly, p22phox protein levels were evaluated by Western blot analysis using an antibody against p22phox. In each experiment the p22phox protein levels under non-stimulated conditions were set equal to 100% (n=3, *p<0.05 versus non-stimulated cells (Ctr)). A representative Western blot is shown.

3.1.4 Thrombin-stimulated ROS production and p22phox expression require kinase activation

To investigate the signaling pathways involved in thrombin-stimulated ROS production and p22phox expression, Ea.hy 926 cells were stimulated with thrombin (3 U/ml) and phosphorylation of protein kinase B (Akt), p38 MAP kinase (p38MAPK) and p42/44 MAP kinase (ERK) was evaluated by Western blot analysis (Fig. 20A). Thrombin transiently increased the phosphorylation of all three kinases peaking at 1 min for Akt and ERK and at 5 min for p38MAPK. To further evaluate the involvement of these protein kinases in thrombin-stimulated ROS production, Ea.hy 926 cells were preincubated with the p38MAPK inhibitor SB220025 (20 μM), the inhibitor of the ERK upstream kinase MEK1, PD98059 (20 μM), or the phosphatidylinositol-3 kinase (PI3K) inhibitor LY294002 (10 μM). Treatment with SB220025 and LY294002, but not with PD98059, significantly decreased thrombin-stimulated ROS production (Fig. 20B). Moreover, application of SB220025 or LY294002 inhibited upregulation of p22phox mRNA by thrombin (Fig. 20B) suggesting that the p38MAPK and PI3 kinase/Akt pathways are involved in the regulation of p22phox expression and NADPH oxidase activity in response to thrombin in endothelial cells.

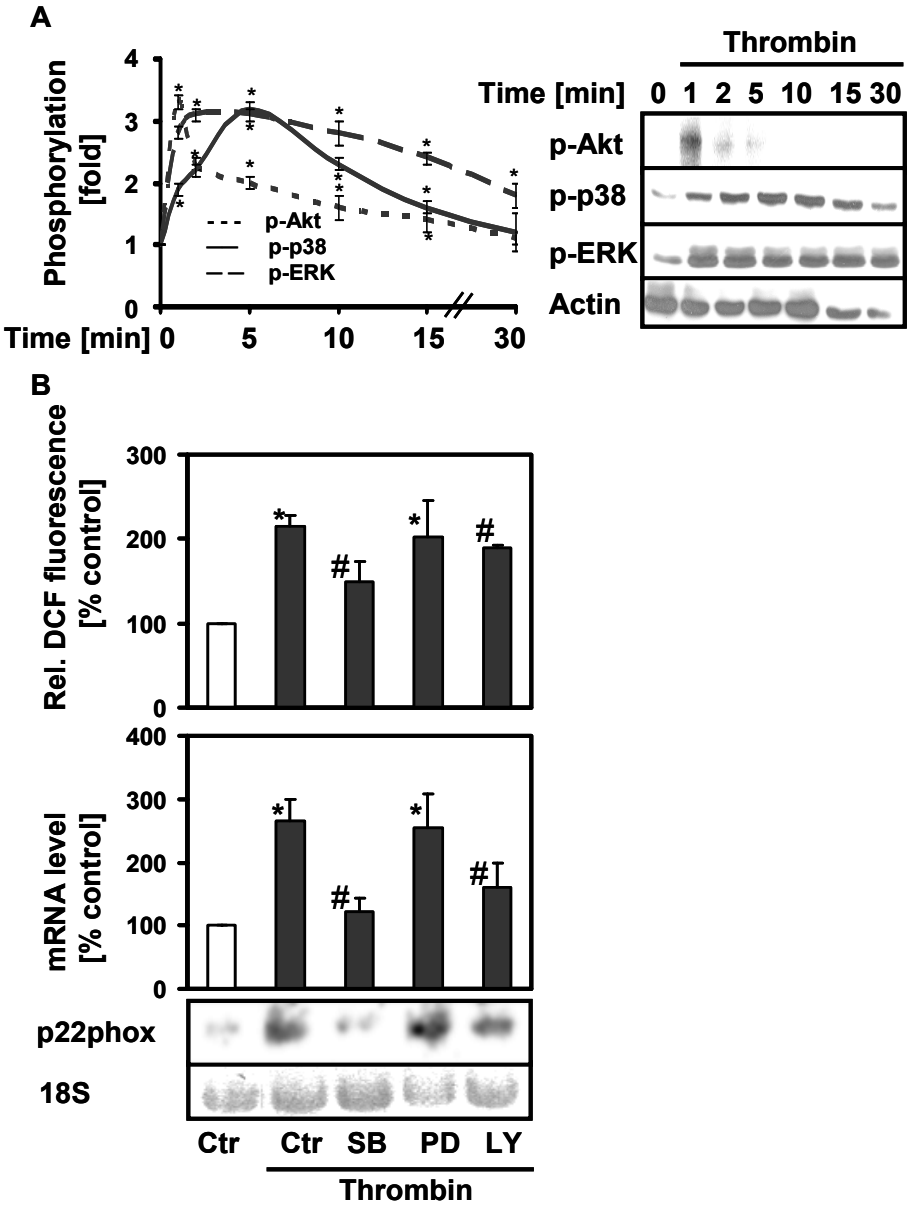


Figure 20. p38 MAP kinase and PI3 kinase mediate increased ROS production and p22phox expression in response to thrombin

A) Ea.hy 926 cells were exposed to 3 U/ml thrombin for different time points (from 1 to 30 min) and Western blot analysis was performed using antibodies against the phosphorylated forms of Akt (p-Akt), p38 MAP kinase (p-p38) and ERK (p-ERK). In each experiment protein levels under non-stimulated conditions were set equal to 1 (n=3, *p<0.05 versus non-stimulated cells (Ctr)). B) Ea.hy 926 cells were preincubated for 30 min with the p38MAPK inhibitor SB220025 (SB, 20 µM), the MEK1 inhibitor PD98059 (PD, 20 µM), and the PI3 kinase inhibitor LY294002 (LY, 10 µM). After stimulation with 3 U/ml thrombin for 3 h, ROS generation was assessed in a microplate reader using DCF fluorescence. Data are presented as relative increase to control (100%) (n=3, *p<0.05 versus non-stimulated cells (Ctr), #p<0.05 versus thrombin-stimulated cells). After exposure to 3 U/ml thrombin for 1 h, p22phox mRNA levels were determined by Northern blot analyses. In each experiment the p22phox mRNA levels under non-stimulated conditions were set equal to 100% (n=3, *p<0.05 versus non-stimulated cells (Ctr), #p<0.05 versus thrombin-stimulated cells).

3.1.5 H_2O_2 induces ROS production and p22phox expression via kinase activation

To further evaluate the signaling pathways activated by H_2O_2, cells were stimulated with H_2O_2 (50 µM) and phosphorylation of Akt, p38MAPK and ERK was determined by performing Western blot analysis (Fig. 21A). Similar to thrombin, H_2O_2 increased the phosphorylation of all three kinases in a time-dependent manner starting between 1 to 5 min of exposure, but staying elevated over the whole observation period (30 min). To investigate the role of these kinase pathways in H_2O_2-stimulated ROS production, cells were pretreated with SB220025, PD98059 or LY294002. Application of SB220025 and LY294002, but not of PD98059, significantly decreased H_2O_2-stimulated ROS generation (Fig. 21B). Similar to the results obtained with thrombin, pretreatment with SB220025 and LY294002 prevented upregulation of p22phox mRNA levels in response to H_2O_2 (Fig. 21B) indicating the involvement of p38MAPK and PI3 kinase/Akt in the regulation of p22phox expression and NADPH oxidase activity in response to low levels of H_2O_2 in endothelial cells.

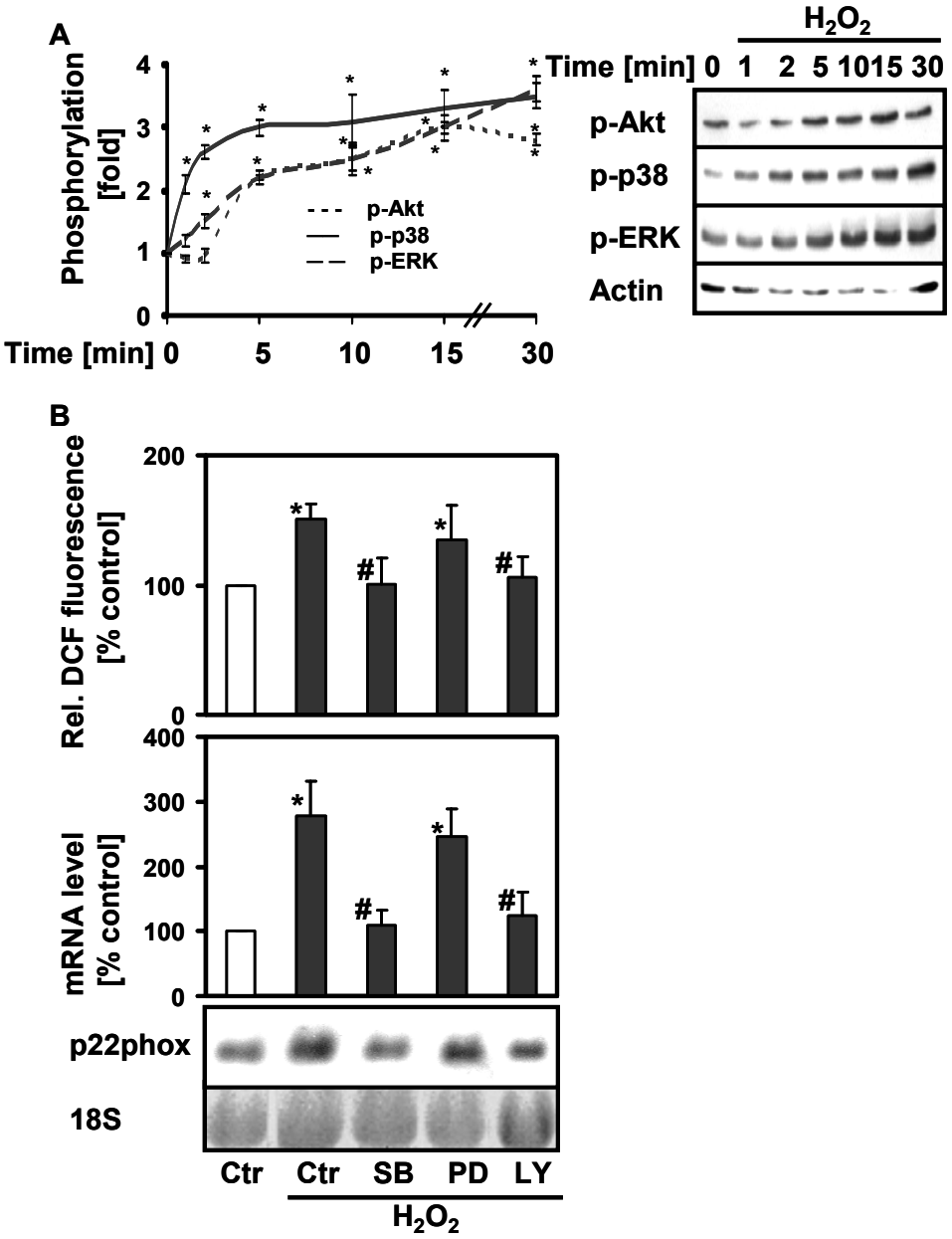


Figure 21. p38 MAP kinase and PI3 kinase mediate increased ROS production and p22phox expression in response to H_2O_2

A) Ea.hy 926 cells were stimulated with 50 µM H_2O_2 from 1 to 30 min. Western blot analysis using antibodies against the phosphorylated forms of Akt (p-Akt), p38 MAP kinase (p-p38MAPK) and ERK (p-ERK) was performed. Protein levels under non-stimulated conditions were set equal to 1 (n=3, *p<0.05 versus non-stimulated cells (Ctr)). B) Ea.hy 926 cells were preincubated for 30 min with the p38MAPK inhibitor SB220025 (SB, 20 µM), the MEK1 inhibitor PD98059 (PD, 20 µM), and the PI3 kinase inhibitor LY294002 (LY, 10 µM). After stimulation with 50 µM H_2O_2 for 3 h, ROS generation was assessed in a microplate reader using DCF fluorescence. Data are presented as relative increase to control (100%) (n=3, *p<0.05 versus unstimulated cells (Ctr), #p<0.05 versus H_2O_2-stimulated cells). After treatment with 50 µM H_2O_2 p22phox mRNA levels were detected by Northern blot analysis. p22phox mRNA levels under non-stimulated, untreated conditions were set equal to 100% (n=3, *p<0.05 versus non-stimulated cells (Ctr), #p<0.05 versus H_2O_2 -stimulated cells).

3.1.6 Thrombin and H_2O_2 regulate p22phox via transcription and de novo synthesis

To further investigate the mechanisms involved in the regulation of p22phox expression by thrombin and H_2O_2, Ea.hy 926 cells were preincubated with actinomycin D (5 µg/ml), an inhibitor of gene transcription, or cycloheximide (100 µM), an inhibitor of protein synthesis, for 30 min, and then exposed to thrombin (3 U/ml) or H_2O_2 (50 µM) for 1 or 2 h, respectively. mRNA levels were evaluated by Northern blot analyses. Actinomycin D significantly blocked the thrombin- or H_2O_2-mediated increase in p22phox mRNA whereas cycloheximide did not prevent the induction of p22phox mRNA (Fig. 22A). These results indicate that thrombin- or H_2O_2-stimulated p22phox mRNA involves transcriptional activation of the p22phox gene.

In addition, preincubation with actinomycin D or cycloheximide for 30 min decreased thrombin- or H_2O_2–stimulated p22phox protein levels (Fig. 22B) suggesting that also de novo protein synthesis is involved in the response to thrombin and H_2O_2.

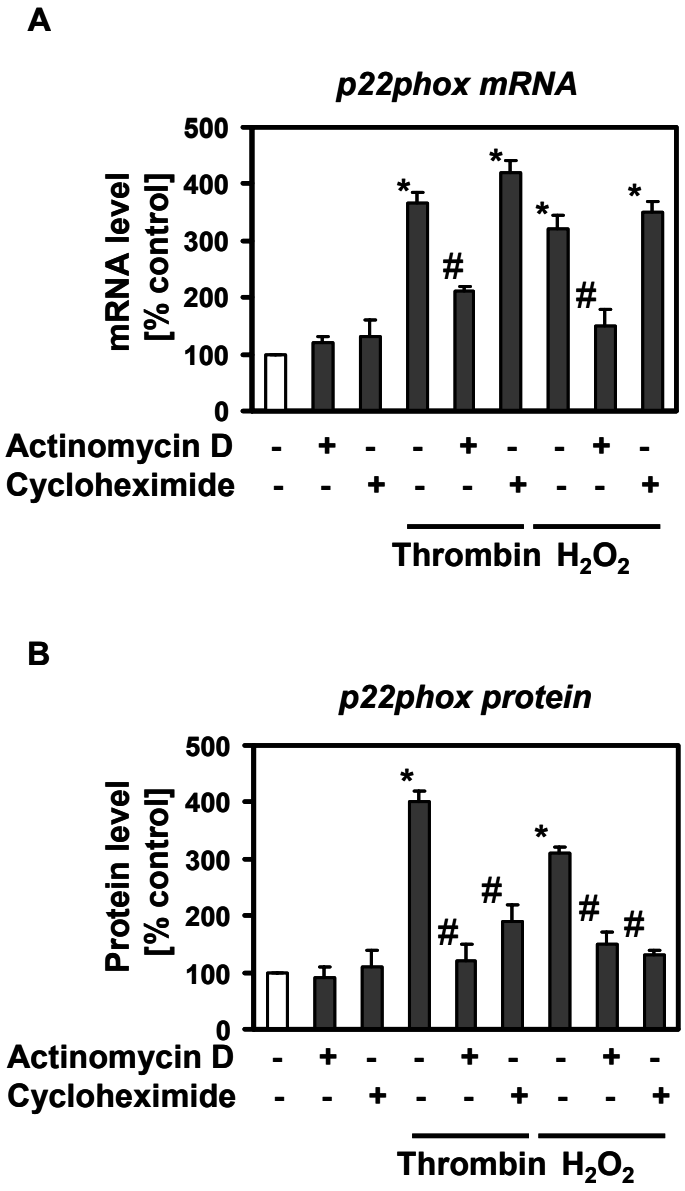


Figure 22. p22phox expression by thrombin and H_2O_2 is regulated at the transcriptional and post-transcriptional levels

A) Ea.hy 926 cells were exposed to actinomycin D (5 µg/ml) or cycloheximide (100 µM) for 30 min prior to stimulation with thrombin (3 U/ml) or H_2O_2 (50 µM) for 1 or 2 h, respectively. p22phox mRNA levels were evaluated by performing Northern blot analysis. In each experiment the p22phox mRNA levels under non-stimulated, untreated conditions were set equal to 100% (n=3, *p<0.05 versus non-stimulated cells (Ctr), #p<0.05 versus thrombin- or H_2O_2-stimulated cells). B) Ea.hy 926 cells were preincubated with actinomycin D (5 µg/ml) or cycloheximide (100 µM) for 30 min and thereafter stimulated with thrombin (3 U/ml) or H_2O_2 (50 µM). p22phox protein levels were evaluated by Western blot analysis using an antibody against p22phox. Loading of equal amounts of proteins was confirmed by reprobing the membranes with a β-actin antibody. In each experiment the p22phox protein levels under non-stimulated, untreated conditions were set equal to 100% (n=3, *p<0.05 versus non-stimulated cells (Ctr), #p<0.05 versus thrombin- or H_2O_2-stimulated cells).

3.1.7 Low levels of H_2O_2 or thrombin stimulate NADPH oxidase-dependent proliferative activity

Low levels of ROS have been suggested to promote proliferation. We therefore determined the effects of increasing concentrations of H_2O_2 and thrombin on the proliferative activity of Ea.hy 926 cells by BrdU incorporation. Stimulation with H_2O_2 at concentrations between 0.1 and 50 µM for 72 h significantly increased the proliferative activity whereas 100 µM H_2O_2 had no effect and higher concentrations such as 500 and 1000 µM significantly decreased the proliferative activity (Fig. 23A). Similarly, exposure to 3 U/ml of thrombin, but not to 5 U/ml, significantly increased proliferation of Ea.hy 926 cells (Fig. 23B). Depletion of p22phox by transfecting an antisense vector completely abrogated thrombin- and H_2O_2-stimulated proliferation (Fig. 23C).

In summary, the data support a model whereby activation of the NADPH oxidase by thrombin leads to a rapid increase in endothelial ROS production which, via activation of p38MAPK and the PI3 kinase/Akt pathway, elicits p22phox expression, subsequently promoting sustained generation of ROS and proliferation of endothelial cells.

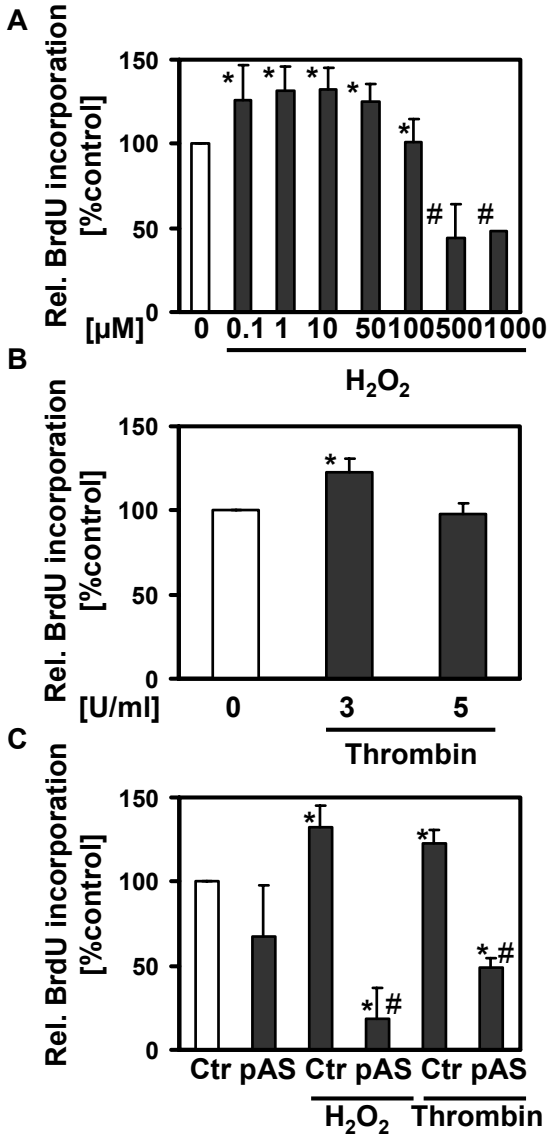


Figure 23. H_2O_2 or thrombin treatment stimulate p22phox-dependent cell proliferation

A) Ea.hy 926 cells were starved from serum for 24 h and then exposed for 72 h to H_2O_2 (0.1 to 1000 µM). Proliferative activity was assessed by using 5-bromo-2'-deoxyuridine (BrdU) incorporation. Data are presented as relative increase to control (100%) (n=3, *p<0.05 versus non-stimulated cells). B) Ea.hy 926 cells were starved from serum for 24 h and then exposed for 72 h to thrombin (3 or 5 U/ml). Proliferation was assessed by using BrdU incorporation. Data are presented as relative increase to control (100%) (n=3, *p<0.05 versus non-stimulated cells). C) Cells were transfected with a p22phox antisense vector (pAS) or control vector (Ctr), starved from serum for 24 h and then stimulated with 10 µM H_2O_2 or 3 U/ml thrombin for 72 h. Proliferation was assessed by using BrdU incorporation. Data are presented as relative increase to control (100%) (n=3, *p<0.05 versus non-stimulated cells transfected with control vector (Ctr), #p<0.05 versus H_2O_2- or thrombin-stimulated cells).

3.2 Rac-1 regulates ROS production and proliferation of pulmonary artery smooth muscle cells in response to thrombin

Thrombosis, ROS generation and proliferation of smooth muscle cells are features of (pulmonary) vascular remodeling. Whereas thrombin has been shown to increase ROS production by activating NADPH oxidases in aortic smooth muscle cells[122], the role of thrombin in the regulation of ROS production and proliferation of PASMC, the primary cell type involved in pulmonary vascular remodeling, is not well understood. Since Rac-1 is an important regulator of NADPH oxidase activity, and can also act as a molecular switch involved in different signaling pathways, it was investigated whether Rac-1 is involved in ROS production and proliferation in response to thrombin in PASMC. Moreover, the effect of Rac-1 on proliferation of PASMC was evaluated.

3.2.1 Rac-1 modulates ROS production in response to thrombin

To investigate the role of Rac-1 in thrombin-stimulated ROS production, PASMC were transfected with expression vectors encoding constitutively active RacG12V or dominant-negative RacT17N or with control vector (Ctr) and stimulated with thrombin for 2 h. Generation of ROS was evaluated by DCF fluorescence. Treatment with thrombin significantly enhanced levels of ROS in PASMC (Fig. 24). Expression of RacG12V further increased ROS production which was abolished by the antioxidant N-acetyl-cysteine (NAC) (Fig. 24). On the other hand, expression of RacT17N prevented thrombin-stimulated ROS generation, suggesting an important role of Rac-1 in controlling ROS production in response to thrombin in PASMC.

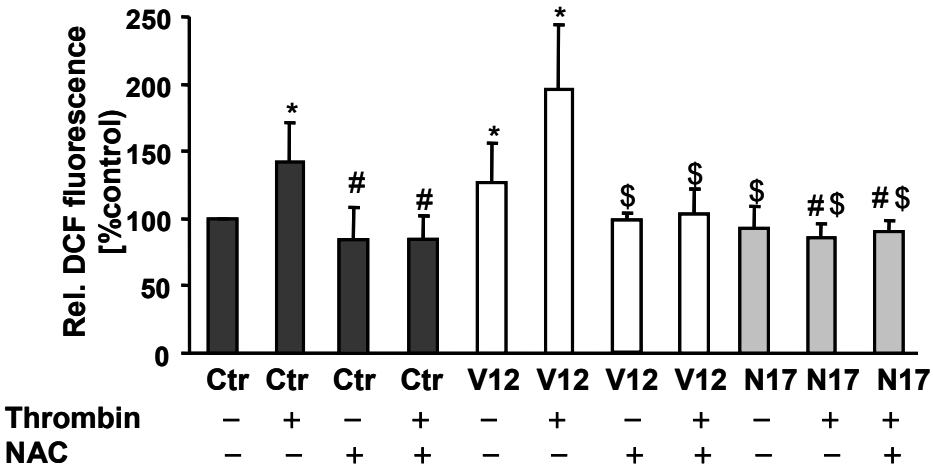


Figure 24. Rac-1 modulates thrombin-stimulated production of reactive oxygen species

PASMC were transfected with control vector (Ctr) or expression vectors for either a constitutively active RacG12V (V12) or a dominant-negative RacT17N (N17) form of Rac-1 and/or preincubated with NAC (10 mM) and exposed to thrombin (3 U/ml) for 2 h. DCF fluorescence was monitored in a microplate reader (n=3; *p<0.05 versus Ctr (unstimulated); #p<0.05 versus Ctr (thrombin-stimulated) $p<0.05 versus V12 transfected cells stimulated with thrombin (V12)).

3.2.2 Rac-1 expression and activity are increased in response to thrombin

Since thrombin-stimulated ROS generation was Rac-1-dependent, it was investigated whether thrombin is able to influence Rac-1 expression and activity. To this end, PASMC were stimulated with thrombin for different time points and Rac-1 protein levels were evaluated by Western blot analyses. Thrombin transiently enhanced Rac-1 protein expression by 1.5 -fold peaking after 2 h of application (Fig. 25A). Moreover, thrombin stimulation for 15 sec resulted in significantly increased activity of Rac-1 to levels about 2-fold higher than in the respective control (Fig. 25B).

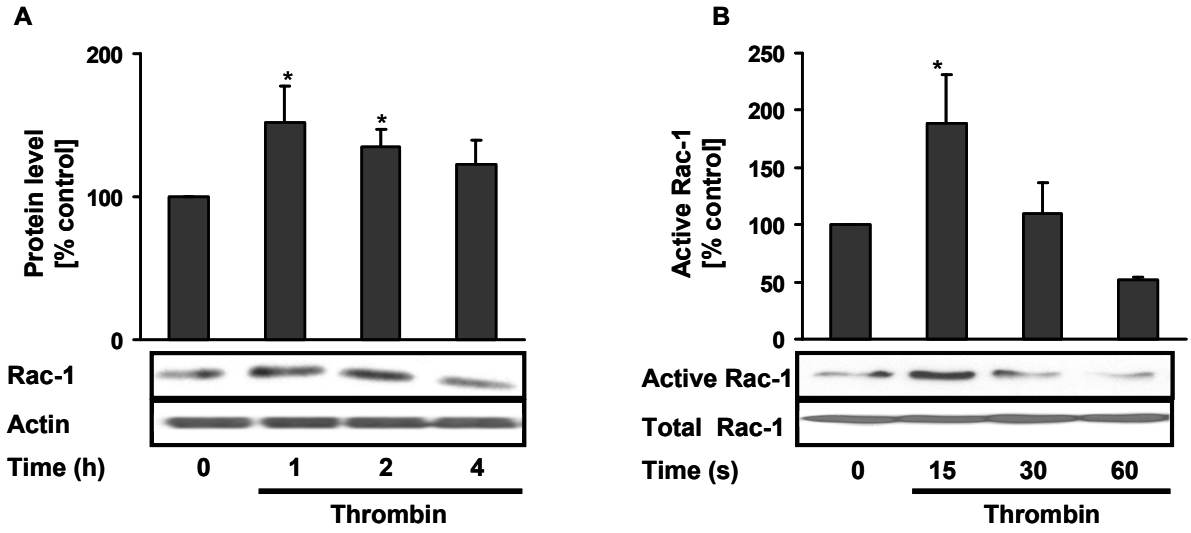


Figure 25. Rac-1 expression and activity are increased in response to thrombin

PASMC were exposed to thrombin (3 U/ml) for different time points and A) Rac-1 protein levels were determined by Western blot analysis. B) Levels of active Rac-1 or total Rac-1 proteins were evaluated by performing a Rac-1 pull down assay. Representative blots are shown. Equal loading of proteins was confirmed by reprobing the membranes with an actin antibody. In each experiment protein levels under non-stimulated conditions were set equal to 100 % (n=4, *$p<0.05$ versus non-stimulated cells (Ctr)).

3.2.3 Rac-1 activity and ROS production in response to thrombin are dependent on calcium

Since thrombin is known to influence calcium currents in smooth muscle cells, it was investigated whether Ca^{2+} could play a role in ROS generation and Rac-1 activation by thrombin. Therefore, PASMC were preincubated with the calcium chelator BAPTA-AM or the antioxidant NAC and treated with thrombin. Application of BAPTA-AM or NAC completely abrogated thrombin-stimulated ROS production (Fig. 26A). Moreover, thrombin-induced activation of Rac-1 was prevented by application of BAPTA-AM (Fig. 26B).

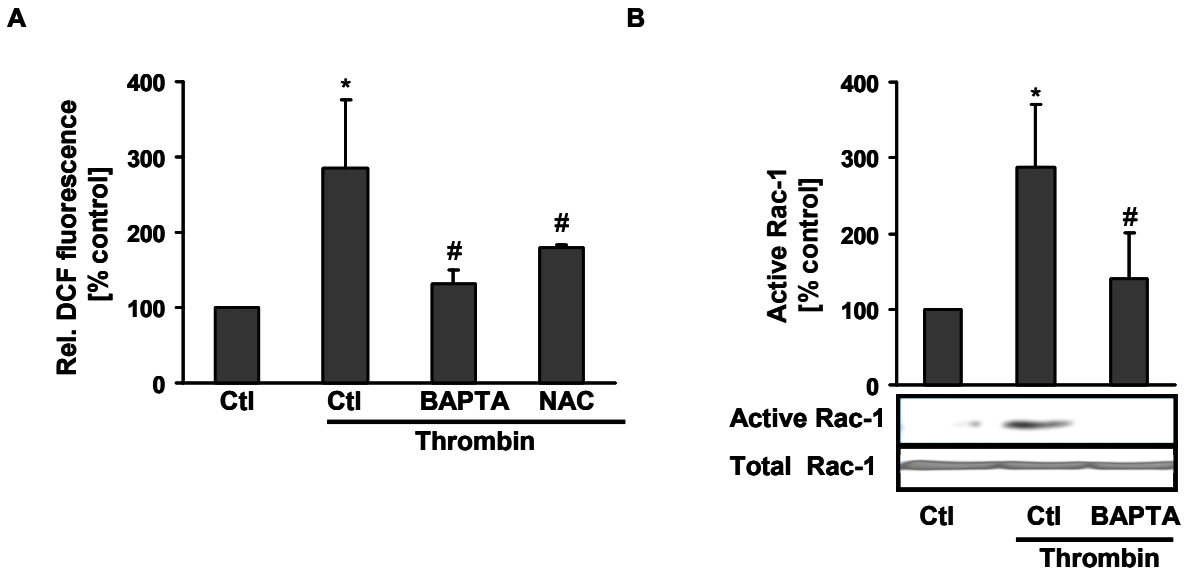


Figure 26. Thrombin-stimulated ROS production and Rac-1 activation are Ca^{2+} sensitive

PASMC were preincubated with the Ca^{2+} chelator BAPTA-AM (BAPTA, 7.5 µM) or the antioxidant NAC (10 mM) for 30 min and A) exposed to thrombin (3 U/ml) for 2 h and ROS production was assessed by DCF fluorescence. Data are presented as relative increase to control (100%) (n=3, *p<0.05 versus unstimulated cells (Ctr); #p<0.05 versus thrombin-stimulated cells (Ctr)) or B) exposed to thrombin for 15 sec, and levels of active Rac-1 were evaluated by performing Rac pull down assay. Data are presented as relative increase to control (100%) (n=3, *p<0.05 versus unstimulated cells (Ctr)).

3.2.4 Rac-1 modulates cell proliferation in response to thrombin

To elucidate the role of Rac-1 in cell proliferation, PASMC were transfected with expression vectors encoding constitutively active RacG12V or dominant-negative RacT17N or with control vector and were exposed to thrombin for 24 h. Thrombin stimulation resulted in a significant increase in proliferative activity of PASMC that was completely abrogated by the antioxidant NAC and the Ca^{2+} chelator BAPTA-AM (Fig. 27). In addition, proliferation of PASMC was further elevated by RacG12V and decreased by treatment with NAC (Fig 27). Overexpression of RacT17N completely prevented proliferation of PASMC in response to thrombin (Fig. 27).

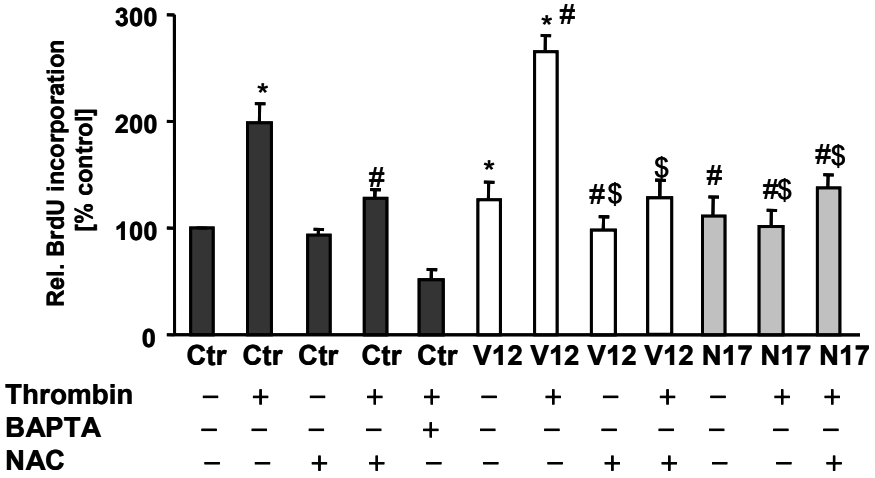


Figure 27. Rac-1 modulates cell proliferation by thrombin

PASMC were transfected with control vector (Ctr), the constitutively active RacG12V (V12) or the dominant-negative RacT17N (N17). On the following day cells were pretreated with the Ca^{2+} chelator BAPTA-AM (7.5 µM) or the antioxidant NAC (10 mM) for 30 min and thereafter stimulated with thrombin (3 U/ml) for 24 h. Proliferative activity was assessed by BrdU incorporation (n=3, *$p<0.05$ versus unstimulated control cells (Ctr), #$p<0.05$ versus thrombin-stimulated control cells (Ctr), \$$p<0.05$ versus V12 transfected cells stimulated with thrombin (V12)).

In summary, thrombin enhanced ROS production in PASMC via the activation and upregulation of Rac-1. Rac-1 activity as well as Rac-1 protein levels, ROS production and cell proliferation were dependent on calcium. Similar to the situation in endothelial cells (3.1), these findings support a model whereby activation of Rac-1 by thrombin could lead to a fast increase in ROS production, while upregulation of Rac-1 may be responsible for prolonged and sustained generation of ROS and proliferation of pulmonary artery smooth muscle cells as observed in vascular remodeling.

3.3 Rac-1 regulates redox-sensitive tissue factor expression by thrombin in pulmonary artery smooth muscle cells: Role of the NFκB pathway

Since thrombin levels are regulated by activation of TF and the coagulation cascade, and TF expression has been shown to be sensitive to ROS and thrombin, it was hypothesized that Rac-1 could play a role in regulation of TF expression in PASMC.

3.3.1 Thrombin enhances tissue factor expression and promoter activity

PASMC were exposed to thrombin for increasing time intervals and TF expression was determined by Northern and Western blot analysis. Thrombin transiently enhanced TF mRNA levels by 4-fold peaking after 2 to 4 h of incubation (Fig. 28A). Elevated TF mRNA levels were followed by enhanced TF protein levels which were continuously increasing to levels about 4-fold higher than in the respective controls after 16 h of exposure to thrombin (Fig. 28B).

To further confirm the transcriptional activation of TF by thrombin, cells were transfected with two human TF promoter luciferase constructs (Fig. 28C). The plasmid pTF111 contained the minimal promoter sequence (-111 to +121 bp) including a proximal enhancer with three overlapping Egr1 and Sp1 sites. The plasmid pTF636 contained the sequence from –636 to +121 bp and harbored in addition a distal enhancer containing a NFκB and two AP1 consensus sequences. Stimulation with thrombin increased luciferase activity of pTF636 by about 4-fold, whereas pTF111-dependent luciferase activity was only elevated by about 2-fold, suggesting that pTF636 contains thrombin-sensitive DNA binding consensus sequences which are not present in pTF111.

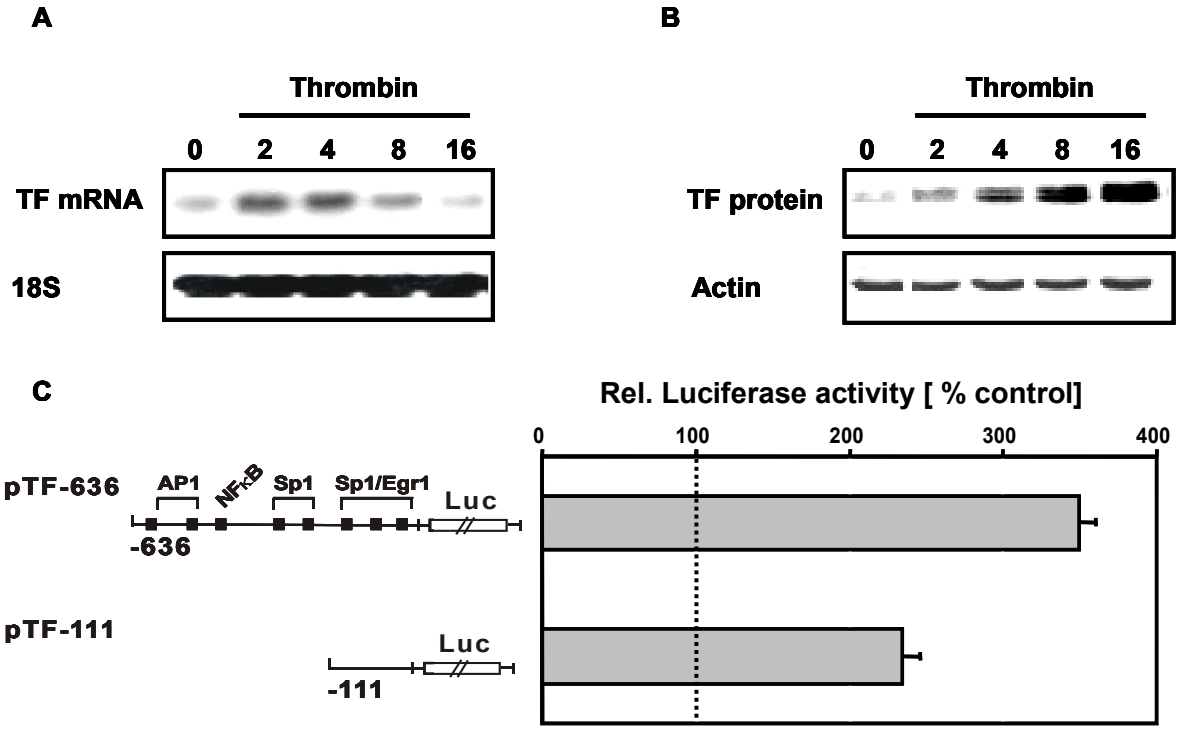


Figure 28. Thrombin stimulates tissue factor expression and promoter activity

PASMC were stimulated with thrombin (3 U/ml) for different time points. A) Tissue factor (TF) mRNA levels were evaluated by Northern blot analysis using a cDNA probe for human TF or for 18S to determine loading efficiency. These blots are representative for three independent experiments. B) TF protein levels were determined by Western blot analysis using an antibody against human TF or against actin. These blots are representative for three independent experiments. C) rSMC were transfected with the human TF promoter luciferase constructs pTF636 and pTF111. After 16 h of serum starvation, cells were stimulated for 8 h with thrombin (3 U/ml). In each experiment, the luciferase activity of the control vector measured under unstimulated conditions was set equal to 100% (represented by the dotted line). Values ± SD represent the % induction by thrombin of luciferase activity of three independent experiments (n=3, *$p<0.05$ versus control (unstimulated)).

3.3.2. Tissue factor expression in response to thrombin is redox-sensitive

To investigate whether ROS are involved in thrombin-stimulated TF expression, PASMC were pretreated with the antioxidant vitamin C or the non-specific NADPH oxidase inhibitor DPI and then stimulated with thrombin for 4 h. Production of ROS was evaluated by using DCF fluorescence. Moreover, TF protein expression was determined by Western blot analysis (Fig. 29). Pretreatment with the antioxidants decreased ROS levels and TF expression by thrombin, indicating that TF expression in response to thrombin is redox-sensitive.

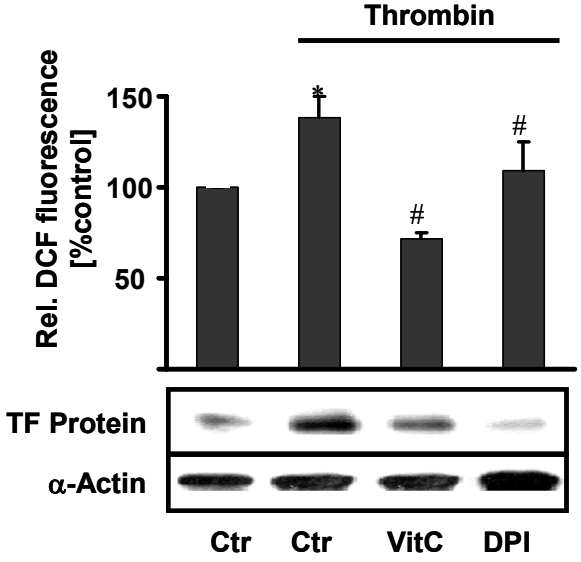


Figure 29. Thrombin-stimulated tissue factor expression is redox-sensitive

PASMC were preincubated with DPI (10 µM) or the antioxidant VitC (100 µM) for 30 min and then exposed to thrombin (3 U/ml) for 4 h. ROS production was assessed by DCF fluorescence. Data are presented as relative increase to control (100%) (n=3, *p<0.05 versus unstimulated cells (Ctr); #p<0.05 versus thrombin-stimulated cells (Ctr)). TF protein levels were determined by Western blot analysis using an antibody against human TF or against actin. These blots are representative for three independent experiments.

3.3.3 Rac-1 mutants modulate thrombin-induced tissue factor expression and procoagulant activity

Since thrombin-stimulated TF expression was redox-sensitive and decreased by the non-specific NADPH oxidase inhibitor DPI, the role of Rac-1 in this pathway was evaluated. Therefore, human PASMC were transfected with expression vectors encoding constitutively active RacG12V (V12) or dominant-negative RacT17N (N17) or with control vector (Ctr) and stimulated with thrombin for 4 h for Northern blot analysis and for 16 h for Western blot analysis. In control cells, stimulation with thrombin resulted in a 2.8-fold increase in TF mRNA levels (Fig. 30A). This response was further enhanced in RacG12V-expressing cells, but was significantly diminished in RacT17N-expressing PASMC. Similarly, TF protein levels were enhanced by 3-fold in control cells after thrombin stimulation, and were further elevated in RacG12V-expressing cells (Fig. 30B). By contrast, in RacT17N-expressing PASMC, TF protein levels were abrogated to levels similar to those from the unstimulated controls.

Since TF protein levels in smooth muscle cells do not necessarily reflect active TF, the role of Rac-1 in modulating surface procoagulant activity derived from PASMC under control conditions and in the presence of thrombin was evaluated using a chromogenic assay (Fig. 30C). PASMC were transfected with RacG12V or RacT17N expression vectors or with control vector and exposed to thrombin for 8 h. Thrombin treatment resulted in a significant increase in surface procoagulant activity. In the presence of RacG12V, procoagulant activity was enhanced under control conditions and further potentiated in the presence of thrombin. In contrast, transfection of RacT17N abrogated thrombin-stimulated surface procoagulant activity. Since addition of an inhibitory antibody against human TF inhibits basal as well as thrombin-stimulated procoagulant activity as was shown previously[71], these data suggest that Rac-1 is involved in regulating TF expression as well as TF activity in response to thrombin in PASMC.

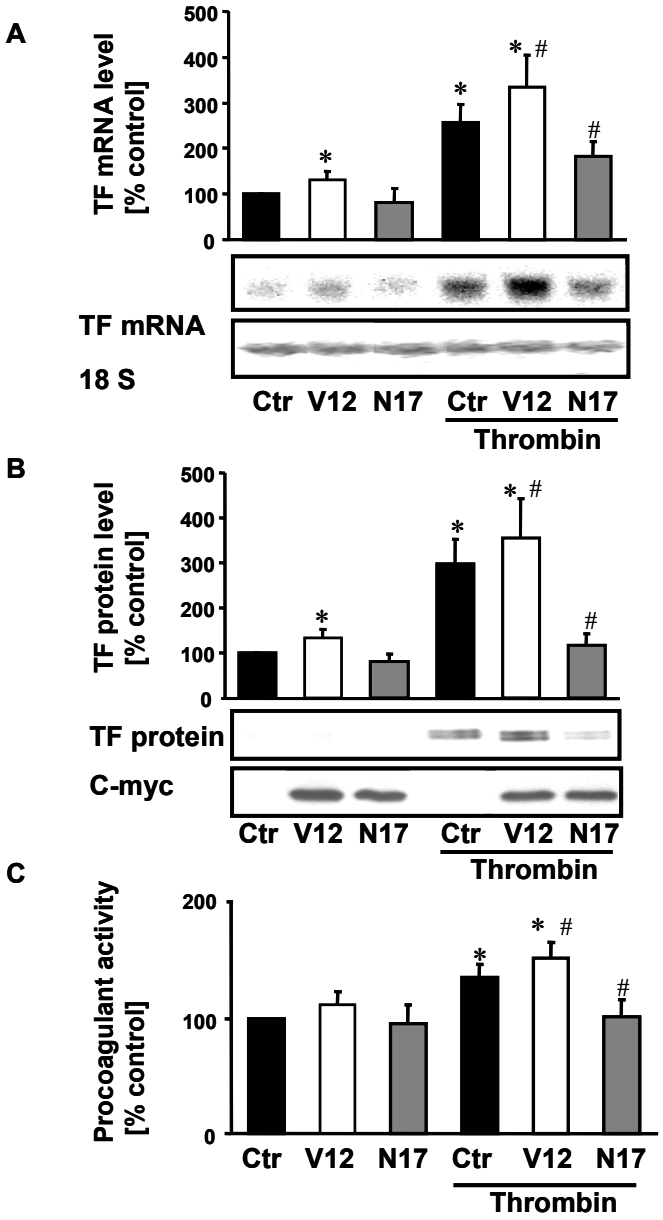


Figure 30. Rac-1 modulates thrombin-induced tissue factor expression and activity

PASMC were transfected with control vector (Ctr) or expression vectors for either the constitutively active RacG12V (V12) or the dominant-negative RacT17N (N17). A) Transfected PASMC were stimulated with thrombin (3 U/ml) for 4 h. Northern blot analyses were performed with a human tissue factor (TF) cDNA or 18S cDNA probe (n=3; *p<0.05 versus Ctr (unstimulated); #p<0.05 versus Ctr (thrombin-stimulated)). B) TF protein levels were determined in transfected PASMC stimulated for 16 h with thrombin (3 U/ml) by Western blot analysis using an antibody against human TF. The expression of the Rac-1 constructs was controlled by Western blot analysis with an antibody against the c-myc epitope (n=3; *p<0.05 versus Ctr (unstimulated); #p<0.05 versus Ctr (thrombin-stimulated)). C) Surface procoagulant activity was investigated in human PASMC transfected with control vector (Ctr) or expression vectors encoding RacG12V (V12) or RacT17N (N17) and exposed to thrombin (3 U/ml) for 8 h. The formation of thrombin was evaluated using a chromogenic assay (n=3; *p<0.05 versus Ctr (unstimulated); #p<0.05 versus Ctr (thrombin-stimulated)).

3.3.4 Rac-1 modulates tissue factor promoter activity via the nuclear factor kappa B pathway

To investigate the role of Rac-1 in thrombin-stimulated TF expression, reporter gene experiments using the two TF promoter constructs pTF111 and pTF636 were performed. Cotransfection of the RacG12V mutant with pTF636 resulted in about 2.8-fold increased luciferase activity compared to control cells, while in RacT17N-expressing cells pTF636-derived luciferase activity was abrogated (Fig. 31A). pTF111-mediated luciferase activity was only increased by 1.5-fold in RacG12V-expressing cells and was not significantly decreased in the presence of RacT17N, suggesting that pTF636 contains DNA sequences sensitive to Rac-1 which are not present in pTF111.

Since the pTF636, but not the pTF111 construct contains a consensus sequence for the redox-sensitive transcription factor NFκB, the role of this transcription factor in Rac-1-regulated human TF promoter activity was determined. In order to evaluate whether Rac-1 is able to modulate NFκB-mediated transcriptional activity, a luciferase construct containing 5 NFκB consensus sites in front of the SV40 promoter was cotransfected with the mutant Rac-1 vectors (Fig. 31B). In the presence of active RacG12V, luciferase activity was significantly enhanced by 1.8-fold while expression of dominant-negative RacT17N resulted in a significant decrease in reporter gene activity compared to control cells. Moreover, exposure to thrombin for 8 h resulted in about 7-fold increased NFκB-dependent luciferase activity (Fig. 31C). These findings demonstrate that thrombin and Rac-1 are able to activate NFκB transcriptional activity in smooth muscle cells.

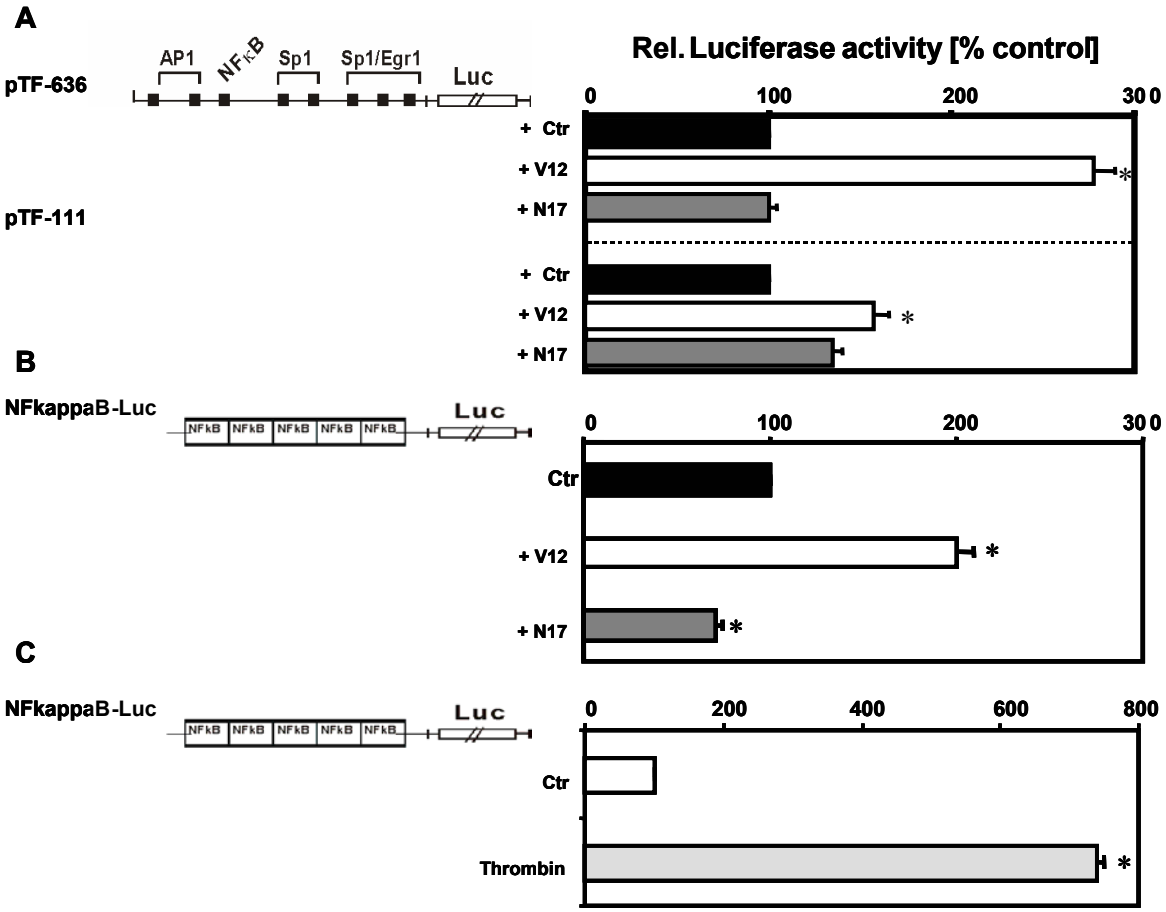


Figure 31. Rac-1 modulates tissue factor promoter activity and nuclear factor kappa B mediated transcriptional activity

A) rSMC were cotransfected with expression vectors encoding constitutively active RacG12V (V12) or dominant-negative RacT17N (N17) or control vector (Ctr) and the human TF promoter luciferase constructs pTF636 or pTF111. Luciferase activity was measured 24 h after transfection. In each experiment the luciferase activity determined in the presence of control vector was set equal to 100%. Values ± SD represent the % induction of luciferase activity of three independent experiments (n=3; *p<0.05 versus Ctr). B) rSMC were cotransfected with expression vectors encoding constitutively active RacG12V (V12) or dominant-negative RacT17N (N17) or control vector (Ctr) and a luciferase construct containing five nuclear factor-κB (NFκB) elements (NFkappaB-Luc) in front of the SV40 promoter. Luciferase activity was measured 24 h after transfection. In each experiment the luciferase activity measured in the presence of control vector was set equal to 100%. Values ± SD represent the % induction of luciferase activity of three independent experiments. (n=3; *p<0.05 versus Ctr). C) rSMC were transfected with the NFkappaB-Luc reporter gene. Cells were then serum-deprived for 16 h and stimulated with thrombin for 8 h. In each experiment the luciferase activity measured under unstimulated conditions was set equal to 100%. Values ± SD represent the % induction of luciferase activity of three independent experiments. (n=3; *p<0.05 versus Ctr).

To evaluate whether NFκB contributes to TF promoter activity, cells were transfected with expression vectors encoding for the NFκB subunits p50 and p65 together with the TF promoter constructs pTF636 or pTF111, respectively. Coexpression of p50/p65 and pTF636 resulted in about 3.5-fold increased luciferase activity (Fig. 32A). Transfection of p50/p65, however, did not significantly influence reporter gene activity of the minimal TF promoter construct pTF111.

To evaluate whether Rac-1 acts on the TF promoter via the NFκB pathway, cells were transfected with the RacG12V expression vector and vectors encoding for either dominant-negative mutant of inhibitory κB protein (IκBdn) or inhibitory κB protein kinase (IκKdn) as well as with pTF636 or pTF111 (Fig. 32B). RacG12V-stimulated luciferase activity of pTF636 was completely abolished in the presence of dominant-negative IκB or IκBK. In contrast, pTF111-dependent luciferase activity in RacG12V-expressing cells was not significantly reduced by dominant-negative IκB or IκBK. These findings indicate that Rac-1 activates NFκB leading to TF upregulation by thrombin.

In summary, thrombin was able to increase redox-sensitive TF expression and activity in PASMC. Upregulation of TF was modulated by Rac-1-dependent ROS generation. Finally, Rac-1 stimulated TF expression involved activation of the NFκB pathway.

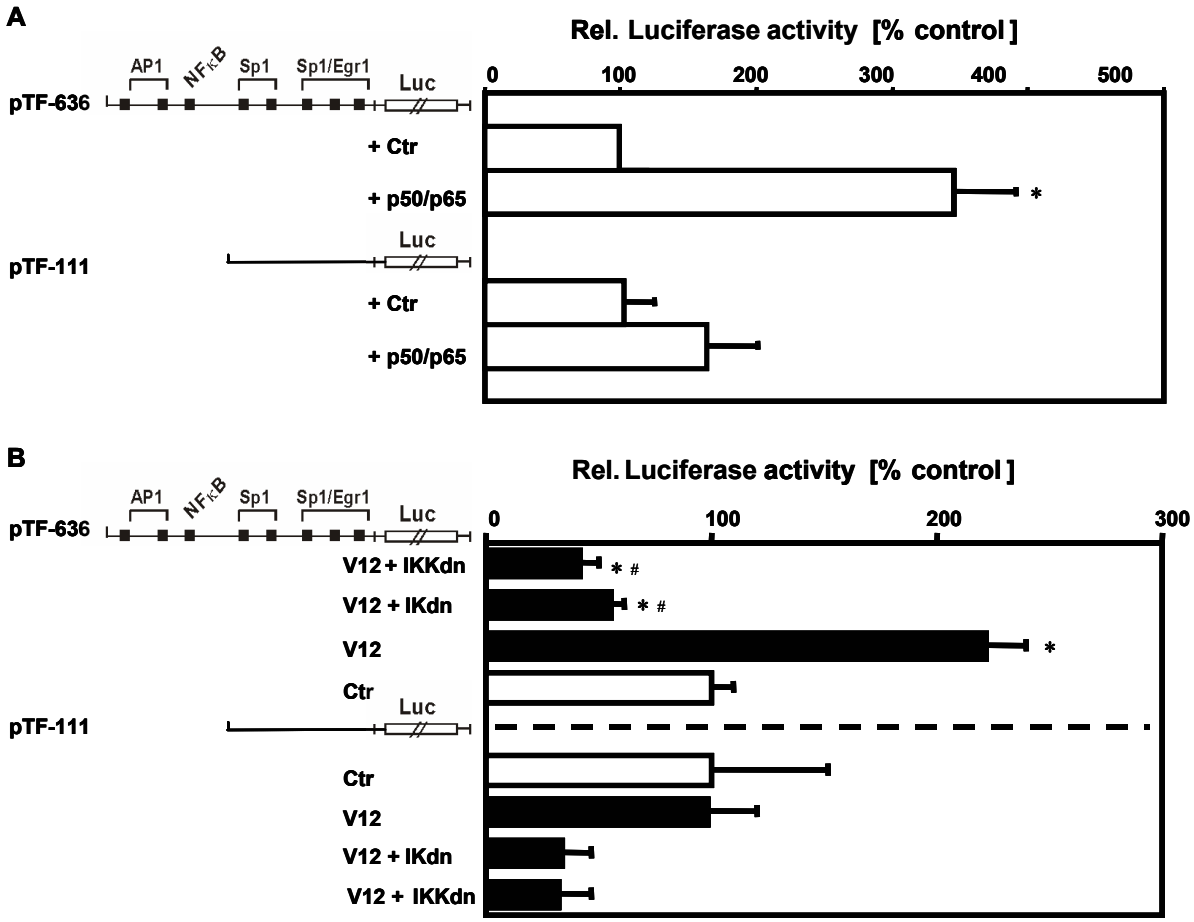


Figure 32. The nuclear factor kappa B pathway is involved in Rac-1-dependent tissue factor promoter activity

A) rSMC were cotransfected with expression vectors encoding the NFκB subunits p50 and p65 or with control vector (Ctr) and the human TF promoter luciferase constructs pTF636 or pTF111. Luciferase activity was measured 24 h after transfection (n=3; *p<0.05 versus Ctr). B) rSMC were cotransfected with the RacG12V expression vector or control vector (pcDNA3) and the pTF636 or pTF111 human TF promoter luciferase constructs and expression vectors encoding for dominant-negative IκB (IKdn) or IκBK (IKKdn). Luciferase activity was measured 24 h after transfection (n=3; *p<0.05 versus Ctr (pcDNA3); #p<0.05 versus RacG12V transfected cells).

3.4 Rac-1 regulates redox-sensitive PAI-1 expression by thrombin in pulmonary artery smooth muscle cells: Role of hypoxia-inducible factor-1

PAI-1 has been associated with tissue remodeling processes in vascular diseases characterized with a prothrombotic state. However, the link between enhanced levels of thrombin and PAI-1 expression has not been determined. Since thrombin enhanced ROS production and Rac-1 activity in PASMC (3.2), it was investigated whether Rac-1 could play a role in the regulation of PAI-1 in PASMC.

3.4.1 Thrombin enhances PAI-1 mRNA and protein levels

PASMC were exposed to thrombin for increasing time intervals and PAI-1 expression was determined by Northern and Western blot analysis. Thrombin significantly enhanced PAI-1 mRNA levels after 4 and 8 h of stimulation (Fig. 33A). Elevated PAI-1 mRNA expression was followed by increased PAI-1 protein levels to about 3-fold higher than in the respective control (Fig. 33B).

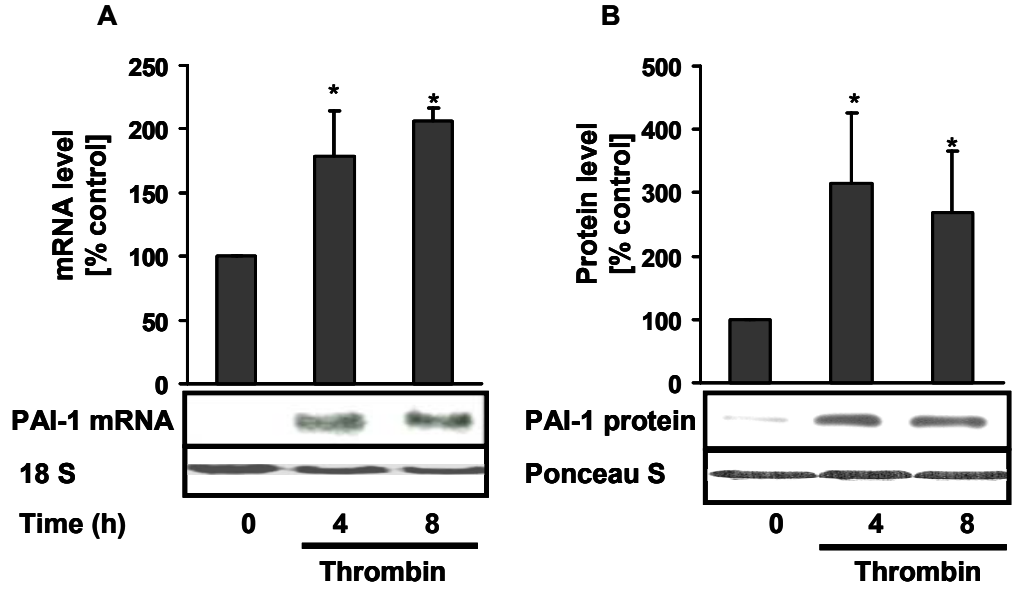


Figure 33. Thrombin stimulates the expression of PAI-l

A) PASMC were stimulated with thrombin (3 U/ml) for 4 and 8 h and PAI-1 mRNA was evaluated by Northern blot analysis. B) PAI-1 protein levels from culture supernatants were determined by Western blot analysis. (n=4, *p<0.05 versus non-stimulated cells (Ctr)).

3.4.2 Thrombin induces PAI-1 by activation of HIF-1

PAI-1 expression has been shown to be dependent on the activation of the transcription factor HIF-1 under hypoxia[134]. To evaluate if thrombin is increasing HIF-1α protein levels, PASMC were stimulated with 3 U/ml thrombin for different time points and Western blot analyses using an antibody against HIF-1α were performed. Thrombin significantly enhanced HIF-1α protein, staying continuously elevated up to 24 h of stimulation (Fig. 34A). To determine the role of HIF-1 in thrombin-stimulated PAI-1 expression, rSMC were transfected with a promoter construct containing the human PAI-1 promoter or a similar construct where the HIF-1 binding site was mutated. Thrombin significantly increased PAI-1 promoter activity (Fig. 34B). However, thrombin-stimulated luciferase activity was completely abrogated after transfection of the mutated plasmid which is unable to bind HIF-1 (Fig.34B), confirming the central role of HIF-1 in PAI-1 expression by thrombin.

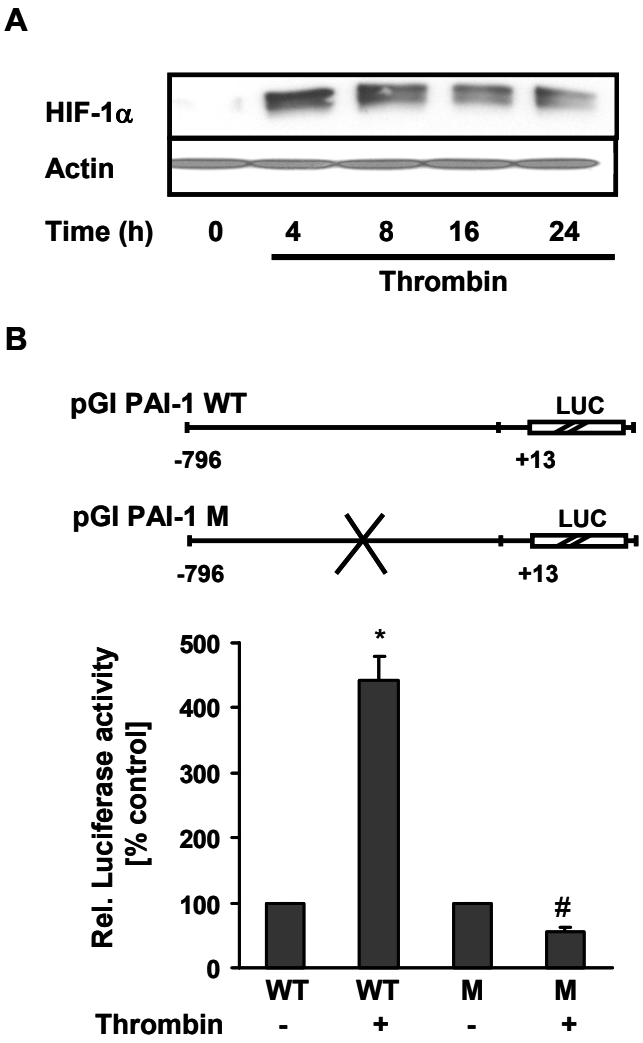


Figure 34. HIF-1 mediates thrombin-induced PAI-1 promoter activity

A) PASMC were stimulated with thrombin for the indicated time points and HIF-1α protein levels were evaluated by Western blot analysis. A representative blot is shown (n=5). B) rSMC were transfected with the human PAI-1 promoter luciferase construct (WT) or a mutated PAI-1 construct (M) and stimulated with thrombin for 8 h. Luciferase activity was measured 24 h after transfection. In each experiment the luciferase activity determined in the presence of control or mutated vector under unstimulated conditions was set equal to 100%. Values ± SD represent the % induction of luciferase activity of three independent experiments (n=3; *p<0.05 versus Ctr (unstimulated WT); #p<0.05 versus Ctr (unstimulated M)).

3.4.3 PAI-1 and HIF-1α expression are redox- and calcium-sensitive

Since thrombin induced ROS production dependent on Ca^{2+}, it was determined whether ROS and Ca^{2+} could play a role in PAI-1 regulation. PAI-1-derived luciferase activity and protein levels induced by thrombin were prevented in PASMC treated with the calcium chelator BAPTA-AM or the antioxidant NAC (Fig. 35A). In addition, thrombin-stimulated HIF-1-dependent luciferase activity and HIF-1α protein levels were significantly decreased after pretreatment with BAPTA-AM and NAC (Fig. 35B), further supporting an important role of Ca^{2+} and ROS in this pathway.

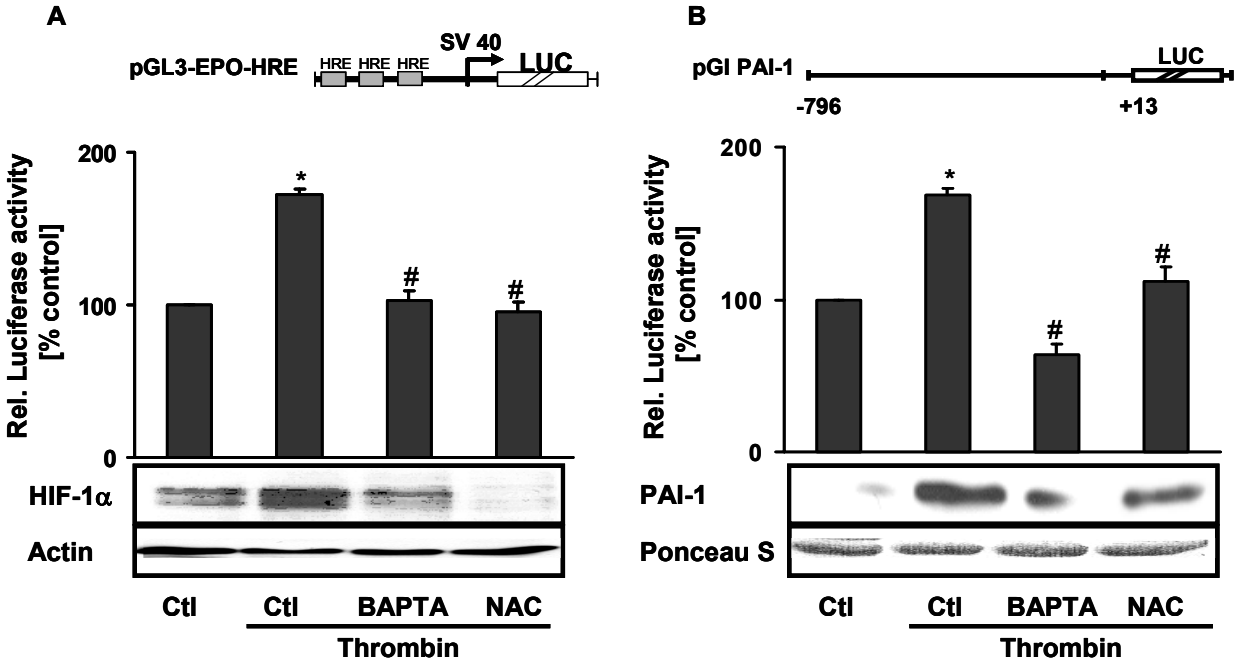


Figure 35. Thrombin-stimulated HIF-1α and PAI-1 expression are redox-sensitive

A) PASMC were preincubated with BAPTA-AM (BAPTA, 7.5 μM) or the antioxidant NAC (10 mM) for 30 min and then exposed to 3 U/ml thrombin for 4 h. HIF-1α protein levels were evaluated by performing Western blot analyses. In addition, rSMC were transfected with the EPO-HRE luciferase construct. Luciferase activity was measured after preincubation with the Ca^{2+} chelator BAPTA-AM (7.5 μM) or the antioxidant NAC (10 mM) for 30 min and stimulation with thrombin for 8 h. B) PASMC were preincubated with BAPTA-AM (BAPTA, 7.5 μM) or the antioxidant NAC (10 mM) for 30 min and then exposed to 3 U/ml thrombin for 4 h. Western blot analyses were performed to determine PAI-1 protein levels. Additionally, rSMC were transfected with the human PAI-1 promoter luciferase construct. Luciferase activity was measured after preincubation with the Ca^{2+} chelator BAPTA-AM (7.5 μM) or the antioxidant NAC (10 mM) for 30 min and stimulation with thrombin for 8 h. In each experiment the luciferase activity determined in the presence of control vector was set equal to 100% (n=3; *p<0.05 versus Ctr (unstimulated); #p<0.05 versus Ctr (thrombin-stimulated)).

3.4.4 Rac-1 modulates thrombin-induced PAI-1 promoter activity and PAI-1 expression

To evaluate whether Rac-1 is involved in PAI-1 expression by thrombin, rSMC were transfected with the human PAI-1 promoter construct and cotransfected with expression vectors encoding constitutively active RacG12V or dominant-negative RacT17N or with control vector and stimulated with thrombin for 8 h. Thrombin significantly increased PAI-1 promoter activity (Fig. 36). Moreover, cotransfection of the RacG12V mutant resulted in about 3.5-fold increased PAI-1 promoter activity compared to control cells, while in RacT17N-expressing cells PAI-1 promoter activity was abrogated.

To evaluate whether Rac-1 mutants modulate PAI-1 mRNA and protein expression after stimulation with thrombin in human PASMC, Northern blot and Western blot analyses were performed. PAI-1 mRNA and protein levels were enhanced by 3-fold in control cells after thrombin stimulation, and were further elevated in RacG12V-expressing cells (Fig. 36). By contrast, in RacT17N-expressing PASMC, PAI-1 mRNA and protein levels were abrogated (Fig.36), further suggesting that Rac-1 is involved in regulating PAI-1 promoter activity and expression in response to thrombin in PASMC.

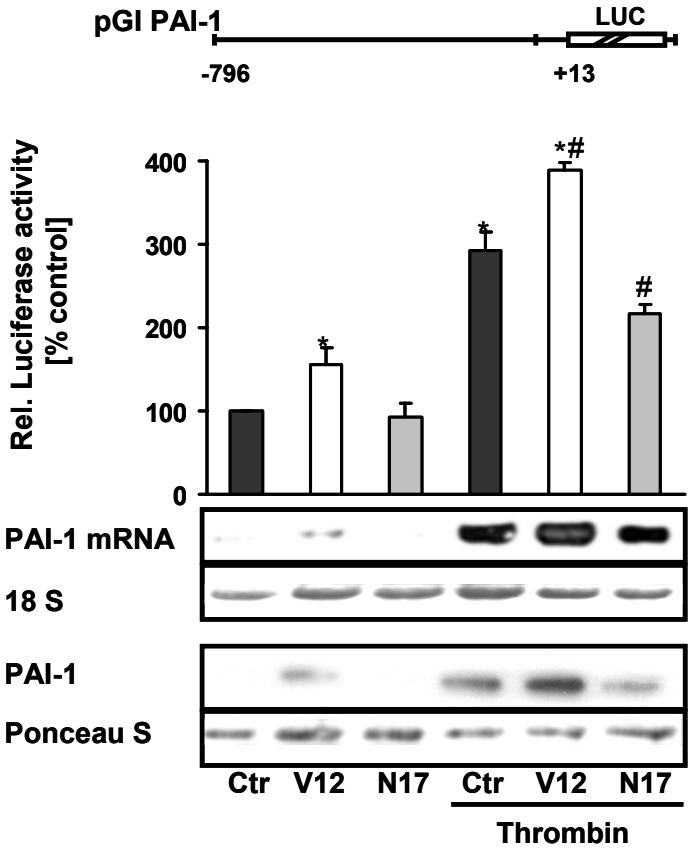


Figure 36. Rac-1 modulates thrombin-induced PAI-1 expression and promoter activity

rSMC were transfected with control vector (Ctr) or expression vectors for either the constitutively active RacG12V (V12) or the dominant-negative RacT17N (N17) and the human PAI-1 promoter luciferase construct. Luciferase activity was measured 24 h after transfection. In each experiment luciferase activity determined in the presence of control vector was set equal to 100%. Values ± SD represent the % induction of luciferase activity of three independent experiments (n=3; *p<0.05 versus Ctr (unstimulated); #p<0.05 versus Ctr (thrombin-stimulated)). PASMC were transfected with control vector (Ctr) or expression vectors for either the constitutively active RacG12V (V12) or the dominant-negative RacT17N (N17). Transfected PASMC were stimulated with thrombin (3 U/ml) for 4 h and mRNA levels were evaluated by performing Northern blot analyses. PAI-1 protein levels were determined in transfected PASMC stimulated for 4 h with thrombin (3 U/ml) by Western blot analysis using an antibody against human PAI-1. Representative blots are shown.

3.4.5 Rac-1 modulates HIF-1α protein levels and HIF-1 activity

To evaluate the role of Rac-1 in thrombin-stimulated HIF-1 activity, the constitutively active RacG12V mutant or the dominant-negative RacT17N were cotransfected with the pGl3-EPO-HRE luciferase construct containing 3 HIF-1-binding sites (Hypoxia Responsive Elements (HRE)) in front of the SV40 promoter. Luciferase activity was 2-fold increased in RacG12V-expressing cells, but remained unchanged in RacT17N-expressing cells (Fig. 37). In thrombin-stimulated cells, RacG12V further enhanced HIF-1-dependent luciferase activity, whereas this response was abrogated in the presence of RacT17N. Similarly, HIF-1α protein levels were increased in cells expressing RacG12V and were further enhanced after thrombin stimulation. However, transfection of RacT17N abrogated HIF-1α expression in response to thrombin (Fig. 37).

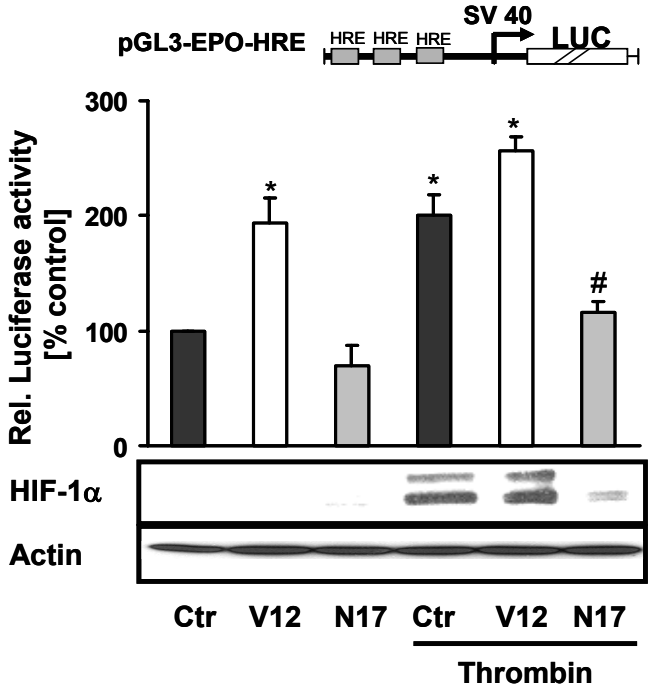


Figure 37. Rac-1 modulates HIF-1 activity and HIF-1α protein levels

rSMC were transfected with control vector (Ctr) or expression vectors for either the constitutively active RacG12V (V12) or the dominant-negative RacT17N (N17) and the human EPO-HRE promoter luciferase construct (pglEPO-HRE). Luciferase activity was measured 24 h after transfection and after stimulation with thrombin for 8 h. In each experiment the luciferase activity determined in the presence of control vector was set equal to 100%. Values ± SD represent the % induction of luciferase activity of three independent experiments (n=3; *p<0.05 versus Ctr (unstimulated); #p<0.05 versus Ctr (thrombin-stimulated)).

PASMC were transfected with control vector (Ctr) or expression vectors for either the constitutively active RacG12V (V12) or the dominant-negative RacT17N (N17). Transfected PASMC were stimulated with thrombin (3 U/ml) for 4 h and HIF-1α protein levels were determined by Western blot analysis using an antibody against human HIF-1α. Representative blots are shown.

3.4.6 Rac-1 activates HIF-1 and increases PAI-1 promoter activity via the activation of PAK in response to thrombin

Since p-21-activated kinase (PAK) is a known downstream effector of Rac-1, it was investigated whether PAK is involved in thrombin-induced PAI-1 expression. To this end, PASMC were exposed to thrombin for different time points and phosphorylation of PAK was determined by Western blot analysis. Thrombin stimulation resulted in rapid PAK phosphorylation peaking at 1 min (Fig. 38). Then, to investigate the effect of Rac-1 on PAK activation by thrombin, PASMC were transfected with RacG12V or RacT17N and exposed to 3 U/ml thrombin for 1 min. In the presence of RacG12V, basal and thrombin-induced phosphorylation of PAK was increased, but was abolished in the presence of RacT17N (Fig. 38).

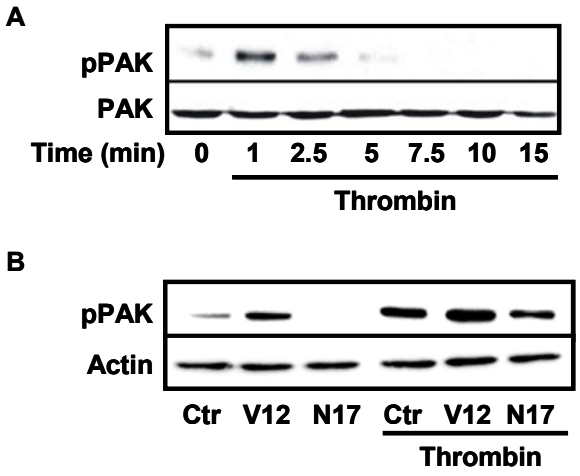


Figure 38. PAK activation by thrombin depends on Rac-1

A) PASMC were stimulated with thrombin (3 U/ml) for the time indicated and levels of pPAK were evaluated by performing Western blot analysis. B) PASMC were transfected with control vector (Ctr) or expression vectors for either the constitutively active RacG12V (V12) or the dominant-negative RacT17N (N17) and stimulated with 3 U/ml thrombin for 1 min. p-PAK protein levels were evaluated by performing Western blot analysis. Representative blots are shown (n=3).

To further evaluate the role of PAK in the regulation of HIF-1, an active PAK mutant (PAK-T423) or a dominant-negative mutant of PAK (PAK-R295), which increased or decreased PAK phosphorylation, respectively, were expressed with the luciferase construct pGl3-EPO-HRE. PAK-T423 increased HIF-1-dependent luciferase activity (Fig. 39A). Moreover, PAK-T423 also enhanced PAI-1 promoter activity whereas PAK-R295 diminished thrombin-induced PAI-1 promoter activity (Fig. 39B).

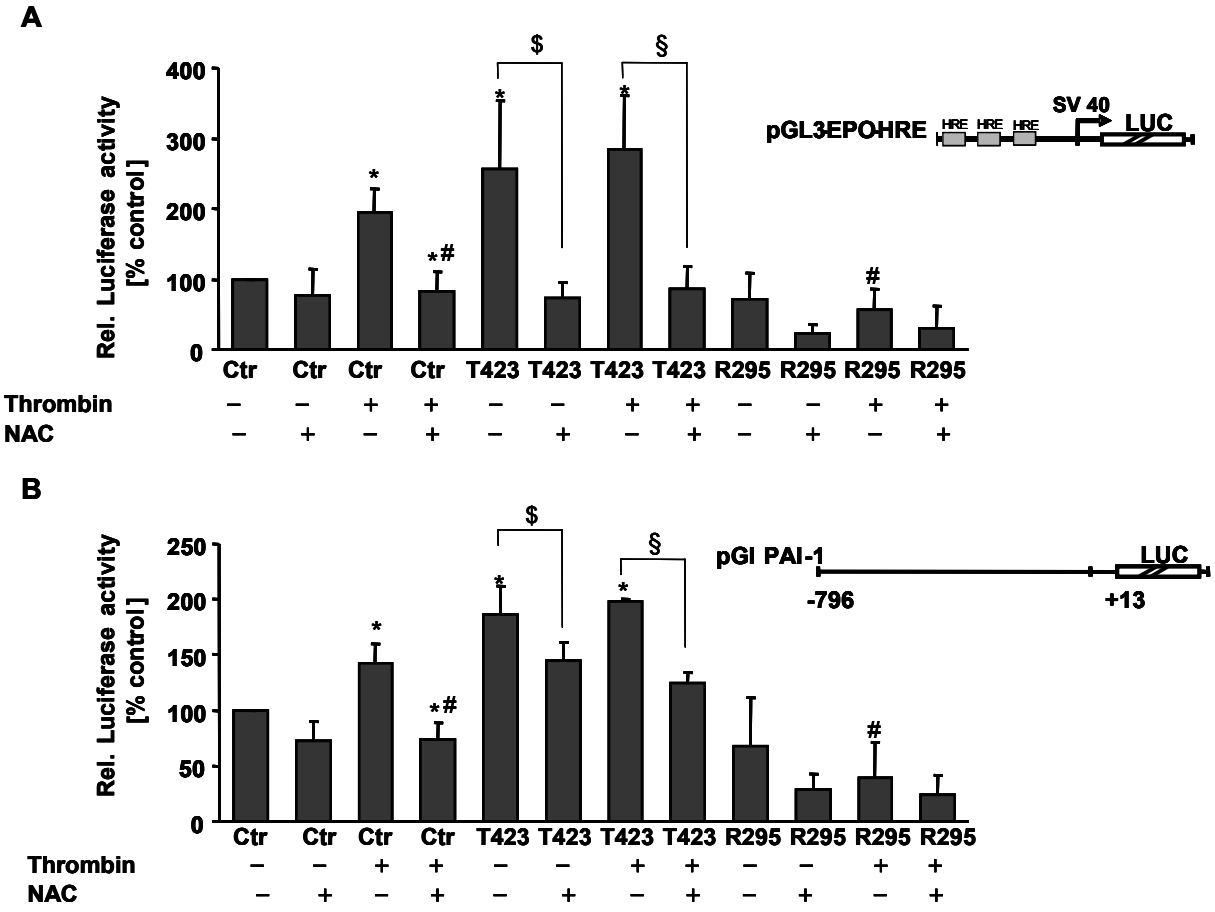


Figure 39. PAK induces HIF-1 activity and PAI-1 promoter activity in a redox-sensitive manner in response to thrombin

rSMC were transfected with expression vectors for either the active PAK-T423 (T432) or the dominant-negative PAK-R295 (R295) and A) the human EPO-HRE or B) PAI-1 promoter luciferase construct. Luciferase activity was measured 24 h after transfection and after preincubation with NAC (10 mM) for 30 min and stimulation with thrombin for 8 h. In each experiment the luciferase activity determined in the presence of control vector was set equal to 100%. Values ± SD represent the % induction of luciferase activity of three independent experiments (n=3; *p<0.05 versus Ctr (unstimulated); #p<0.05 versus Ctr (thrombin-stimulated)).

Application of the antioxidant NAC significantly decreased PAK-T423-induced HIF-1 activity and PAI-1 promoter activity (Fig. 39), indicating that PAK-mediated responses are redox-sensitive and that PAK could be upstream of ROS production. To test this hypothesis, the effect of PAK mutants on ROS production was evaluated. Active PAK significantly increased ROS production under control conditions as well as after thrombin stimulation (Fig. 40), whereas the dominant-negative mutant prevented thrombin-stimulated ROS generation.

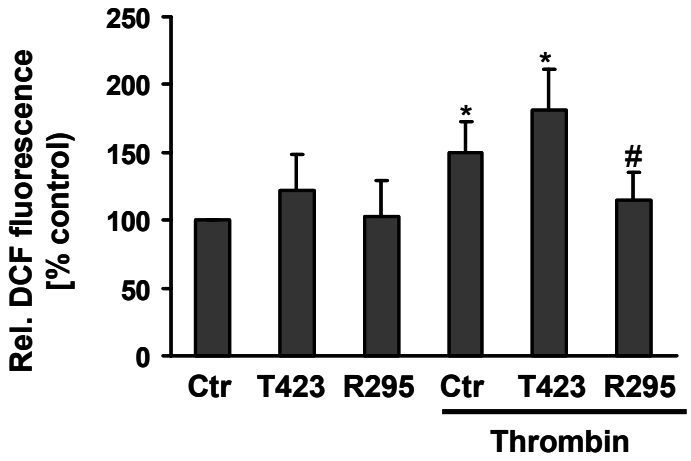


Figure 40. PAK modulates thrombin-stimulated ROS production

PASMC were transfected with expression vectors for either active PAK-T423 (T432) or the dominant-negative PAK-R295 (R295) and stimulated with thrombin for 4 h. ROS production was assessed by performing DCF measurements. Data are presented as relative increase compared to control (100%) (n=3, *p<0.05 versus non-stimulated cells (Ctr), #p<0.05 versus Ctr (thrombin-stimulated), $p<0.05 versus thrombin-stimulated PAK-T423-transfected cells)).

3.4.7 PAI-1 regulates cell proliferation in response to thrombin

Finally, to determine the role of secreted PAI-1 in regulating proliferation of PASMC, cells were stimulated with thrombin for 8 h. The conditioned cell culture medium was then transferred to another set of serum-starved PASMC. In order to deplete PAI-1, a PAI-1 inhibitory antibody or control mouse IgG were added and PASMC were incubated for 16 h. The conditioned medium from thrombin-stimulated PASMC increased the proliferation of PASMC, whereas in the presence of the inhibitory antibody against PAI-1 this response was decreased (Fig. 41), confirming the important role for PAI-1 in proliferation of PASMC.

In summary, Rac-1 modulates ROS-dependent PAI-1 expression in PASMC in response to thrombin by the activation of its substrate PAK and HIF-1 in a Ca^{2+}-dependent manner. Moreover, PAI-1 secretion promoted PASMC proliferation in response to thrombin.

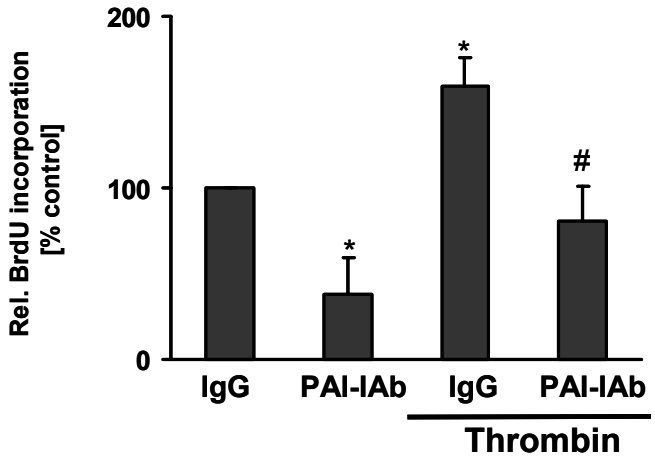


Figure 41. PAI-1 modulates cell proliferation by thrombin

PASMC were stimulated with thrombin (3 U/ml) for 8 h. The conditioned medium was transferred to another set of serum-starved PASMC. An antibody directed against PAI (PAI-Iab) or mouse IgG as a control were added to the supernatant. Cells were further incubated for 16 h in the presence of BrdU (10 µM). Proliferative activity was assessed by evaluating BrdU incorporation. Stimulated proliferative activity in control cells was set equal to 100% (n=3; *p<0.05 versus Ctr (unstimulated IgG treated); #p<0.05 versus Ctr (thrombin-stimulated IgG treated))

3.5 p22phox and NOX4 regulate redox-sensitive PAI-1 expression and proliferation of pulmonary artery smooth muscle cells in response to the vasoactive peptide urotensin-II

Urotensin-II (hU-II) has been suggested to play a role in the pathogenesis of several cardiovascular diseases, including pulmonary hypertension[135]. However, the signaling mechanisms linking hU-II to vascular remodeling are unclear. Since enhanced levels of ROS and PAI-1 expression may play a role in promoting vascular remodeling, the role of hU-II in the formation of ROS by NADPH oxidases, in the regulation of PAI-1 expression and in the control of proliferation of PASMC was investigated.

3.5.1 Urotensin-II elevates ROS

To investigate whether the vasoactive peptide hU-II can increase ROS levels, PASMC were exposed to 100 nM hU-II for 2 h and stained with dihydroethidium (DHE). A strong increase in fluorescence in stimulated PASMC compared to controls indicated increased ROS levels (Fig. 42A). Alternatively, PASMC were treated with hU-II (100 or 200 nM) for 2 h and ROS levels were measured by DCF fluorescence in a flow cytometer. Significantly increased levels of ROS compared to control cells were observed after challenging with both concentrations (Fig. 42B). Pretreatment with the flavin inhibitor diphenyleneiodonium (DPI, 10 nM) for 30 min significantly decreased hU-II-induced ROS levels, as was measured by DCF fluorescence in a microplate reader (Fig. 42C).

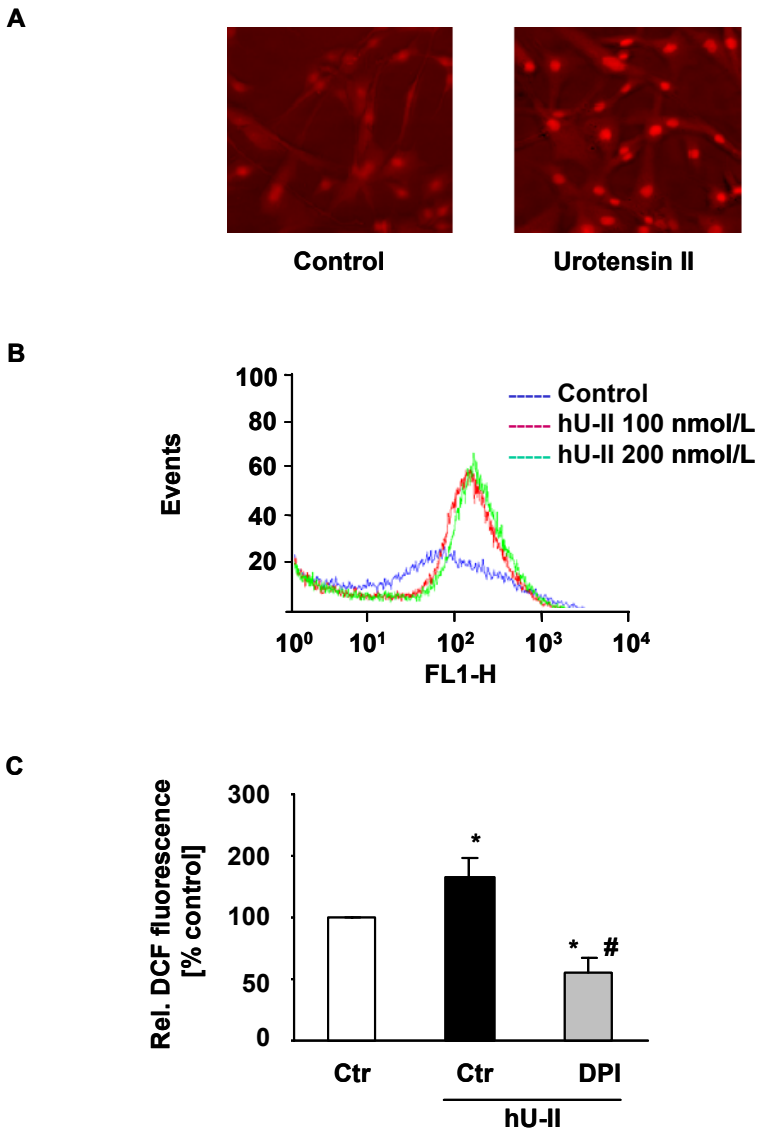


Figure 42. hU-II increases levels of ROS

A) ROS levels were visualized in PASMC treated with 100 nM hU-II for 2 h by dihydroethidium staining (n=3). B) PASMC were stimulated with 100 and 200 nM hU-II for 2 h. ROS levels were assessed by DCF fluorescence using flow cytometry (n=3). C) PASMC cells were exposed to 10 µM diphenyleneiodonium (DPI) for 30 min prior to hU-II stimulation for 2 h. ROS levels were evaluated by DCF fluorescence in a microplate reader. Data are presented as relative increase compared to control (100%) (n=3, *p<0.05 versus unstimulated cells (Ctr), #p<0.05 versus hU-II-stimulated cells (Ctr)).

3.5.2 p22phox and NOX4 contribute to urotensin-II-induced ROS levels

Since DPI is known to (unspecifically) inhibit NADPH oxidases, we evaluated the role of p22phox and NOX4 in ROS generation in response to hU-II. PASMC were transfected with sense or antisense vectors for p22phox or NOX4 and exposed to hU-II for 2 h. Depletion of p22phox or NOX4 abolished the hU-II-stimulated increase in ROS, whereas ROS levels were slightly enhanced in p22phox- and NOX4-overexpressing PASMC (Fig. 43A). Transfection of p22phox or NOX4 antisense constructs prevented the increase of both subunits in response to hU-II (Fig. 43A, B).

To further investigate the mechanisms underlying p22phox- and NOX4-dependent ROS production by hU-II, PASMC were stimulated with 100 nM hU-II for increasing time periods (from 1 to 16 h) and the protein levels of the NADPH oxidase subunits p22phox and NOX4 were determined by Western blot analysis (Fig. 44). Application of hU-II resulted in strong upregulation of p22phox and NOX4 protein levels peaking at 2 h after stimulation.

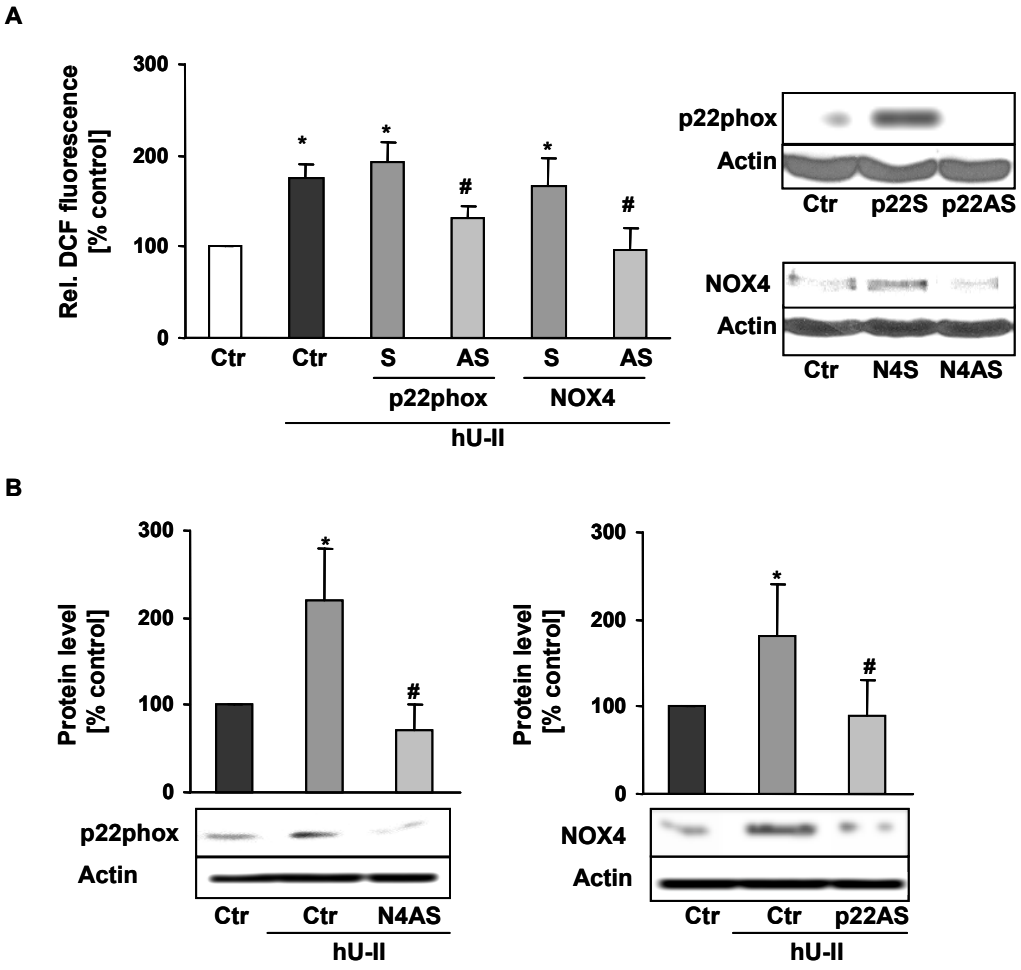


Figure 43. hU-II increases ROS production dependent on p22phox and NOX4

A) PASMC were transfected with control vector (Ctr), p22phox or NOX4 sense (S) or antisense (AS) vectors. Transfection efficiency was controlled by Western blot using antibodies against p22phox and NOX4. After treatment with hU-II (100 nM) for 2 h, ROS levels were assessed by DCF fluorescence. (n=3, *p<0.05 versus unstimulated controls (Ctr), #p<0.05 versus hU-II stimulated cells (Ctr)). B) PASMC were transfected with control vector (Ctr), p22phox antisense (p22AS) or NOX4 antisense (N4AS) constructs and exposed to hU-II (100 nM) for 2 h. Western blot analysis was performed using antibodies against p22phox and NOX4 (n=4, *p<0.05 versus unstimulated cells (Ctr)). All data are presented as relative increase compared to control (100%).

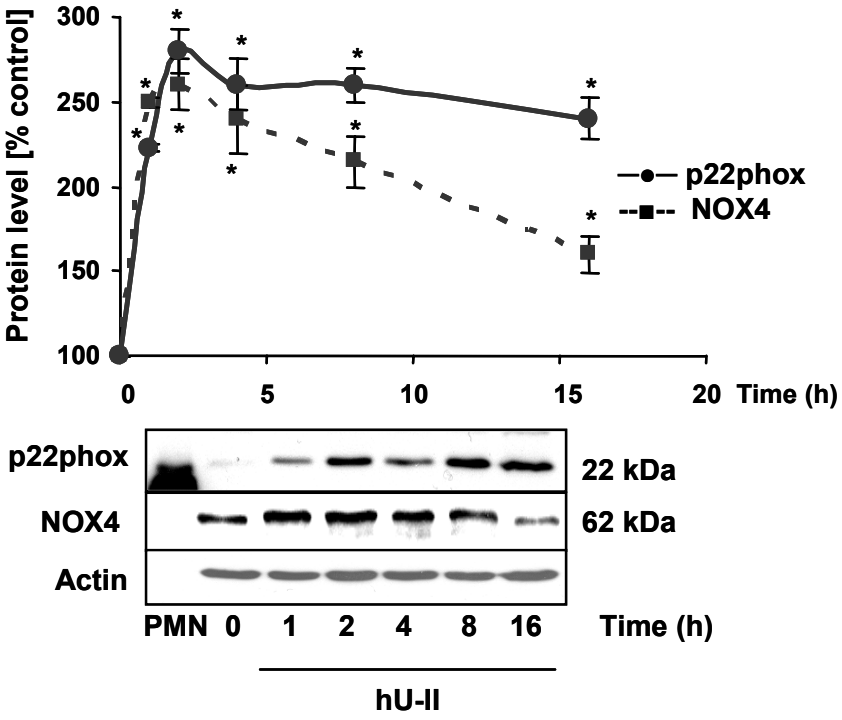


Figure 44. hU-II increases p22phox and NOX4 protein levels

PASMC were stimulated with 100 nM hU-II for increasing time points and Western blot analysis was performed. Lysates of polymorphonuclear leukocytes (PMN) were used as control (n=4, *p<0.05 versus unstimulated cells (Ctr)). Western blot analysis was performed using antibodies against p22phox and NOX4 (n=4, *p<0.05 versus unstimulated cells (Ctr)). All data are presented as relative increase compared to control (100%).

3.5.3 Urotensin-II increases protein kinase activity

To investigate the signaling pathways activated by hU-II, PASMC were treated with hU-II (100 nM) for increasing time periods. Phosphorylation of ERK, p38 MAP kinase (p38MAPK), c-Jun N-terminal kinase (JNK) and protein kinase B (Akt) was determined by Western blot analysis. hU-II increased the phosphorylation of all kinases peaking at 5 min (Fig. 45). Phosphorylation of all kinases was observed with 10 nM and stayed elevated up to 100 or 500 nM of hU-II, while higher concentrations of this peptide decreased kinase phosphorylation (Fig. 46).

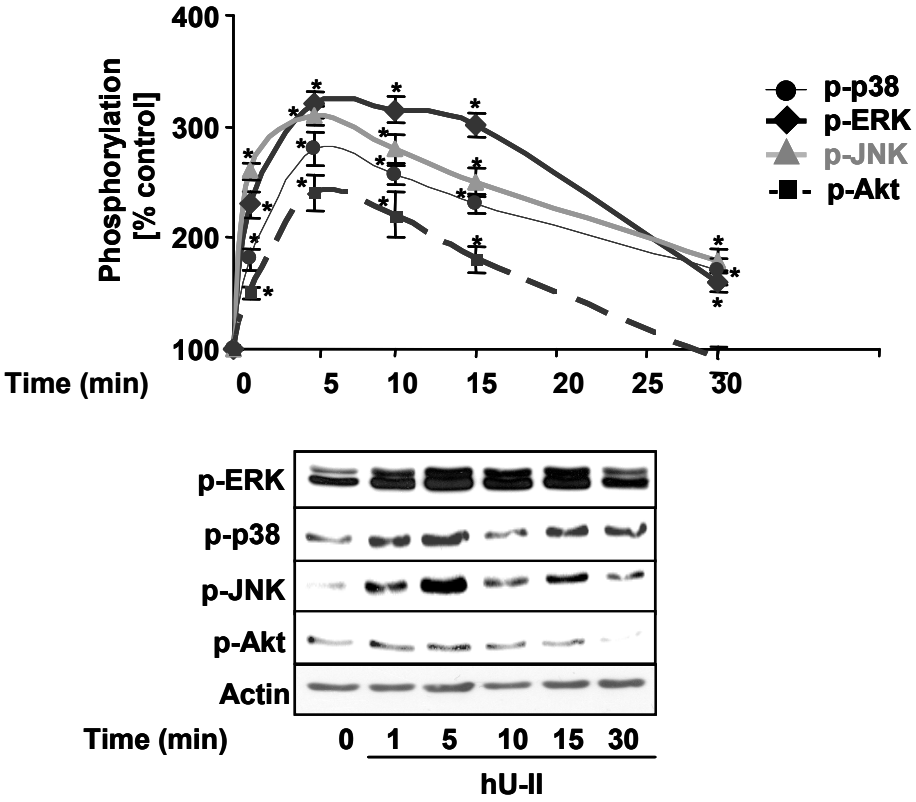


Figure 45. hU-II activates protein kinases

PASMC were exposed to 100 nM hU-II from 1 to 30 min and Western blot analysis using antibodies against the phosphorylated forms of ERK (p-ERK), p38 MAP kinase (p-p38), c-Jun N-terminal kinase (p-JNK) and Akt (p-Akt) were performed. Equal loading was confirmed by reprobing the membranes with an anti-α-actin antibody. In each experiment the protein levels of protein kinases under non-stimulated conditions were set equal to 100% (n=4, *p<0.05 versus non-stimulated cells (Ctr)).

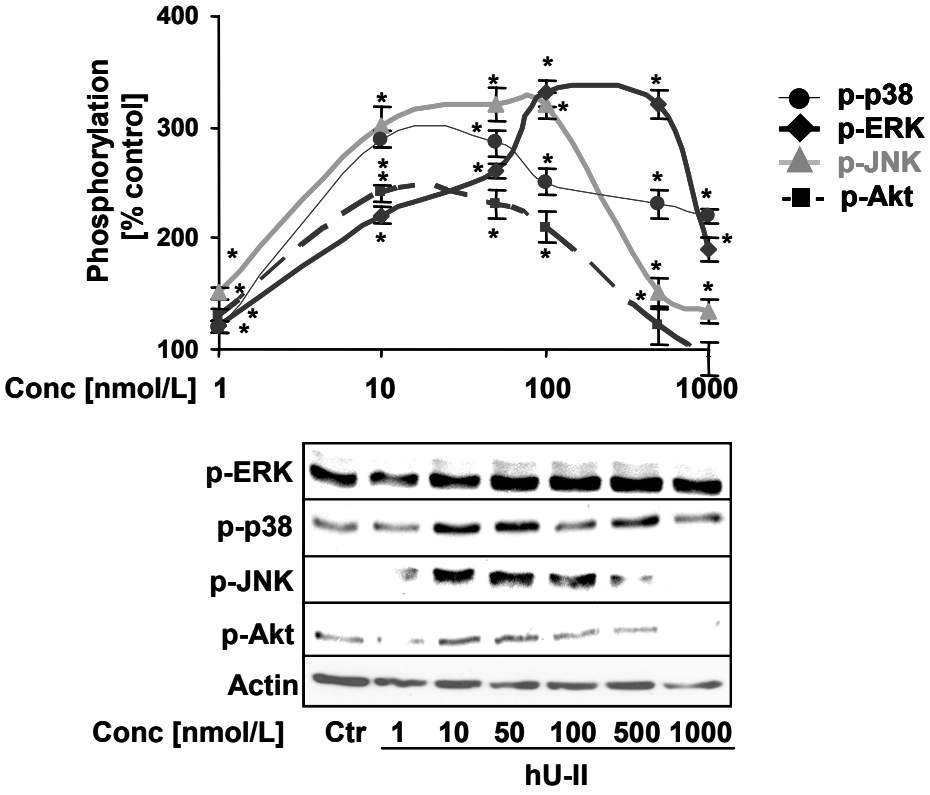


Figure 46. Different concentrations of hU-II activate protein kinases

PASMC were exposed to different concentrations of hU-II (1-1000 nM) for 5 min. Western blot analysis using antibodies against the phosphorylated forms of ERK (p-ERK), p38 MAP kinase (p-p38), c-Jun N-terminal kinase (p-JNK) and Akt (p-Akt) were performed. Equal loading was confirmed by reprobing the membranes with an anti-α-actin antibody. In each experiment the protein levels of protein kinases under non-stimulated conditions were set equal to 100% (n=4, *p<0.05 versus non-stimulated cells (Ctr)).

3.5.4 p22phox and NOX4 are involved in urotensin-II-stimulated protein kinase activity

To test the role of NADPH oxidases in the activation of kinases by hU-II, PASMC were treated with DPI (10 µM) or transfected with p22phox or NOX4 antisense vectors prior to stimulation with hU-II. DPI as well as depletion of p22phox or NOX4 abrogated hU-II-induced phosphorylation of ERK, p38MAPK, JNK and Akt (Fig. 47A, B), indicating that hU-II can stimulate MAP kinases and Akt in PASMC via a redox-sensitive pathway involving NADPH oxidases.

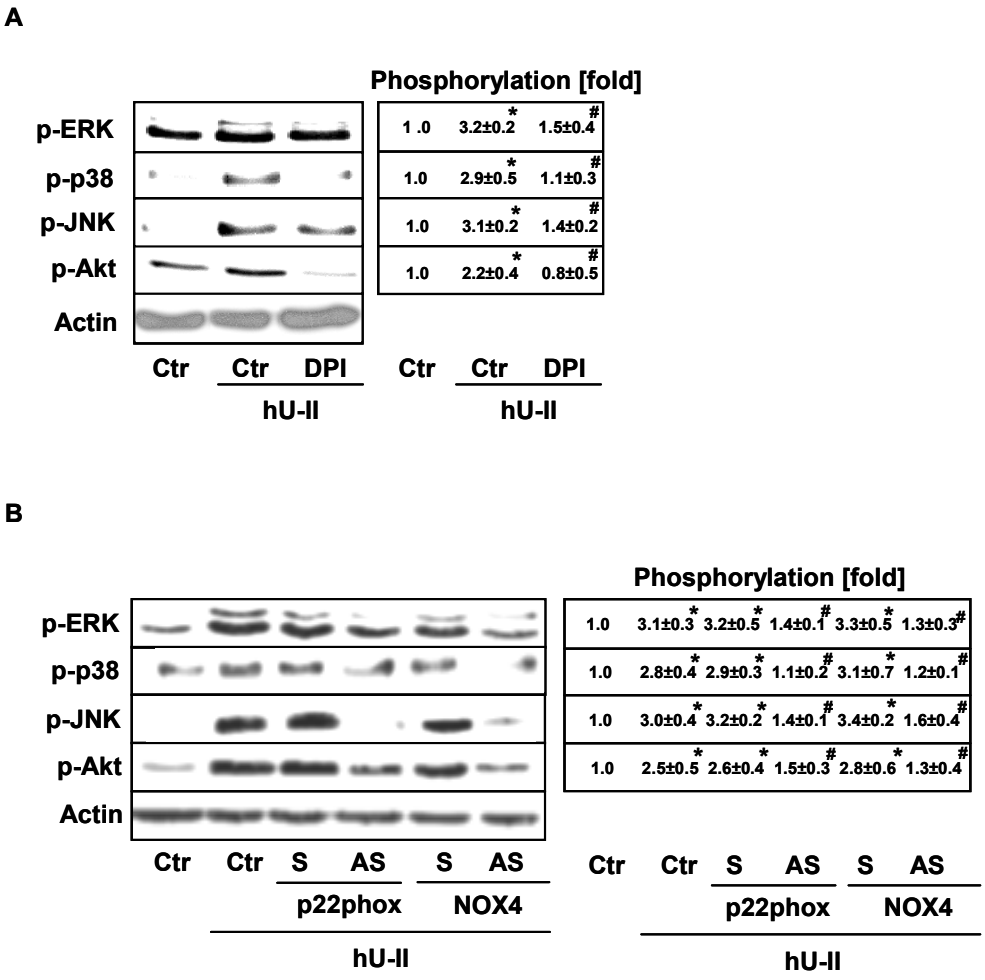


Figure 47. The NADPH oxidase mediates protein kinase activation by hU-II

PASMC were A) pretreated with diphenyleneiodonium (DPI, 10 µM) for 30 min or B) transfected with sense (S) or antisense (AS) vectors against p22phox or NOX4 and stimulated with hU-II (100 nM) for 5 min. Western blot analysis using antibodies against the phosphorylated forms of ERK (p-ERK), p38 MAP kinase (p-p38), c-Jun N-terminal kinase (p-JNK) and Akt (p-Akt) were performed. In each experiment the protein levels under non-stimulated conditions were set equal to 1 (n=4, *p<0.05 versus non-stimulated cells (Ctr); #p<0.05 versus hU-II stimulated cells).

3.5.5 Urotensin-II induces PAI-1 expression

Since hU-II has been suggested to contribute to vascular remodeling and has been associated with cardiovascular diseases such as PH, the role of hU-II in the regulation of PAI-1 was investigated.

Exposure to 100 nM hU-II increased PAI-1 mRNA levels peaking at 4 h and PAI-1 protein levels, determined in culture supernatants, after 4 and 8 h (Fig. 48), suggesting that hU-II is able to stimulate gene expression in PASMC.

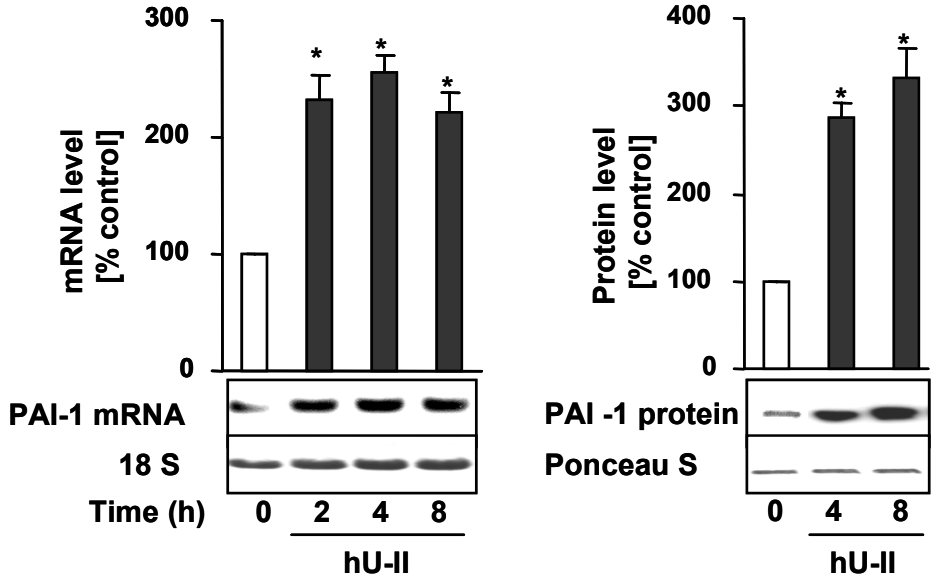


Figure 48. hU-II stimulates the expression of PAI-I

PASMC were stimulated with hU-II (100 nM) and PAI-1 mRNA levels were evaluated by Northern blot analysis and PAI-1 protein levels from the culture supernatants were determined by Western blot analysis. Equal loading of proteins was confirmed by Ponceau S staining. In each experiment the mRNA or protein levels under non-stimulated conditions were set equal to 100% (n=4, *p<0.05 versus non-stimulated cells (Ctr); #p<0.05 versus hU-II stimulated cells).

3.5.6 Urotensin-II induces PAI-1 expression by MAP kinases and the PI3 kinase/Akt pathway involving NADPH oxidases

The involvement of MAPK and PI3K/Akt pathway, as well as the role of p22phox and NOX4 in PAI-1 expression was then evaluated. Application of inhibitors of p38MAPK (SB220025, 20 µM), MEK1 (PD98059, 20 µM or U0126, 20 µM), PI3 kinase (PI3K) (LY294002, 10 µM), Akt (SH-5, 10 µM)[136] or JNK (SP600125, 25 µM) or pretreatment with DPI decreased hU-II-induced PAI-1 protein levels (Fig. 49A). Since in test experiments all inhibitors prevented the phosphorylation of their respective target kinases, these data suggest that hU-II regulates PAI-1 expression by stimulating NADPH oxidase-dependent ROS generation and subsequent activation of MAP kinases and the PI3 kinase/Akt pathway. In support of these findings, transfection of p22phox or NOX4 antisense vectors prevented the increase in PAI-1 protein by hU-II (Fig. 49B).

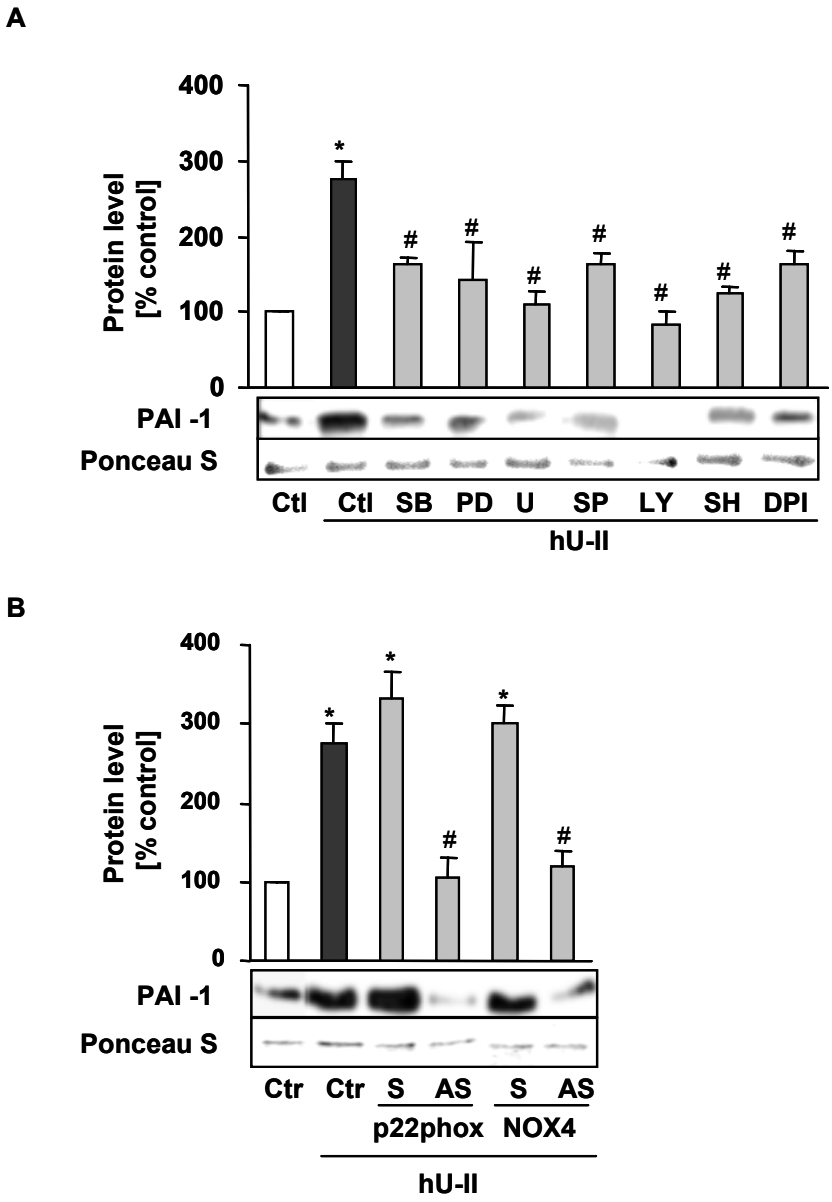


Figure 49. hU-II stimulates the expression of PAI-I involving NADPH oxidases, MAP kinases and PI3 kinase/Akt

A) PASMC were pretreated with the p38 MAP kinase inhibitor SB220025 (SB, 20 μM), the MEK1 inhibitors PD98059 (PD, 20 μM) or U0126 (U, 20 μM), the c-Jun N-terminal kinase inhibitor SP600125 (SP, 25 μM), the PI3 kinase inhibitor LY294002 (LY, 10 μM) or the Akt inhibitor SH-5 (SH, 10 μM), or exposed to diphenyleneiodonium (DPI, 10 μM) for 30 min prior to stimulation with hU-II for 4 h, or B) transfected with control vector (Ctr), p22phox or NOX4 sense (S) and antisense (AS) vectors. PAI-1 protein levels from the culture supernatants were determined by Western blot analysis. Equal loading of proteins was confirmed by Ponceau S staining. In each experiment the mRNA or protein levels under non-stimulated conditions were set equal to 100% (n=4, *p<0.05 versus non-stimulated cells (Ctr); #p<0.05 versus hU-II stimulated cells).

3.5.7 Urotensin-II stimulates proliferation via protein kinases and NADPH oxidases

To evaluate whether hU-II can regulate proliferation of PASMC, cells were stimulated with 100 and 1000 nM hU-II and the proliferative activity of PASMC was determined using BrdU incorporation. Stimulation with hU-II for 48 h significantly increased proliferative activity of PASMC (Figure 50A). In addition, specific inhibition of MAP kinases and the PI3K/Akt pathway completely blocked proliferation of PASMC in response to hU-II (Fig. 50B).

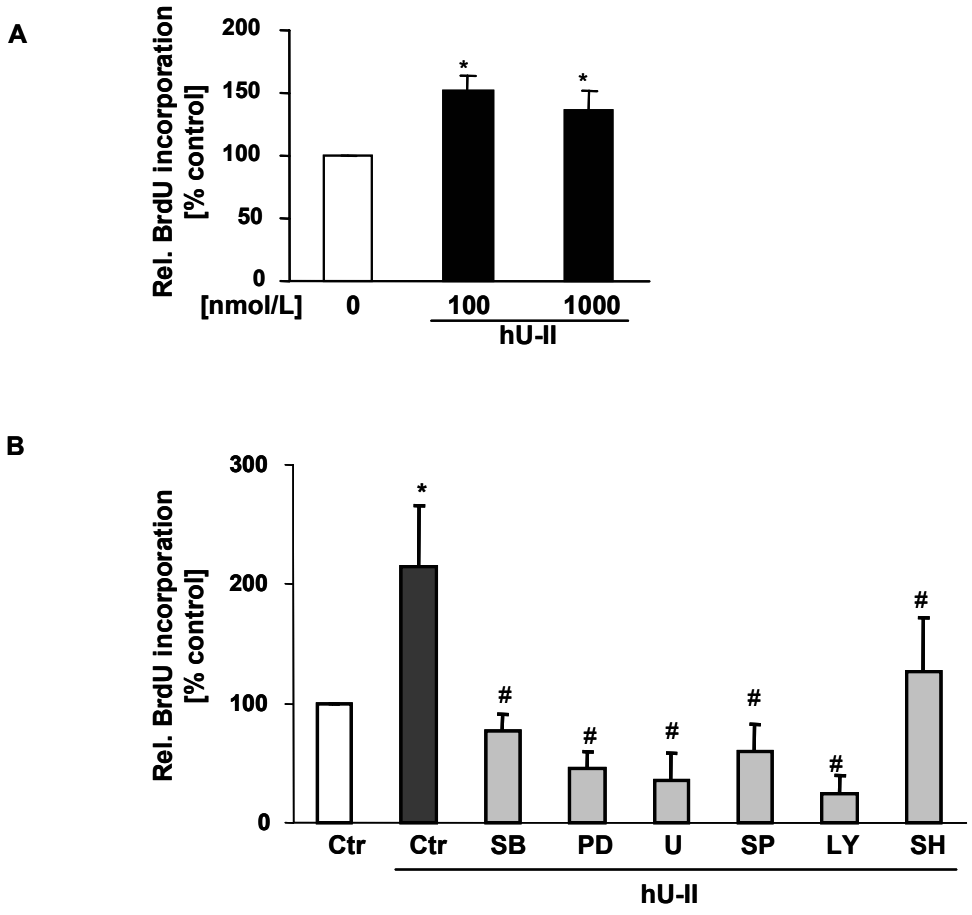


Figure 50. hU-II stimulates cell proliferation involving MAP kinases and PI3 kinase/Akt

A) PASMC were exposed to hU-II (100 or 1000 nM) for 48 h. Proliferative activity was assessed by labeling with BrdU (n=3, *p<0.05 versus unstimulated cells (0 nM)). B) PASMC were pretreated with the inhibitors of p38MAP kinase (SB220025 (SB), 20 µM), MEK1 (PD98059 (PD), 20 µM or U0126 (U), 20 µM), c-Jun N-terminal kinase (SP600125 (SP), 25 µM), PI3 kinase (LY294002 (LY), 10 µM) or Akt (SH-5 (SH), 10 µM) 30 min prior to stimulation with hU-II for 48 h. Proliferative activity was assessed by labeling with BrdU (n=3, *p<0.05 versus unstimulated cells (Ctr), #p<0.05 versus hU-II stimulated control cells (Ctr)).

To determine whether proliferation of PASMC by hU-II is mediated by NADPH oxidases, p22phox and NOX4 were depleted by using antisense vectors. Depletion of p22phox or NOX4 specifically abrogated hU-II-stimulated proliferation of PASMC (Fig. 51A), whereas the proliferation in response to sphingosine-1-phosphate (S1P, 1 μM), known to promote proliferation of smooth muscle cells[137], was not significantly reduced by depletion of p22phox or NOX4 (Fig. 51B).

In summary, these findings identify hU-II as a novel agonist of NADPH oxidases in PASMC and demonstrate that enhanced levels of ROS and the subsequent redox-sensitive activation of MAP kinases and Akt increases PAI-1 expression and PAI-1-dependent proliferation.

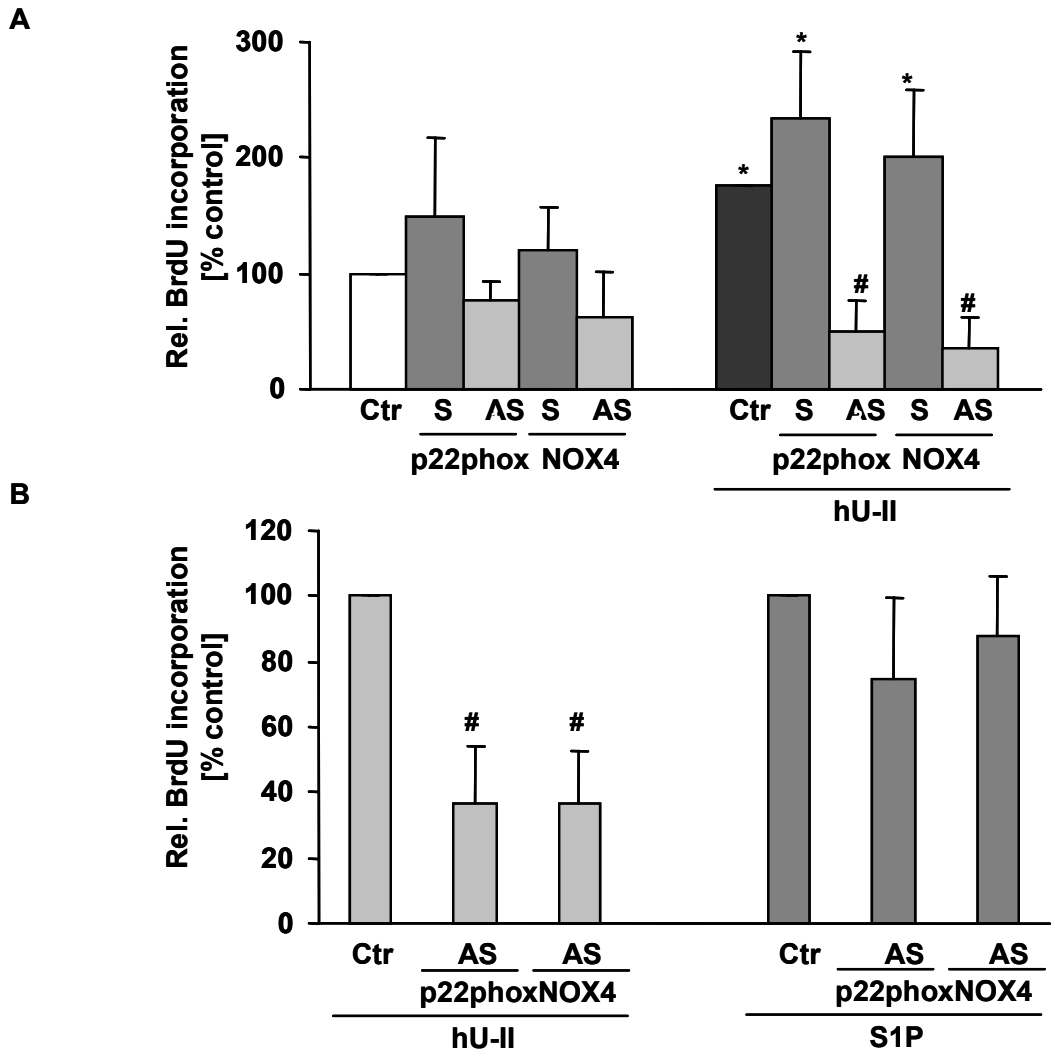


Figure 51. hU-II stimulates cell proliferation involving NADPH oxidases

A) PASMC were transfected with control vector (Ctr), p22phox or NOX4 sense and antisense vectors. After stimulation with hU-II for 48 h, proliferative activity was assessed by BrdU incorporation (n=3, *p<0.05 versus unstimulated control cells (Ctr), #p<0.05 versus hU-II stimulated control cells (Ctr)). B) PASMC were transfected with control vector (Ctr), p22phox or NOX4 antisense (AS) vectors and stimulated with hU-II or sphingosine-1-phosphate (S1P, 1 µM) for 48 h. Proliferative activity was assessed by BrdU incorporation. Stimulated proliferative activity in control cells was set equal to 100% (n=3, #p<0.05 versus stimulated control cells (Ctr)).

4. Discussion

4.1. Thrombin induces NADPH oxidase-dependent ROS production in endothelial and pulmonary artery smooth muscle cells

4.1.1 p22phox mediates thrombin-stimulated sustained elevation of ROS and proliferation of endothelial cells

Endothelial dysfunction is one of the early events in vascular remodeling. It is characterized by an imbalance between nitric oxide (NO) and superoxide anion radical production leading to a decrease in NO bioavailability and is frequently associated with enhanced thrombin formation and a procoagulant state[75]. Whereas in smooth muscle cells thrombin has been shown to potently increase ROS production via activation and/or induction of NADPH oxidases[122], the role of thrombin in regulating endothelial ROS production is less clear.

In the first part of the study, it was demonstrated that thrombin is able to induce a biphasic increase in ROS production in the endothelial cell line Ea.hy 926. A rapid but transient elevation in ROS generation peaking at 15 min was followed by a delayed response peaking at 3 to 4 h of stimulation. This delayed response was caused by a redox-sensitive, p38MAPK- and PI3 kinase-dependent increase in the expression of the NADPH oxidase subunit p22phox since a) exposure to thrombin resulted in a time-dependent increase in ROS production which was closely matched by an increase in p22phox mRNA and protein levels, b) antioxidants or the flavin inhibitor DPI decreased ROS production as well as p22phox mRNA and protein levels induced by thrombin, c) exposure to low concentrations of H_2O_2 resulted in an increase in ROS production and concomitantly elevated p22phox expression, d) increased levels of p22phox achieved by overexpression of p22phox elevated intracellular ROS production, and e) redox-sensitive activation of p38MAPK and the PI3

kinase/Akt pathway was involved in ROS production and upregulation of p22phox in response to thrombin and H_2O_2.

NADPH oxidases have now been established as important sources of ROS production in the vascular wall. In endothelial cells, a functionally active NADPH oxidase closely resembling the leukocyte NADPH oxidase has been described[81, 138]. Indeed, the results of the present study show that overexpression of p22phox increased ROS production in endothelial cells indicating that enhanced levels of this NADPH oxidase subunit allow increased ROS generation. Expression of a p22phox fusion protein was found perinuclear and was closely linked to sites of intracellular ROS production, as shown by 2 photon confcal microscopy. This is in contrast to the situation in neutrophils where it is generally accepted that p22phox is mainly located at the plasma membrane[52]. However, in support of our findings, it was shown that p22phox is present in nuclear-enriched fractions and localized in a perinuclear zone in endothelial cells[139]. Moreover, part of the enzyme was found to be present as a preassembled intracellular complex in endothelial cells[139] suggesting that a) low levels of ROS production may occur under unstimulated conditions and b) stimulation with agonists may allow a rapid activation of the NADPH oxidase. Indeed, exposure to thrombin resulted in a fast increase in endothelial ROS production, starting at 5 min and peaking after 15 min of stimulation, thereafter declining again. Similarly, thrombin has been shown to rapidly elicit ROS formation in rat aortic smooth muscle cells within 10 min of exposure[122, 140], whereas in endothelial cells, angiotensin II stimulation evoked a rapid activation of ROS production by the NADPH oxidase[141]. Interestingly, in smooth muscle cells, NADPH oxidase-dependent production of ROS in response to angiotensin II was shown to be biphasic[142]. The first phase peaked at 30 sec and was dependent on PKC, whereas the second phase started at 1 min and was sustained for 30 min and more.

Whereas a rapid increase in ROS production may be sufficient to initiate a primary cellular response to injury, many cardiovascular disorders including pulmonary hypertension are characterized by a chronic dysfunction of the endothelium and sustained elevation of ROS levels[62]. Indeed, prolonged exposure to thrombin resulted in a delayed, time-dependent

increase in ROS production. This response was closely accompanied by an elevation of p22phox mRNA and protein levels. Moreover, depletion of p22phox by transfection of an antisense vector diminished thrombin-induced ROS production, confirming that p22phox is required for thrombin-stimulated ROS production. The importance of p22phox is further confirmed by *in vivo* studies showing increased p22phox protein levels in the endothelium and in the smooth muscle cell layer of atherosclerotic vessels compared to non-atherosclerotic vessels[143]. These findings also support the view that a hypercoagulant state as it is frequently observed in cardiovascular diseases may contribute to ROS generation, and that upregulation of p22phox may participate in this response.

In addition to p22phox, thrombin has also been shown to increase the levels of p47phox protein in VSMC, and this has been associated with increased ROS production[73]. In preliminary studies we also observed increased p47phox protein levels in endothelial cells in response to thrombin, suggesting that elevated levels of more than one subunit may contribute to the delayed response to thrombin in endothelial cells and that the regulation of the different subunits may be controlled in a complex manner.

To date only limited information is available with regard to the signaling mechanisms involved in the regulation of NADPH oxidase subunits. Inhibition of ROS formation by the antioxidant vitamin C or the flavin inhibitor DPI prevented thrombin-induced p22phox mRNA and protein expression suggesting a redox-sensitive mechanism involved in the regulation of p22phox in endothelial cells. Consistently, exposure to low doses of H_2O_2 up to 50 µM increased ROS production and p22phox mRNA and protein levels.

Our findings that pretreatment with actinomycin D blocks p22phox expression by thrombin or H_2O_2 indicated that p22phox is regulated at the transcriptional level. This is further supported by recent studies reporting the presence of functional polymorphisms in the p22phox promoter that resulted in higher transcriptional activity of the p22phox gene in hypertensive rats[144]. Moreover, NADPH-derived ROS generation has been shown to be blocked by actinomycin D and cycloheximide[145, 146]. Consistent with these observations, pretreatment with cycloheximide prevented upregulation of p22phox by thrombin and H_2O_2, whereas

application of cycloheximide after treatment with thrombin or H_2O_2 did not increase p22phox protein levels suggesting the involvement of *de novo* protein synthesis rather than altered stability in this response.

To further evaluate the signaling pathways mediating redox-sensitive regulation of p22phox, we investigated the role of MAP kinases and the PI3 kinase/Akt pathway in this response. Exposure to thrombin transiently enhanced phosphorylation of p38MAPK, ERK and Akt, while treatment with H_2O_2 resulted in increased phosphorylation of the same kinases over the whole observation period, indicating sustained activation of the PI3 kinase/Akt pathway and MAP kinases. Similar observations have been made in smooth muscle cells[140] and may relate to differences between receptor-mediated activation of kinases as for thrombin compared to "redox-sensitive" activation of kinases occurring in the presence of elevated ROS levels as for H_2O_2. These findings are also in agreement with our suggestion that thrombin causes a rapid induction of NADPH oxidase activity whereas H_2O_2 causes a sustained increase in NADPH oxidase-mediated ROS production. Furthermore, inhibition of p38MAPK or PI3 kinase, an upstream kinase of Akt, prevented thrombin- and H_2O_2-stimulated ROS production and p22phox upregulation whereas inhibition of ERK was not effective. Consistently, in smooth muscle cells thrombin-induced stimulation of ROS production via the NADPH oxidase was able to specifically activate p38MAPK and Akt, but not ERK[71, 122, 140].

These findings support the view that ROS are involved in the regulation of p22phox expression and suggest that activation of the NADPH oxidase by thrombin stimulates specific redox-dependent pathways leading to the upregulation of p22phox and thus to a sustained generation of ROS in endothelial cells. Very recently, it has been shown that antioxidants reduced ROS production and p22phox protein levels in response to intermittent high glucose treatment in endothelial cells[147] further supporting a prominent role of ROS in regulating NADPH oxidase expression and activity in endothelial cells.

The sustained ROS generation in many cardiovascular disorders, including pulmonary hypertension, has been described to result on one hand in a stimulatory effect on the

proliferation of vascular cells[56, 148], on the other hand it has been associated with toxic effects and increased cell death[149]. Indeed, exposure to low concentrations of H_2O_2 (between 0.1 and 50 µM) increased endothelial cell proliferation whereas doses of 100 µM or higher reduced or even prevented the proliferative response. Similar observations have been made in bovine aortic endothelial cells[150]. Consistently, p22phox protein levels were upregulated in response to H_2O_2 concentrations up to 50 µM whereas higher concentrations decreased p22phox protein levels indicating that the amount of H_2O_2 leading to the activation of signaling cascades, gene expression and proliferation is tightly regulated in endothelial cells. Very similar to the situation with H_2O_2, stimulation with 3 U/ml of thrombin, but not 5 U/ml, increased proliferation of endothelial cells. Moreover, depletion of p22phox diminished proliferation in response to H_2O_2 and thrombin, pointing towards an important role of the NADPH oxidases in regulating proliferative responses in endothelial cells. In contrast, in smooth muscle cells, much higher doses of H_2O_2 (200 µM) were required to stimulate NADPH oxidase-dependent ROS production suggesting that endothelial and smooth muscle cell NADPH oxidases may differ in their redox sensitivity and their activity[151]. However, in support of our results, depletion of p22phox resulted in decreased proliferation of vascular smooth muscle cells[152], suggesting that NADPH oxidases play a crucial role in regulating proliferative responses in endothelial and smooth muscle cells.

4.1.2 Rac-1 and PAK mediate thrombin-stimulated elevation of ROS in pulmonary artery smooth muscle cells

In the second part of the study, it was demonstrated that thrombin is able to rapidly increase Rac-1 activity within 15 s in PASMC. Since Rac-1 is known to be required for activation of the NADPH oxidase, these results suggest that thrombin is, similar to the situation in endothelial cells, rapidly stimulating ROS production in PASMC. In support of these findings, NADPH oxidase-dependent production of ROS in response to angiotensin II was shown to be biphasic in smooth muscle cells[142]. The first phase peaked at 30 sec and was dependent

on PKC, whereas the second phase was dependent on Rac-1 activation and translocation and started at 1 min. The differences in the kinetics may be related to stimulus- and cell type specific variations as well as to different NADPH oxidase homologues.

Moreover, thrombin also elevated Rac-1 protein levels within 2 h. Consistently, Rac-1 mutants modulated ROS levels in response to thrombin in PASMC. Together with the results that thrombin induced a biphasic increase in ROS production in endothelial cells, where the delayed phase was paralleled by upregulation of p22phox, these findings suggest that thrombin elicits a biphasic production of ROS also in PASMC.

To date only limited information is available with regard to the signaling events involved in the regulation of ROS production in response to thrombin. The data of the present study show that Rac-1 is activated by thrombin involving a Ca^{2+} dependent mechanism in PASMC, since treatment with the Ca^{2+} chelator BAPTA-AM decreased thrombin-stimulated Rac-1 activity. Similarly, in prostate carcinoma cells, Rac-1 activation was shown to be dependent on Ca^{2+} levels in response to thrombin receptor activating peptide (TRAP)[153]. In endothelial cells, sphingosine-1 phosphate (S1P)-induced Rac-1 activation was mediated by Ca^{2+}[154]. Since the increase in intracellular calcium levels is a common mechanism in coupling the signals from activated G protein-coupled receptors to signaling pathways, Ca^{2+} could be the messenger linking PAR signaling after thrombin stimulation to activation of NADPH oxidases. Indeed, BAPTA-AM prevented thrombin-induced ROS production. In addition to NOX5 and DUOX that contain Ca^{2+} binding moieties and appear to be directly activated by Ca^{2+} [90, 155, 156], it has been suggested that other NOX homologues could also be regulated by Ca^{2+} [156, 157]. It was demonstrated that NOX2 can be activated *in vitro* by Ca^{2+}-binding proteins[158]. Moreover, ROS are important regulators of intracellular Ca^{2+} homeostasis[12, 159], suggesting that there is a tight regulation between levels of Ca^{2+} and redox status of the cells.

Furthermore, this study showed that thrombin stimulation of PASMC resulted in PAK phosphorylation peaking after 1 min, and this response was modulated by Rac-1 mutants. These results show that PAK activation in response to thrombin is downstream of Rac-1 that was activated after only 15 sec. Consistently, PAK has been identified as a target of Rac-1,

where Rac-1 is directly binding to the PBD domain of PAK[110]. In support of these findings, PAK has been shown to be rapidly activated by thrombin and to play a central role in thrombin-mediated TF expression in PASMC[160].

In addition, expression of an active mutant of PAK significantly increased ROS production. In support to this data, PAK was shown to directly interact with p47phox and to phosphorylate this NADPH oxidase subunit, and to subsequently increase ROS production in endothelial cells[161]. However, in VSMC PAK phosphorylation in response to Ang-II was not only dependent on Ca^{2+} and blocked by a dominant-negative Rac mutant, but was also inhibited by antioxidants[162], suggesting that reactive oxygen species are also involved in the upstream signaling of PAK. Similarly, it was reported that phosphorylation of PAK in response to PDGF was attenuated by inhibition of ROS[163], further suggesting that PAK is not only able to increase ROS production, but is also sensitive to ROS. These data support the view that a positive feed back loop exists, where Rac-1-dependent ROS production leads to activation of PAK, which is able to further stimulate NADPH oxidase and additionally increase ROS levels.

In summary, the data presented support a model whereby activation of the NADPH oxidase by thrombin in endothelial or PASMC leads to a biphasic increase in ROS production. The first, rapid phase is probably due to the activation of a preassembled NADPH oxidase complex or due to the activation of regulatory oxidase components, such as Rac-1. The second, prolonged phase of ROS production is allowed by the increased protein expression of p22phox or Rac-1, subsequently promoting sustained generation of ROS. Whereas a rapid increase in ROS production in response to thrombin may be beneficial for the initial wound healing at sites of vascular injury, the initiation of a vicious circle promoting ROS generation over a long time period by upregulating p22phox or Rac-1 may be important in promoting vascular remodeling in the pathogenesis of many cardiovascular diseases, including pulmonary hypertension.

4.2 NADPH oxidases mediate cell proliferation and tissue factor and plasminogen activator inhibitor-1 expression by thrombin in pulmonary artery smooth muscle cells: Role of Rac-1

4.2.1 Rac-1 mediates tissue factor expression involving the NFκB pathway

Enhanced thrombin levels and procoagulant activity have been associated with vascular remodeling. In this study it was demonstrated that activation of Rac-1 by thrombin leads to the activation of the transcription factor NFκB and induction of tissue factor in PASMC. This was based on the findings that a) thrombin increased Rac-1 activity and expression, b) constitutively active and dominant-negative Rac-1 mutants increased or decreased, respectively, thrombin-induced TF expression and TF-dependent procoagulant activity, c) thrombin and Rac-1 were able to activate NFκB-dependent transcriptional activity, d) overexpression of NFκB enhanced TF promoter activity, and e) inhibition of the NFκB pathway prevented Rac-1-dependent TF promoter activity.

Using constitutively active and dominant-negative Rac-1 mutants, TF expression and procoagulant activity in PASMC by thrombin could be enhanced or decreased, respectively, indicating that Rac-1 and NADPH oxidases are involved in thrombin-induced TF expression in PASMC. These findings also confirm previous observations in VSMC demonstrating the involvement of p22phox and Rac-1 in the regulation of TF by thrombin[71]. Moreover, in endothelial cells, TF expression by thrombin was decreased by treatment with statins which prevent activation of the RhoGTPases Rho, Rac and cdc42[164] further supporting an important role of Rac-1 in the control of TF expression and activity by thrombin.

Rac-1 and thrombin stimulated TF promoter activity only in the presence of a distal enhancer containing AP1 and NFκB sites. In contrast, in smooth muscle cells stimulated with activated platelets or in endothelial cells activated by VEGF, luciferase activity of the pTF111 construct was not significantly different to the activity of promoter constructs containing also the distal enhancer[129, 165]. However, in endothelial cells, pTF111-mediated luciferase activity in

response to lipopolysaccharides (LPS), TNF-α and IL-1ß was significantly reduced compared to the luciferase activity of constructs containing also the distal enhancer[166-168]. The LPS- and cytokine-stimulated response in endothelial cells and in monocytes has been shown to require constitutive AP1 DNA binding and inducible activation of the NFκB pathway[166, 168]. The involvement of NFκB in Rac-1 and thrombin-induced TF regulation was further demonstrated by experiments where overexpression of the NFκB subunits p50 and p65 or dominant-negative mutants of IκB or IκBK increased or decreased TF promoter activity, respectively. These findings are consistent with previous observations that RacG12V induced NFκB activity in HepG2 cells and that Rac-1 is involved in phosphorylation of IκB and nuclear translocation of NFκB[126, 169]. However, a role for AP1 in Rac-1-induced TF expression can not be completely ruled out. Possibly, the interaction of AP1 and NFκB is also required for maximal induction of TF in PASMC similar to the situation in endothelial cells[35]. Moreover, the modest induction of pTF111-derived luciferase activity by thrombin and RacG12V suggests that additional transcription factors, such as Sp1 and/or Egr1 may also contribute to the regulation of TF expression by thrombin and Rac-1 in PASMC.

In summary, in PASMC thrombin increased TF expression and activity via Rac-1 involving activation of the NFκB pathway. Since enhanced NFκB activity has been observed in various cardiovascular diseases[170], and statins which inhibit Rac and Rho kinases decrease procoagulant activity in the vascular wall[171], Rac-1-dependent activation of the NFκB pathway may be a critical element promoting thrombin-induced TF activity.

Thrombin and TF have been shown to directly interact with the vascular wall and to activate signaling pathways and gene expression thus being key players in promoting vascular remodeling[37, 172]. Moreover, since TF activity determines the levels of thrombin, Rac-1 and NFκB may promote a positive feed back loop where thrombin-induced upregulation of TF expression and TF-dependent procoagulant activity in PASMC lead to the increased thrombogenicity of the vascular wall. Such a thrombogenic cycle may then promote vascular

remodeling that is associated with the pathogenesis of various vascular diseases, such as pulmonary hypertension[173, 174].

4.2.2 Rac-1 mediates PAI-1 expression and cell proliferation involving HIF-1

In addition to thrombin and TF, elevated levels of PAI-1 have been associated with vascular remodeling in vascular disorders characterized with a prothrombotic state, such as pulmonary hypertension. PAI-1 is a matrix protein and the major inhibitor of fibrinolysis and has a central role in tissue remodeling by degrading extracellular matrix and activating growth factors[48].

This study now demonstrates that thrombin upregulates PAI-1 expression in PASMC via a redox-sensitive, Ca^{2+}-dependent pathway involving Rac-1 and the transcription factor HIF-1 since a) PAI-1 mRNA and protein levels were elevated in response to thrombin; b) constitutively active and dominant-negative Rac-1 mutants increased or decreased, respectively, PAI-1- promoter activity as well as PAI-1 mRNA and protein expression; c) Rac-1 mutants were able to modulate thrombin-stimulated HIF-1-dependent transcriptional activity and HIF-1α protein levels; d) pretreatment with the antioxidant NAC or the Ca^{2+} chelator BAPTA-AM prevented Rac-1-dependent PAI-1 and HIF-1α expression; e) HIF-1 was required for PAI-1-induced promoter activity by thrombin; f) constitutively active and dominant-negative Rac-1 mutants increased or decreased, respectively, thrombin-induced cell proliferation, and this response was prevented by treatment with NAC or BAPTA-AM and g) PAI-1 promoted proliferation of PASMC.

Together with the findings that thrombin activated and induced Rac-1 via a Ca^{2+}-dependent pathway, and Rac-1 modulated ROS generation in response to thrombin, this study now identifies a pathway whereby thrombin enhances proliferation of PASMC via activation of Rac-1, ROS generation, and HIF and PAI-1 induction.

Our findings identify Ca^{2+}- and Rac-1-dependent ROS as key players in thrombin-induced PAI-1 expression in PASMC, since BAPTA-AM or the antioxidant NAC and RacT17N

decreased PAI-1 mRNA and protein levels as well as PAI-1 promoter activity, whereas constitutively active RacG12V had opposite effects. Consistently, p22phox-dependent ROS generation has been shown to contribute to thrombin-induced PAI-1 expression in aortic smooth muscle cells[122]. Moreover, PAI-1 upregulation by AngII was prevented by BAPTA-AM in vascular smooth muscle cells[175]. In contrast, hypoxic induction of PAI-1 was reduced by RacG12V but enhanced by BAPTA-AM in HepG2 cells[126, 176]. Although the reasons for this conflicting data are not resolved to date, they may relate to cell type specific differences in the role of Ca^{2+}-activated Rac-1.

PAI-1 is known as the target gene of the transcription factor HIF-1 under hypoxia. Similarly, HIF-1 is also required for thrombin-induced PAI-1 expression, since mutation of a HIF-1 binding site in the PAI-1 promoter prevented reporter gene activity by thrombin. Furthermore, Rac-1 regulated HIF-1α protein levels as well as HIF-1 activity by thrombin in a redox-sensitive manner. Moreover, an active mutant of PAK, which is a downstream target of Rac-1 (see 4.1.2) increased transcriptional activity of HIF-1 as well as PAI-1 promoter activity by thrombin. Supporting these findings, it was previously shown that thrombin increased HIF-1 activity and expression in a redox-sensitive manner involving p22phox, further indicating the important role of NADPH oxidase in regulating HIF-1[122]. Similarly, AngII has been shown to induce HIF-1α in a redox-sensitive manner[125].

In addition, BAPTA-AM also decreased thrombin-induced HIF-1α levels. In support of these findings it was reported that elevation of intracellular Ca^{2+} by calcium ionophores leads to enhanced HIF-1α protein levels[177] and that induction of HIF-1 transcriptional activity by intermediate hypoxia was prevented by BAPTA-AM[178]. However, it was also demonstrated that the Ca^{2+} chelators BAPTA-AM or EGTA-AM can transiently increase hypoxia-induced HIF-1α accumulation in HepG2 or Hep3B cells by inhibiting PHD activity and thereby the interaction between HIF-1α and VHL[177, 179]. Taken together, these data show that Ca^{2+} plays an important role in controlling HIF-1 activity by acting at different levels of regulation in a cell type-specific and stimulus-dependent manner.

The sustained ROS generation in vascular remodeling has been described to have stimulatory effects on the proliferation of vascular cells[56, 148]. This study clearly shows that Rac-1 activation by thrombin increases proliferation of PASMC, while a dominant-negative mutant of Rac-1 or treatment with the antioxidant NAC prevented thrombin-stimulated proliferation of PASMC. Rac-1 has been described to also play an important role in proliferation of tumor cells[180]. In a recent study, transgenic mice that express the cDNA of a constitutively active mutant of human Rac-1 showed accelerated wound healing associated with efficient proliferation of vascular cells[181]. Moreover, expression of a dominant-negative Rac-1 mutant in endothelial cells inhibited VEGF-induced ROS production and proliferation[182], again supporting the model that NADPH oxidases play a crucial role in regulating proliferative responses in vascular cells.

Interestingly, proliferation of endothelial cells treated with tumor supernatants was inhibited by targeting Rac-1 GTPase with small interference RNA in tumor cells[183]. These data would support the mechanism where different secreted proteins from tumor cells (such as PAI-1) exhibit also paracrine effects on proliferation of vascular cells.

In addition, not only ROS production but also cell proliferation was modulated by Ca^{2+}. Proliferation was inhibited after removal of Ca^{2+} by the Ca^{2+} chelator BAPTA-AM. Supporting these findings, it has been demonstrated that thrombin-induced proliferation of tracheal smooth muscle cells was modulated by Ca^{2+} [184] and that Ca^{2+} plays an important role in proliferative responses of PASMC[185].

Various studies reported a role for PAI-1 in proliferation of vascular cells, but results from these investigations have been controversial. The data of this study clearly imply that PAI-1, which is induced via Ca^{2+}, Rac-1, and HIF-1-dependent pathway, is leading to increased proliferation of PASMC in response to thrombin, since cell proliferation after addition of conditioned medium from PASMC stimulated with thrombin was attenuated when incubated with an inhibitory antibody against PAI-1 (which decreases PAI-1 levels in the supernatant). Therefore, since PAI-1 is a secreted protein, our data suggest that it could exhibit an autocrine proliferative effect in PASMC. In support of our findings, using the rat carotid

balloon injury model, induction of modest, transient increase in PAI-1 was associated with increased cell proliferation[186]. In addition, PAI-1 was shown to promote neointima formation in mouse, rat and murine carotid arteries[186-188]. In contrast, Ploplis et al demonstrated that endothelial cells isolated from arterial trees of PAI-1-deficient mice showed increased proliferation *in vitro* and that neointima formation was attenuated following arterial injury in PAI-1 deficient mice[189, 190]. These opposite findings may be explained by differences in the models used and their genetic background, variations in the vascular bed as well as by different endogenous levels of PAI-1. However, consistent with our studies in human PASMC, several studies showed that enhanced levels of PAI-1 in patients were associated with increased remodeling of the vascular wall and increased thickening of the intimal and medial layers, and with the pathogenesis of vascular diseases including pulmonary hypertension [48, 191-194].

In summary, in PASMC thrombin increased PAI-1 expression via Rac-1 by activation of its substrate PAK and by inducing HIF-1. PAI-1 expression was not only redox-sensitive, but also dependent on Ca^{2+}. In addition, PAI-1 stimulated proliferative responses of PASMC. Therefore, since PAI-1 and Ca^{2+} have been associated with remodeling processes, these findings provide a mechanism that may link redox-sensitive and calcium-dependent expression of PAI-1 with elevated levels of thrombin and proliferation of PASMC.

4.3 NADPH oxidase-derived ROS mediate urotensin-II-induced signaling and proliferation of pulmonary artery smooth muscle cells

Urotensin-II has been described as a potent vasoactive peptide and has been suggested to contribute to several cardiovascular diseases including pulmonary hypertension. Herein, we showed that hU-II increases the levels of NADPH oxidase-derived ROS leading to the activation of MAP kinases and Akt followed by enhanced PAI-1 expression and increased proliferation of PASMC. This conclusion is supported by the findings that: a) hU-II strongly enhanced ROS levels which were blocked by DPI or depletion of the NADPH oxidase subunits p22phox or NOX4, b) hU-II elevated protein levels of p22phox and NOX4, c) hU-II increased the phosphorylation of ERK, p38MAPK, JNK and Akt, which was prevented by treatment with DPI and by depletion of p22phox or NOX4, d) hU-II upregulated the expression of PAI-1 in a redox-sensitive, NADPH oxidase- and kinase-dependent manner, e) hU-II increased the proliferative activity of PASMC which was abrogated by depletion of p22phox or NOX4.

4.3.1 Urotensin-II stimulates ROS production by NADPH oxidases

In this study it was demonstrated that hU-II significantly increased ROS levels in PASMC. This response was accompanied by elevated protein levels of the NADPH oxidase subunits p22phox and NOX4, but was abrogated by the flavin inhibitor DPI and by depletion of p22phox or NOX4. These findings show that hU-II is able to activate ROS generation and identify the NADPH oxidases as the source of ROS in PASMC. Interestingly, depletion of either p22phox or NOX4 also downregulated the expression of the other subunit (NOX4 or p22phox, respectively), but did not affect expression levels of the p47phox subunit (data not shown). These findings suggest that, similar to the neutrophil NADPH oxidase, protein expression of p22phox or NOX4 requires concomitant expression of NOX4 or p22phox, respectively.

Whereas vasoactive peptides such as AngII and ET-1 have been shown to activate ROS production by NADPH oxidases in aortic smooth muscle cells and endothelial cells, respectively[23, 77], and a role for ROS in pulmonary vascular remodeling has been suggested[69], only limited data are available with regard to ROS production in pulmonary artery smooth muscle cells. In fetal pulmonary artery smooth muscle cells endothelin-1 increased ROS production which was sensitive to treatment with the unspecific NADPH oxidase inhibitors DPI and apocynin[195]. Similarly, serotonin enhanced ROS production in murine pulmonary arteries which was inhibited by apocynin[196] suggesting that both peptides may, similar to hU-II, induce ROS production via activation of NADPH oxidases. Whereas p22phox has been described to contribute to ROS production in pulmonary endothelial and airway smooth muscle cells[69], the involvement of NOX4 in the ROS production of pulmonary vascular cells has not been reported so far. In support of our study, it was recently shown that NOX4 and p22phox co-localize in smooth muscle cells[197]. However, in contrast to our study where hU-II increased NOX4 protein, stimulation with AngII decreased NOX4 levels in rat aortic smooth muscle cells[198]. Although the reasons for these differences are not clear to date, they may relate to stimulus- and/or cell type-specific signaling mechanisms and/or intracellular localization as has been suggested recently[197]. However, in favor of our data, NOX4 was upregulated in vessels from hypertensive rats[199]. Together with the findings that in animals with pulmonary hypertension hU-II levels are elevated[31], the data that hU-II increases ROS levels via the activation of NADPH oxidases in PASMC suggest that enhanced NADPH oxidase expression and activity in response to hU-II may contribute to the development of vascular remodeling processes.

4.3.2 Urotensin-II activates redox-sensitive signaling cascades and PAI-1 expression

It was further demonstrated that hU-II rapidly increases the phosphorylation of ERK, p38MAPK, JNK and Akt in PASMC. In vascular smooth muscle cells, hU-II has been reported to activate ERK[200]. Thus, hU-II appears to be an effective activator of PASMC since these kinases have been linked to various cellular processes including proliferation,

differentiation, cell growth and apoptosis. Activation of the kinases by hU-II was redox-sensitive and was inhibited by depletion of p22phox or NOX4, suggesting the involvement of NADPH oxidase-derived ROS. Whereas p38MAPK, JNK and Akt have been shown to be activated by thrombin or angiotensin II in a redox-sensitive, NADPH oxidase-dependent manner in vascular smooth muscle cells from different beds and species, ERK has been found to be rather insensitive to ROS in response to thrombin[71, 107, 122]. However, conflicting data exist regarding the role of ROS in ERK activation by angiotensin II, indicating a stimulus-dependent involvement of the NADPH oxidase and ROS in the activation of ERK. Interestingly, hU-II-stimulated NADPH oxidase-dependent kinase activation was faster than the increase in ROS levels measured, indicating that in addition to increased ROS levels due to enhanced NADPH oxidase expression, hU-II may also activate the NADPH oxidase. Similar observations have been made with thrombin[140] or angiotensin II[142] in smooth muscle cells and endothelial cells (see 4.1.1)[201].

In this study it was further shown that hU-II strongly elevates PAI-1 expression in PASMC. Depletion of NADPH oxidase subunits or inhibition of MAP kinases and PI3K/Akt prevented this response, indicating that similar to thrombin, hU-II induces PAI-1 expression by a NADPH oxidase-dependent increase in ROS levels and the subsequent activation of MAP kinases and Akt. In vascular smooth muscle cells, MAP kinases and PI3K/Akt have been shown to be involved in the regulation of PAI-1 by thrombin, PDGF and angiotensin II[122, 175, 202]. Since findings of the present study indicate that enhanced levels of PAI-1 stimulate proliferation of PASMC, this novel pathway activated by hU-II can be relevant in promoting vascular remodeling.

In summary, our findings demonstrate that hU-II is a novel agonist of NADPH oxidases in PASMC. Elevated levels of ROS due to the increased expression of p22phox and NOX4 lead to the subsequent activation of MAP kinases and Akt that induce PAI-1 expression and PAI-1-dependent proliferation of PASMC. Since PAI-1 is essentially involved in vascular remodeling processes possibly by facilitating migration and/or proliferation of PASMC, such a mechanism may be highly relevant in promoting pulmonary vascular remodeling processes.

5. Summary

Vascular remodeling processes are characterized by increased proliferation of vascular cells and a prothrombotic state. Coagulation factors and vasoactive peptides have been suggested to contribute to these processes. Reactive oxygen species (ROS) have been implicated to act as vascular signaling molecules, and NADPH oxidases have been identified as a major source of vascular ROS production. The aims of the present study were therefore to identify signaling pathways linking factors promoting remodeling processes such as thrombin and the vasoactive peptide urotensin-II to ROS production derived from NADPH oxidases as well as subsequent proliferation and procoagulant activity.

Thrombin induced a biphasic increase in ROS levels. A rapid activation of NADPH oxidases was followed by a second delayed response. This increase was paralleled by ROS-dependent induction of p22phox involving p38MAPK and PI3K/Akt in endothelial cells, and by enhanced Rac-1 levels in pulmonary artery smooth muscle cells, and followed by enhanced proliferation.

Activation of Rac-1-dependent ROS production by thrombin resulted in elevated levels of the prothrombotic factors tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1) in PASMC. Rac-1-dependent induction of TF was mediated by the transcription factor NFκB and resulted in enhanced procoagulant activity. Since active TF results in thrombin generation, this pathway could play an important role in promoting a feedback loop leading to a thrombogenic cycle.

Rac-1-dependent induction of PAI-1 was mediated by elevated Ca^{2+}, the Rac-1 downstream target PAK and the transcription factor HIF-1. Since PAI-1 inhibition diminished proliferation of PASMC, these findings identify PAI-1 as an important mediator of thrombin-stimulated NADPH oxidase-dependent proliferation.

Finally, the vasoactive peptide U-II was identified as a novel activator of NADPH oxidases and ROS-dependent activation of MAP kinases and Akt which subsequently induced PAI-1 expression and proliferation.

Taken together, these findings support a model where enhanced levels of thrombotic and vasoactive factors, as found in an early stage of vascular remodeling, activate and induce NADPH oxidases and ROS generation. ROS-dependent signaling cascades link and amplify these signals and promote proliferative and thrombotic events which can lead to progression of vascular remodeling.

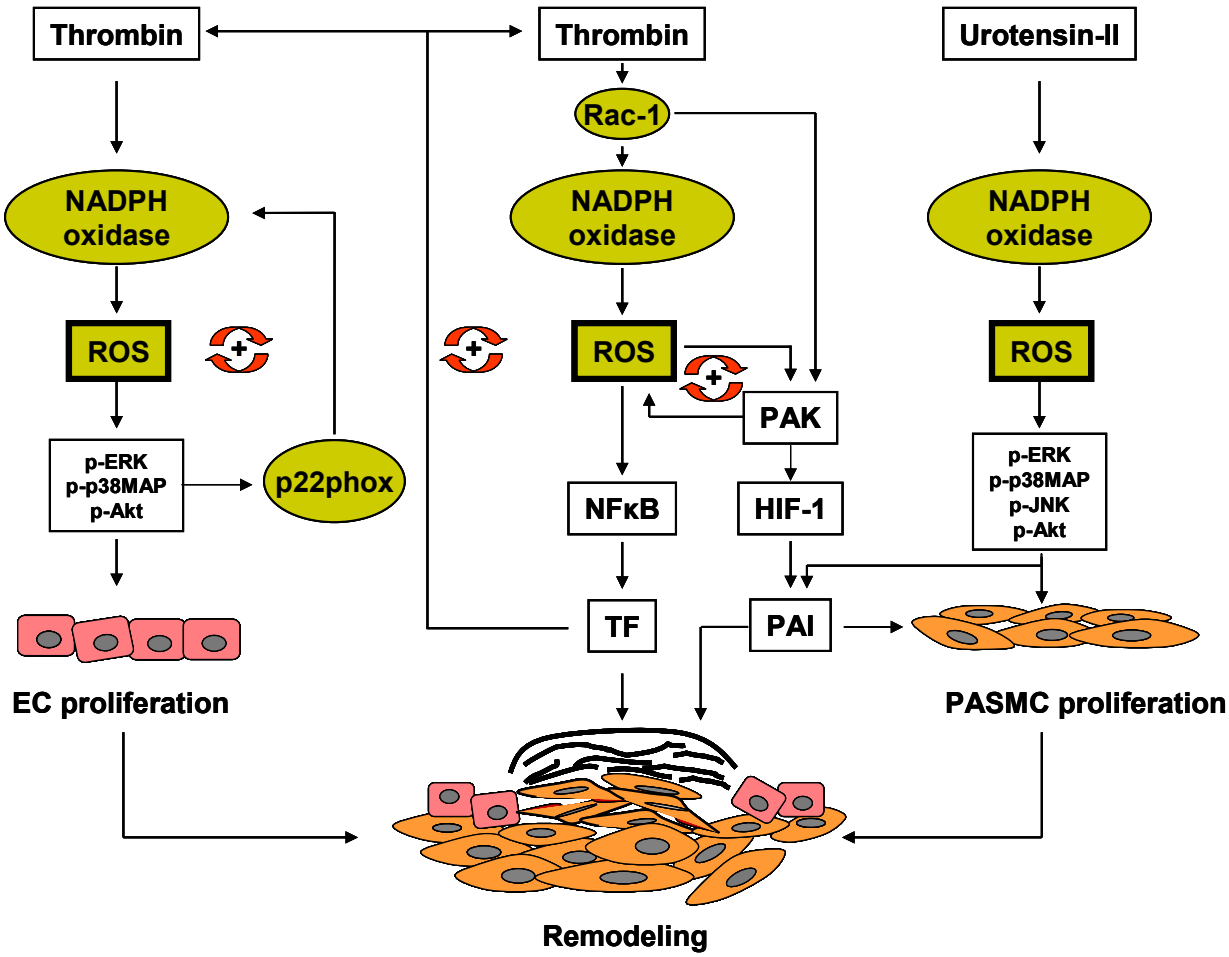


Figure 52. Summary of the pathways described in this study

Explanations are given in the text.

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7. Appendix

Abbreviations

2P-CLSM	2- photon confocal laser scanning microscopy
Akt	Protein kinase B (PKB)
ARNT	Arylhydrocarbon receptor nuclear translocator
BrdU	5-bromo-2'deoxyuridine
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CFP	Cyan fluorescent protein
CGD	Chronic granulomatous disease
DAF-2	Diaminofluorescein –2 Diacetate
DCF	5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA)
DHE	dihydroethidium
DHR	Dihydrorhodamine 123
DMEM	Dulbecco's Modified Eagl's Medium
DMSO	Dimethylsulfoxide
DPI	Diphenyleneiodonium
DTT	Dithiothreitol
EC	Endothelial cells
eNOS	Endothelial nitric oxide synthase
EPO	Erythropoietin
ERK	p41/42 extracellular signal regulated kinase
ET-1	Endothelin-1
ET_A	Endothelin receptor A
ET_B	Endothelin receptor B
GAPs	GTPase activating proteins
GDIs	Guanine nucleotide exchange inhibitors
GEFs	Guanine nucleotide exchange factors
GPR	G protein-coupled receptors
GPx	Glutathione-peroxidase
GTT	Guanidinethiocyanate
h	Hours
H_2O_2	Hydrogen peroxide

HAT	Hypoxanthine, Aminopterin, Thymidin
HBSS	Hank's balanced salt solution
HIF-1	Hypoxia inducible factor-1
HRE	Hypoxia-responsive elements
hU-II	Human urotensin-II
ICAM–1	Intercellular adhesion molecule
IGF-1	Insulin growth factor
IL1-α	Interleukin-1 alpha
IκBKα	Inhibitory κB protein alpha kinase
IκBKβ	Inhibitory κB protein beta kinase
IκBα	Inhibitory κB protein alpha
IκBβ	Inhibitory κB protein beta
JNK	c-jun N-terminal kinase
LDL	Low density lipoproteins
L-NAME	N-ω-nitro-L-arginine methyl ester
LPS	Lipopolysaccharides
MAPK	Mitogen activated protein kinases
MEK1	Mitogen activated protein kinase kinase 1
Min	Minutes
NAC	N-Acetyl-Cysteine
NFκB	Nuclear factor kappa B
NO	Nitric oxide
$O_2^{-\cdot}$	Superoxide anion radical
$OH^{\cdot}$	Hydroxyl radicals
$ONOO^-$	Peroxynitrite
oxLDL	Oxidised low density lipoproteins
PAGE	Polyacrylamide gel electrophoresis
PAI-1	Plasminogen activator inhibitor-1
PAK	p21-activated kinase 1
PAR	Protease-activated receptors
PASMC	Pulmonary artery smooth muscle cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PH	Pulmonary hypertension
Phox	Phagocytic oxidase
PI3K	Phosphatidyl-inositol-(3) kinase

PKB	Protein kinase B
PMA	Phorbol 12-Myristate 13 Acetate
PMN	Polymorphonuclear leukocytes
PPP	Platelet poor plasma
pVHL	Von Hippel-Lindau protein
RH123	Rhodamine123
ROS	Reactive oxygen species
rSMC	Rat smooth muscle cells
S1P	Shingosine1 Phosphate
SHR	Spontaneously hypertensive rats
SOD	Superoxide dismutase
TF	Tissue factor
TGF-β	Transforming growth factor-beta
TMB	Tetramethyl-benzidine
TNFα	Tumor necrosis factor alpha
TRAP	Thrombin receptor activating peptide
tPA	Tissue-type plasminogen activator
U-II	Urotensin-II
uPA	Urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor
Vit C	Vitamin C
VSMC	Vascular smooth muscle cells
vWF	von Willebrand factor

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Education

April 2002: I started the PhD project about the NADPH oxidases as sources of oxidative stress in vascular cells and the role of reactive oxygen species (ROS) as signaling molecules in the processes of vascular remodeling observed in diseases such as pulmonary hypertension at German Heart Center, Department of Experimental Pediatric Cardiology (Head PD Dr. med. Agnes Görlach). In August 2002 I was formally registrated as a PhD student under the supervision of Prof. Dr. Angelika Vollmar at Ludwig Maximilans University Munich, Faculty of Chemistry and Pharmacy, Department of Pharmacy-Center for Drug Research.

1996 till 2001: I graduated at the Chemical Faculty, Department of Biochemistry, University of Belgrade with average mark 9.09 out of 10, and mark 10 out of 10 for diploma thesis „Distribution of selenium in dry inactive cells of selenium enriched yeast and commercially available drugs of organic bonded selenium".

1992 till 1996: I finished the High School „Dimitrije Tucovic", Belgrade, with graduation mark 5 out of 5.

1984 till 1992: I finished the Elementary School „Cirilo i Metodije" with average mark 5 out of 5.

International experience

May-July 2001: As a member of IASTE (International Association for Student Exchange) I worked on several projects at the Institute of Biochemical Physics, Russian Academy of Sciences and at Department of Microbiology, Lomonosov University, Moscow, Russia.

Awards

Oktober 2003: I was one of the finalists selected for the competition „Young Investigator Award" organized by the Society for Microcirculation and Vascular Biology, on 18th October 2003 in Munich, where I successfully presented my project „Urotensin-II is a novel activator of the NADPH oxidase in pulmonary artery smooth muscle cells", Munich, Germany. The abstract was published in the Journal of Vascular Research 2004;41:91-120.

May 2002: I was awarded from Kostic Foundation for Chemical Sciences for the most successful diploma work in Yugoslavia in the field of biochemistry.

Oktober 2002: I was awarded from Serbian Chemical Society for special contributions during undergraduate studies.

Grants

In the period from December 2002 till November 2003 I was selected to receive the scholarship from Ludwig-Maximilians University in Munich (Frauenbeauftragte der LMU).

I received the travel grant to American Heart Scientific Sessions in New Orleans, 7-11 November 2004, from pharmaceutical company Aventis, Frankfurt am Main, Germany (Aventis [i]lab award).

I received the travel grant to 3rd European Meeting on Vascular Biology and Medicine in Hamburg, 28-30 September 2005 from pharmaceutical company Glaxo-Smith-Kline, Munich, Germany.

List of publications

Manuscripts

1. **Djordjevic T**, Hess J, Herkert O, Gorlach A, Belaiba RS. Rac regulates thrombin-induced tissue factor expression in pulmonary artery smooth muscle cells involving the nuclear factor-kappaB pathway. *Antioxid Redox Signal.* 2004;6:713-720.

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7. BelAiba RS, **Djordjevic T**, Bonello S, Artunc F, Lang F, Hess J, Gorlach A. The serum- and glucocorticoid-regulated kinase is involved in pulmonary vascular remodeling: Role in redox-sensitive regulation of tissue factor by thrombin. *Circ Res*; In revision.

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1. **T Djordjevic**, A Pogrebniak, C Zähringer, S BelAiba,S Bonello, T Kietzmann , H Acker, J Hess, A Görlach„ The NADPH oxidase subunit p22phox is regulated by redox-sensitive pathway in non-phagocytic cells“ European Journal of Physiology 2003; 445;47.

2. **T Djordjevic**, S BelAiba, A Pogrebniak, S Bonello, J Hess,A Görlach „Urotensin-II is a novel activator of the NADPH oxidase in pulmonary artery smooth muscle cells“ European Journal of Physiology 2003; 445;23.

3. S BelAiba, **T Djordjevic**, C Zähringer, S Bonello, A Pogrebniak, J Hess, T Kietzmann, A Görlach „Reactive oxygen species regulate the hypoxia-inducible factors HIF-alpha in pulmonary artery smooth muscle cells“ European Journal of Physiology 2003; 445;47.

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5. A Görlach, **T Djordjevic**, RS BelAiba, A Pogrebniak, S Bonello, O Herkert, T Kietzmann, J Hess „Tissue factor expression is promoted by thrombin via Rac and p21-activated kinase PAK in pulmonary artery smooth muscle cells“ Cardiology in the Young 2003; 13;40.

6. RS BelAiba, C Zähringer, **T Djordjevic**, S Bonello, A Pogrebniak, T Kietzmann, J Hess, A Görlach „Reactive oxygen species regulate the hypoxia-inducible transcription factor HIF and its target genes VEGF and PAI-1 in pulmonary artery smooth muscle cells“ Cardiology in the Young 2003; 13;68.

7. A Görlach, S BelAiba, **T Djordjevic**, S Bonello, A Pogrebniak, D Männel, A Samoylenko, O Herkert, J Hess, T Kietzmann „The p21- activated kinase regulates the expression of tissue factor by thrombin in pulmonary artery smooth muscle cells“ Z. Kardiologie 2003; 92; 69.

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9. **T. Djordjevic**, R.S. BelAiba, S. Bonello, J. Hess, A. Görlach „Urotensin-II Is a Novel Activator of the NADPH Oxidase in Pulmonary Artery Smooth Muscle Cells“. J Vasc Res 2004;41:91–120.

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14. R.S BelAiba, **T Djordjevic**, S Bonello, J Hess, T Kietzmann, A Görlach „VEGF expression is modulated by the redox-modifying enzymes catalase, gluthatione peroxidase and superoxide dismutase in pulmonary artery smooth muscle cells: role of reactive oxygen species”. Cardiology in the Young 2004; 14, 16.

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27. S Bonello, A Petry, RS BelAiba, **T Djordjevic**, C Zaehringer,J Hess, A Görlach „HIF-3alpha inhibits the hypoxic response in vascular cells". European Journal of Physiology 2005; 449, 1, 40

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30. S Bonello, Chr Zaehringer, RS Belaiba, **T Djordjevic**, T Kietzmann, C Michiels, J Hess, A Görlach „Oxidative stress and thrombin induce HIF-1α via activation of nuclear factor κB". Journal of Vascular Research 2005; 42, II/41

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34. **T Djordjevic**, R.S BelAiba, I Diebold, T Kietzmann, J Hess, A Görlach „Rac and PAK promote plasminogen activator inhibitor-1 expression and proliferation of pulmonary artery smooth muscle cells involving hypoxia-inducible factor-1". Journal of Vascular Research 2005; 42, II/116

Selected presentations on international conferences

1. Poster presentation on the Annual Meeting of the Society for Microcirculation and Vascular Biology, 10-12. October 2002 in Munich, Germany.

2. Oral presentation on the 82. Annual Meeting of German Physiological Society, 2-5. March 2003, in Bochum, Germany.

Poster presentation on the 82. Annual Meeting of German Physiological Society, 2-5. March 2003, in Bochum, Germany.

3. Oral presentation in the final of the Young Investigator Award on the Annual Meeting of the Society for Microcirculation and Vascular Biology, 16-18. October 2003 in Munich, Germany.

4. Poster presentation on the 83. Annual Meeting of German Physiological Society, 14-17. March 2004 in Leipzig, Germany.

5. Oral presentation on the 70. Annual Meeting of German Cardiac Society, 15-17. April 2004 in Mannheim, Germany.

6. Oral presentation on the XXXIX Annual Meeting of The Association for European Paediatric Cardiology, 19-22. May 2004 in Munich, Germany.

Oral presentation on the XXXIX Annual Meeting of The Association for European Paediatric Cardiology, 19-22. May 2004 in Munich, Germany.

Poster presentation on the XXXIX Annual Meeting of The Association for European Paediatric Cardiology, 19-22. May 2004 in Munich, Germany.

7. Poster presentation on the Scientific Sessions of American Heart Association, 7-10 November 2004 in New Orleans, Louisiana, USA.

8. Oral presentation on the 84. Annual Meeting of German Physiological Society, 6-9. March 2005, in Göttingen, Germany.

9. Oral presentation on the 5th European Conference on Endothelial cells, 23-25. September 2005 in Dresden, Germany.

10. Oral presentation on the 3rd European Meeting on Vascular Biology and Medicine, 28-30. September 2005 in Hamburg, Germany.

11. Poster presentation on the 3rd European Meeting on Vascular Biology and Medicine, 28-30. September 2005 in Hamburg, Germany.

12. Poster presentation on the 3rd European Meeting on Vascular Biology and Medicine, 28-30. September 2005 in Hamburg, Germany.