Inhibition of endothelial nitric oxide synthase increases capillary formation via Rac1-dependent induction of hypoxia-inducible factor-1α and plasminogen activator inhibitor-1

Andreas Petry*1; Rachida S. BelAiba*1; Michael Weitnauer1; Agnes Görlach1,2

1Experimental and Molecular Pediatric Cardiology, German Heart Center Munich at the Technical University Munich, Munich, Germany; 2Munich Heart Alliance, Munich, Germany

Summary
Disruption of endothelial homeostasis results in endothelial dysfunction, characterised by a dysbalance between nitric oxide (NO) and reactive oxygen species (ROS) levels often accompanied by a prothrombotic and proproliferative state. The serine protease thrombin not only is instrumental in formation of the fibrin clot, but also exerts direct effects on the vessel wall by activating proliferative and angiogenic responses. In endothelial cells, thrombin can induce NO as well as ROS levels. However, the relative contribution of these reactive species to the angiogenic response towards thrombin is not completely clear. Since plasminogen activator inhibitor-1 (PAI-1), a direct target of the proangiogenic transcription factors hypoxia-inducible factors (HIFs), exerts prothrombotic and proangiogenic activities we investigated the role of ROS and NO in the regulation of HIF-1α, PAI-1 and capillary formation in response to thrombin. Thrombin enhanced the formation of NO as well as ROS generation involving the GTPase Rac1 in endothelial cells. Rac1-dependent ROS formation promoted induction of HIF-1α, PAI-1 and capillary formation by thrombin, while NO reduced ROS bioavailability and subsequently limited induction of HIF-1α, PAI-1 and the angiogenic response. Importantly, thrombin activation of Rac1 was diminished by NO, but enhanced by ROS. Thus, our findings show that capillary formation induced by thrombin via Rac1-dependent activation of HIF-1 and PAI-1 is limited by the concomitant release of NO which reduced ROS bioavailability. Rac1 activity is sensitive to ROS and NO, thereby playing an essential role in fine tuning the endothelial response to thrombin.

Keywords
Thrombin, angiogenesis, nitric oxide, reactive oxygen species, PAI-1

Introduction
The maintenance of vascular wall integrity depends on endothelial homeostasis as characterised by endothelial cell quiescence and the presentation of an inert antithrombotic and anticoagulative surface to circulating cells. Disturbance of endothelial homeostasis can result in endothelial dysfunction at the early stage of all cardiovascular diseases. It comprises a shift of the vascular tone to a vasoconstrictive phenotype, mainly due to a reduction in the availability of nitric oxide (NO), which acts vasoprotective and vasodilatory. In addition, a prothrombotic state which includes initiation of clotting, inhibition of fibrinolysis and induction of endothelial proliferation and angiogenesis has been associated with endothelial dysfunction (1–3), and has been found in several cardiovascular diseases such as atherosclerosis or pulmonary hypertension (4–6).

While thrombin is the major activator of the coagulation cascade it can also activate intracellular pathways by binding and cleaving protease activated receptors (PARs) resulting in changes in gene expression, proliferation of smooth muscle and endothelial cells and even angiogenic responses (7, 8).

In endothelial cells, thrombin signalling exerts a Janus face: on the one hand, thrombin can enhance NO levels. On the other hand, thrombin has been shown to increase the generation of reactive oxygen species (ROS).

While endothelial NO synthase (eNOS) is the main source of NO in endothelial cells (9), and has been considered to exert a protective effect on the vessel wall, NADPH oxidases have been shown to contribute to endothelial ROS generation (10, 11). This family of multiprotein enzymes is dependent on the presence and function of a transmembrane catalytically active NOX subunit, which requires in the majority of cases to be stabilised and/or activated by several cofactors (12). Several family members, including NOX2 which is importantly involved in endothelial ROS generation (10, 12), require activation of the small G-protein Rac1 for generation of ROS. ROS can reduce the bioavailability of NO by either un...
coupling of eNOS or by formation of peroxynitrite, thereby promoting activation and dysfunction of the endothelium (13).

Interestingly, NO as well as ROS have been implicated in the modulation of angiogenesis (14, 15), although their exact contribution in controlling the angiogenic response towards thrombin is not fully resolved.

Under hypoxic conditions, hypoxia-inducible transcription factors (HIFs) are major regulators of angiogenesis by controlling the expression of proangiogenic and antiapoptotic factors (16). In addition to the HIF target vascular endothelial growth factor (VEGF) which is critically involved in controlling vessel formation, plasminogen activator inhibitor 1 (PAI-1), has also been reported to modulate angiogenesis (15). This serine protease inhibitor, which is also a HIF target, prevents activation of plasmin, and thus fibrinolysis, thereby acting as a prothrombotic factor. In addition, it can also cleave and activate matrix modulating factors, which may contribute to the reported proangiogenic effects (17). Thrombin has been previously shown to increase PAI-1 levels in endothelial cells (18), although the role of NO and ROS in the regulation of PAI-1 and the angiogenic response towards thrombin is not well resolved.

In this study we investigated the relative contribution of NO and ROS to the regulation of HIF-1α and PAI-1 and to angiogenesis in thrombin-stimulated endothelial cells. We found that thrombin enhanced ROS generation in endothelial cells involving Rac1, concomitantly supporting NO formation by eNOS. While inhibition of Rac1-dependent ROS formation prevented induction of HIF-1α and of its target PAI-1 and subsequently angiogenesis induced by thrombin, inhibition of NO synthase enhanced HIF-1α, PAI-1 and angiogenesis under control and thrombin-stimulated conditions. Our findings indicate that formation of NO is a limiting factor to the angiogenic response by thrombin, which affects activation of Rac1 and subsequently ROS bioavailability as well as induction of HIF-1α and PAI-1 and suggest Rac1 as an essential redox-sensitive target important for fine tuning the endothelial proliferative responses in cardiovascular disorders associated with reduced NO bioavailability and enhanced levels of thrombin.

Materials and methods

Reagents

N-omega-nitro-L-arginine (L-NNA) and NG-nitro-L-arginine methyl ester (L-NAME) were from Calbiochem (Darmstadt, Germany). Human α-thrombin was from Haemochrom Diagnostika (Essen, Germany). All other chemicals were from Sigma (Taufkirchen, Germany).

Cell culture

The human microvascular endothelial cells HMEC-1, purchased from CDC (Atlanta, GA, USA), were grown in MCDB131 medium supplemented with 2 mM L-glutamine, 10 ng/ml EGF, 1 μg/ml hydrocortisone (all Invitrogen, Karlsruhe, Germany), 10% fetal calf serum (FCS) (PAN Biotech, Aidenbach, Germany), and 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen). The human embryonic kidney cell line HEK293 (ATCC CRL-1573) was cultured in DMEM (Invitrogen) with 1 g/l glucose, 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells were grown at 37°C and 5% CO₂ and passaged twice a week. Prior to stimulation cells were starved for 16 hours (h). Spermine-NONOate was dissolved in 0.01 M NaOH. All other reagents have been dissolved in double distilled water, and adequate amounts of solvent have been added to controls. Ascorbic acid or spermine-NONOate in the concentrations and applications used did not substantially change pH in buffered culture media.

Plasmids

The luciferase reporter gene construct EPO-HRE, the luciferase reporter gene constructs containing wild-type and HRE mutated PAI-1 promoter (kindly provided by Dr. Kietzmann, University of Oulu, Finland) and the expression plasmids encoding Rac1 G12V and T17N mutants were previously described (19–21).

Cell transfections and luciferase assay

HMEC-1 cells were transfected at a confluency of 60–70% using Superfect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. 24 h after transfection, cells were serum deprived for 16 h and then stimulated with thrombin (3 U/ml). For the luciferase activity assay, HMEC-1 or HEK293 cells were transfected in 24 well plates (25,000 cells/well). After 2 h the medium was changed and cells were cultured for 7 h. Cells were serum starved for 16 h and then stimulated with thrombin for 4 h. In some experiments cells were pre-incubated with inhibitors or antioxidants for 30 minutes (min) and then exposed to stimuli.

Measurement of ROS production

HMEC-1 cells were pretreated or not with inhibitors for 30 min and stimulated with thrombin as previously described (22). Generation of ROS was then detected using the fluoroprobe dihydroethidium (DHE, Invitrogen) or 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein di-acetate acetyl ester (CM-H2DCFDA, Invitrogen) as described (23). After stimulation cells were washed with HBSS (Invitrogen), loaded with DHE (50 μM) or CM-H2DCFDA (8.5 μM) for 10 min and fluorescence was analysed using a microplate reader (Tecan, Crailsheim, Germany). Fluorescence signal was normalised to viable cells using AlamarBlue assay (Invitrogen).
Visualisation of ROS production

Generation of ROS was visualised by the fluorophore dihydroethidium (DHE) as described (23). Serum starved HMEC-1 cells were pretreated or not with inhibitors for 30 min prior to thrombin stimulation. Cells were then washed with HBSS and incubated in the dark with DHE (50 μM) for 10 min at 37°C. Thereafter, cells were washed with HBSS to remove excess dye. Fluorescence was monitored in a fluorescence microscope (Olympus, Hamburg, Germany) and images were obtained using the Openlab Modular Software for Scientific Imaging (Improvison, Tübingen, Germany).

NO measurements

Cells were made quiescent in serum-free medium for 16 h prior to thrombin stimulation. Generation of NO was then detected using the fluoroprobe 4,5-diaminofluoresceine diacetate (DAF2, Invitrogen). Cells were incubated in the dark with DAF2 (10 μM) for 10 min at 37°C. Thereafter, cells were washed with PBS to remove excess dye and fluorescence was monitored in a microplate reader (Tecan). At the end of the experiment, DAF2 fluorescence was normalised to viable cells using the AlamarBlue assay (Invitrogen).

Western blotting

Fifty μg of isolated proteins (cell lysates or culture supernatant) were separated by SDS-PAGE and transferred to nitrocellulose membranes as described (12). After blocking for 2 h in TBS containing 5% non-dry milk and 0.3% Tween 20, membranes were incubated overnight at 4°C with specific antibodies against Rac1, HIF-1α, eNOS (all from BD Transduction, Heidelberg, Germany), PAI-1 (American Diagnostica, Pfungstadt, Germany), c-myc (Santa Cruz, Karlsruhe, Germany), GFP (Roche Diagnostics, Mannheim, Germany) or serine 1177-phosphorylated eNOS (Cell Signaling, Danvers, MA, USA). After incubation with a horseradish peroxidase-conjugated secondary antibody (Calbiochem) for 1 h, proteins were visualised by performing luminol-enhanced chemiluminescence. Loading of equal amounts of proteins was confirmed by reprobing the membranes with a β-actin antibody (Santa Cruz) or by ponceau S staining.

Northern blot analysis

Total RNA from HMEC-1 was isolated and subjected to Northern blot analysis using a digoxigenin-labeled antisense RNA probe for human PAI-1 detection as described (24). Probe was detected using an anti-digoxigenin antibody (Roche).

Rac activity assays

Rac1 activity was evaluated with an affinity precipitation assay by using the PAK-1 PBD conjugated glutathione agarose beads, according to the manufacturer’s instructions (Upstate, Hamburg, Germany), as described previously (25).

Proliferation assays

DNA synthesis in HMEC-1 cells was assessed by 5-bromo-2’-deoxyuridine (BrdU) labelling (Roche) as described previously (12). In brief, HMEC-1 cells were seeded in 96-well plates at a density of 2,000 cells/well and deprived from serum for 16 h prior to inhibitor pre-treatment and stimulation with thrombin. Thereafter, cells were grown in the presence of BrdU (10 μM) for 16 h, fixed for 30 min and incubated for 1 h with the peroxidase-conjugated antibody against BrdU. Immunodetection was performed by adding the colorimetric substrate tetramethylbenzidine (TMB) and by measuring the absorbance in an ELISA reader (Tecan).

Angiogenesis assays

Transfected or wildtype HMEC-1 cells were plated in 6-well plates and incubated in 2% serum containing medium for 16 h prior to stimulation with thrombin (3 U/ml) in presence or in absence of inhibitors as previously described (23). A total of 50,000 cells were seeded per well of a 48-well plate mounted with growth factor reduced Matrigel (BD Biosciences). Cells were then incubated for 8 h at 37°C and stained with calcein AM (BD Biosciences) according to the manufacturer’s instructions. The formation of capillary-like structures was assessed by fluorescence microscopy (Olympus) using the Openlab Modular Software for Scientific Imaging (Improvison) and was quantified using Image J software (Wright cell imaging Facility, Toronto, ON, Canada).

For the spheroid sprouting assay, endothelial cell spheroids of defined cell number were generated as described previously (26). In brief, 750 HMEC-1 cells per spheroid were mixed with HMEC-1 full medium containing 20% methocel (Sigma), seeded in non-adherent round bottom 96-well plates (Greiner, Frickenhausen, Germany) and cultivated for 24 h. Then spheroids were embedded in growth factor reduced Matrigel (BD Biosciences) containing thrombin (3 U/ml) in the presence or absence of L-NAME (100 μM). Cell sprouting was analysed after 48 h using phase contrast microscopy.

Statistical analysis

Optimal sample sizes were calculated by power analysis using power.t.test function in R 2.15 (The R foundation, Vienna, Austria). The sample size (number of independent experiments)
required to reach an experimental power of 0.8 at a p-value threshold of 0.05 ranged for the different types of experiments between 2 and 4. Independent endothelial cell preparations adjusted to the required sample size were then used. If not stated otherwise in the text, three independent experiments were performed. Values presented are means ± standard deviation. Results were compared by ANOVA for repeated measurements followed by Student-Newman-Keuls t-test. A value of \( p < 0.05 \) was considered statistically significant as stated in the text.

Results

Rac1 and ROS mediate thrombin-induced capillary formation

A prothrombotic state has been associated with changes in angiogenesis. Previously we could show that thrombin is able to enhance angiogenic responses of endothelial cells in a redox-dependent manner (23, 27). However, thrombin has been shown not only to increase ROS formation in endothelial cells (22), but also to enhance the levels of NO, and both reactive species have been implicated in the regulation of angiogenesis. We therefore were interested in the relative contribution of ROS and NO to control angiogenic responses towards thrombin and investigated capillary tube formation as part of the angiogenic process.

In this aspect, NADPH oxidases have been identified as important sources of ROS in endothelial cells. We have previously shown that thrombin can rapidly activate the GTPase Rac1 in endothelial cells (27) which is required for activation of several NADPH oxidases including NOX1 and NOX2. In fact, expression of a dominant-negative Rac1 mutant (RacT17N) reduced thrombin-stimulated ROS generation determined by DCF fluorescence (Fig. 1A), whereas expression of constitutively active Rac1 enhanced ROS levels (see Suppl. Fig. 1, available online at www.thrombosis-online.com).

Subsequently, thrombin not only activated Rac1 as has been shown previously (27) but also enhanced the levels of Rac1 (Fig. 1B).

![Figure 1: Thrombin-stimulated ROS generation and capillary formation is mediated by Rac1.](image)

A) Human microvascular endothelial cells (HMEC-1) were transfected with plasmids coding for a dominant negative mutant of Rac1 (T17N) or control vector (Ctr) prior to thrombin stimulation (3 U/ml) for 2 h and ROS generation was analysed by DCF fluorescence. Data are presented as relative change to unstimulated cells transfected with control vector (Ctr) set to 100% (n=3; *p<0.05 thrombin stimulated cells vs. control cells, #p<0.05 T17N transfected cells vs. control vector transfected cells, STD). B) HMEC-1 cells were stimulated with thrombin (3 U/ml) for the indicated time points and Rac1 expression was analysed by Western blot. Actin served as loading control. A representative blot from three independent experiments is presented. C) HMEC-1 cells were transfected with dominant negative Rac1 (T17N), constitutively active Rac1 (G12V) or control vector (Ctr) prior to thrombin stimulation (3 U/ml) for 4 h. Formation of capillary like structures was analysed using matrigel assay. A representative experiment from 3 independent experiments is shown. D) HMEC-1 cells were pretreated for 30 min with ascorbate (Asc, 100 μM) prior to stimulation with thrombin for 4 h, and formation of capillary like structures was analysed in a matrigel assay. A representative experiment from three independent experiments is shown.
Stimulation with thrombin significantly increased capillary formation in the matrigel assay by 177.7 ± 4.1% compared to untreated controls while expression of dominant negative Rac1 abolished thrombin-induced capillary formation to 89.8 ± 8.1% of control levels (Fig. 1C). Similarly, application of the antioxidant ascorbate significantly reduced thrombin-stimulated capillary formation (165.8 ± 15.7% of control) to 107.8 ± 4.9% of control levels (Fig. 1D). In contrast, expression of a constitutively active Rac1 mutant significantly increased capillary formation by 177.5 ± 31.6% similar to the situation with thrombin (Fig. 1C) further indicating that activation of Rac1 and subsequent ROS generation promotes capillary formation in thrombin-stimulated endothelial cells.

Rac1-dependent ROS promote capillary formation to thrombin by upregulating the HIF target PAI-1

PAI-1, which inhibits fibrinolysis and therefore promotes a prothrombotic state, has been previously associated with an angiogenic response (17). In line, application of an inhibitory antibody against PAI-1 diminished endothelial proliferation in response to thrombin to a similar extent as ascorbate (Fig. 2A). Subsequently, thrombin significantly stimulated capillary formation to 180.1 ± 8.7% over control levels while application of the inhibitory antibody against PAI-1 abolished capillary formation to 89.0± 21.3% of control levels (Fig. 2B) indicating that PAI-1 contributes to ROS-dependent capillary formation in response to thrombin.

Concomitantly, thrombin was able to induce PAI-1 mRNA levels as well as the release of PAI-1 protein in a time dependent manner in endothelial cells (Fig. 2C, D).

The upregulation of PAI-1 by thrombin was inhibited in the presence of the antioxidants ascorbate and N-acetyl-cysteine indicating an involvement of ROS (Fig. 2E, F). In addition, expression of dominant-negative Rac1 blocked thrombin-induced PAI-1 expression (Fig. 2G).

Furthermore, thrombin-induced PAI-1 expression was dependent on activation of HIF transcription factors, since mutation of the hypoxia response element in the PAI-1 promoter blocked thrombin-induced PAI-1 promoter activity (Fig. 3A).

In line, thrombin was able to upregulate HIF-1α protein levels (Fig. 3B) and to enhance HIF activity (Fig. 3C) in endothelial cells. Concomitantly, thrombin induction of HIF-1α protein was abolished in cells expressing the dominant negative Rac1 mutant (Fig. 3D) while expression of the constitutively active Rac1 mutant enhanced HIF-1α levels. This latter response was blocked by treatment with ascorbate (Fig. 3E) indicating that Rac1-dependent ROS formation contributes to induction and activation of HIF-1α and subsequent upregulation of PAI-1 expression resulting in an angiogenic response towards thrombin.

NO production modulates endothelial cell responses to thrombin

Next we aimed to evaluate the role of NO in the thrombin-induced angiogenic response. First we determined DAF2 fluorescence as an indicator of NO production (28) (Fig. 4A). As has been shown previously, thrombin enhanced DAF2 fluorescence. In line, thrombin rapidly increased phosphorylation and thus activation of endothelial NO synthase (eNOS) (Fig. 4B). As expected L-NNA, known to inhibit coupled and uncoupled endothelial NO synthase (29), decreased DAF2 fluorescence. Pre-treatment with the antioxidants ascorbate or N-acetyl-cysteine or expression of the dominant negative Rac1 mutant also decreased thrombin-induced phosphorylation of eNOS (Fig. 4C) indicating that ROS derived from Rac1 are involved in activation of eNOS.

To investigate the role of eNOS inhibition on capillary formation, we then treated endothelial cells with L-NNA (Fig. 4D). Interestingly, L-NNA did not inhibit but enhanced tube formation. Similarly, L-NAME enhanced capillary formation in a sphere sprouting assay (see Suppl. Fig. 2, available online at www.thrombosis-online.com). In contrast, treatment with the NO donor spermineNONOate reduced capillary formation (Fig. 4E). The ability to form capillary like structures was also reduced when a NO donor was added to cells treated with L-NAME (see Suppl. Fig. 3, available online at www.thrombosis-online.com).

In line, treatment with L-NNA also increased the levels of active Rac1 as well as of HIF-1α and PAI-1 under basal conditions and in the presence of thrombin (Fig. 4F-H).

We then determined the effects of L-NNA treatment on ROS levels using DHE fluorescence. However, L-NNA did not affect thrombin-induced ROS generation suggesting that uncoupled eNOS did not contribute to increased ROS levels by thrombin. Interestingly, L-NNA increased basal ROS levels, and this response was reduced by treatment with the antioxidant ascorbate similar to the situation with thrombin (Fig. 5A, B). In line, ascorbate reduced capillary formation not only towards thrombin (Fig. 1E), but also towards L-NNA (Fig. 5C) and prevented activation of Rac1 by L-NNA (Fig. 5D). Together, these findings suggested that decreased bioavailability of ROS may contribute to the effects observed upon treatment with eNOS inhibitors.

In this regard the formation of peroxynitrite which can rapidly occur in the presence of superoxide and NO could not only decrease the bioavailability of NO similar to the situation with eNOS inhibitors, but also the availability of ROS as with dominant negative Rac1 or antioxidants. Interestingly, thrombin stimulation enhanced the levels of 3-nitro tyrosinated proteins (see Suppl. Fig. 4, available online at www.thrombosis-online.com) as a surrogate marker for peroxynitrite action. However, application of the peroxynitrite donor SIN-1 significantly decreased capillary formation by 18.4 ± 7.9% (10 μM) and 50 ± 5.3% (100 μM) compared to controls (Fig. 5E) while treatment with uric acid, which can scavenge peroxynitrite, enhanced the formation of capillary like structures (Fig. 5F).

These results suggest that under basal conditions, NO is diminishing the levels of ROS and this limits tube formation either di-
Figure 2: Upregulation of PAI-1 involving Rac1 and ROS mediates thrombin-induced capillary formation and proliferation. A) Human microvascular endothelial cells (HMEC-1) were pretreated for 30 min with an inhibitory PAI-1 antibody (PAI-1 AB) or with control IgG (Ctr) in the presence or absence of ascorbate (Asc, 100 μM). Proliferation after thrombin stimulation (3 U/ml) for 14 h using BrdU incorporation assay was analysed. Data are presented as relative change to control (Ctr) set to 100% (n=3; *p<0.05 thrombin stimulated cells vs. control cells, #p<0.05 ascorbate or PAI-1 antibody treated cells vs. thrombin-only treated cells). B) HMEC-1 cells were pretreated for 30 min with an inhibitory PAI-1 antibody (PAI-1 AB) or control IgG (Ctr), and formation of capillary like structures was analysed in a matrigel assay after 4 h of thrombin stimulation. A representative experiment from three independent experiments is shown. C, D) HMEC-1 cells were stimulated with thrombin for indicated time points and Northern blot analysis for PAI-1 expression (C) or Western blot analysis for secreted PAI-1 (D) were performed. Data are presented as relative change to unstimulated cells (0 h) set to 100% (n=3; *p<0.05 thrombin stimulated cells vs. unstimulated cells). Staining of 18S (C) or Ponceau S staining (D) worked as loading control. E, F) HMEC-1 cells were pretreated with either ascorbate (Asc, 100 μM) (E) or N-acetyl-cysteine (NAC, 5 mM) (F) for 30 min prior to stimulation with thrombin for 4 h. Western blot analysis for secreted PAI-1 was performed. Ponceau S staining served as loading control. A representative blot from three independent experiments is presented. G) HMEC-1 cells were transfected with dominant negative Rac1 (T17N) or control vector (Ctr) prior to thrombin stimulation for 16 h. Western blot analysis of secreted PAI-1 was performed. Ponceau S staining served as loading control. A representative blot from three independent experiments is presented.
rectly or via formation of peroxynitrite (Fig. 6A). Stimulation with thrombin increases ROS generation which might overwhelm the limiting actions of peroxynitrite thus allowing capillary formation (Fig. 6B). NO-dependent inhibition of Rac1 activation and ROS-mediated activation of Rac1 and eNOS might help to fine tune the angiogenic response to thrombin.

Discussion

Enhanced levels of thrombin have been described in a variety of cardiovascular diseases. Thrombin has been implicated to modulate endothelial homeostasis in addition to its prothrombotic effects, and to activate various signalling pathways either protecting or activating the endothelial cell layer. In this study we showed that the concomitant release of NO and ROS by thrombin is able to control and fine tune capillary formation by modulating activation of the GTPase Rac1 which is central in the control and subsequent activation of the transcription factor HIF-1α and the proangiogenic target PAI-1. This assumption is supported by our findings that 1) thrombin activated and induced Rac1 in a ROS-dependent manner, leading to enhanced levels of ROS, HIF-1α and PAI-1. 2) Rac1-dependent ROS formation, HIF-1α and PAI-1 were able to promote capillary formation by thrombin. 3) Inhibition of NO formation by eNOS increased Rac1 activity, ROS generation, induction of HIF-1α and PAI-1 as well as capillary formation towards thrombin. 4) Application of NO or peroxynitrite donors decreased, while treatment with a peroxynitrite scavenger enhanced capillary formation.

Figure 3: Thrombin-induced PAI-1 expression is mediated by HIF-1α.
A) Human microvascular endothelial cells (HMEC-1) were transfected with luciferase constructs containing the proximal PAI-1 promoter (PAI-1) or the PAI-1 promoter mutated at the hypoxia responsive element (HRE) (PAI-1 mut) and luciferase activity was measured after thrombin stimulation (3 U/ml) for 8 h. Data are presented as relative change to unstimulated cells transfected with pGL-PAI-1 (PAI-1) set to 100% (n=3; *p<0.05 thrombin stimulated vs. unstimulated cells, #p<0.05 PAI-1 mut vs. PAI-1). B) HMEC-1 cells were stimulated with thrombin (3 U/ml) for 4 or 8 h and Western blot analysis for HIF-1α was performed. Actin served as loading control. A representative blot from three independent experiments is presented. C) HMEC-1 cells were transfected with a luciferase construct driven by three EpoHRE sites to determine HIF activity. Luciferase assay was performed after thrombin stimulation (3 U/ml) for 8 h. Data are presented as relative change to unstimulated cells (Ctr) (n=3; *p<0.05 Thrombin stimulation vs. control cells). D) HMEC-1 cells were transfected with a vector coding for dominant negative Rac1 (T17N) or with control vector (Ctr) prior to stimulation with thrombin (3 U/ml) for 4 h. Western blot analysis for HIF-1α was performed. Actin served as loading control. A representative blot from three independent experiments is presented. E) HMEC-1 cells were transfected with a constitutively active mutant of Rac1 (G12V) prior to treatment with ascorbate (Asc, 100 μM) for 1 h and Western blot analysis for HIF-1α was performed. Actin served as loading control. A representative blot from three independent experiments is presented.
Thrombin-activation of Rac1 is redox-sensitive and increases HIF-1α and PAI-1 levels

In this study we demonstrated that thrombin enhanced ROS generation by activation and induction of Rac1 in human microvascular endothelial cells, similar to the situation in human umbilical vein endothelial cells (HUVEC) (27) or smooth muscle cells (19). Rac1-mediated ROS generation has been related to activation of NADPH oxidases containing NOX1, NOX2 or NOX3. In particular, NOX2 has been found to be important for endothelial ROS generation (10, 12, 30), and was also here importantly involved in thrombin-induced ROS generation (data not shown) similar to the situation described in thrombin-stimulated HUVEC (27). In addition, NOX4 and NOX5 have been associated with endothelial ROS generation (12, 23). While Rac1 does not seem to be required for NOX4-dependent ROS generation, it can interact with NOX5.
and might contribute to NOX5-dependent ROS generation as well (data not shown) further confirming the importance of this small protein in controlling endothelial ROS generation.

Interestingly, Rac1 activation by thrombin was diminished by antioxidants indicating that ROS promote activation of this GTPase in a feed forward loop similar to a situation previously described in the setting of hypoxia-reoxygenation (31). Although the exact mechanisms of ROS-dependent Rac1 activation are not completely elucidated, our previous observation that thrombin can increase cGMP levels in endothelial cells, and that 8-bromo-cGMP can activate Rac1 and elevate ROS levels in these cells (27), may also contribute to increased Rac1 activity in this study.

We could previously show in endothelial EaHy cells that thrombin elicits a biphasic increase in ROS generation with a fast response possibly mediated by initial activation of Rac1, and a sustained prolonged response (22) which may account for further activation of Rac1 as well as for the induction of the Rac1 protein itself. In support, we could previously show that thrombin transcriptionally induces Rac1 via HIF-1 (25) suggesting that in addition to the redox-sensitive activation of Rac1, HIF-1-dependent upregulation of Rac1 further contributes to maintaining the availability of Rac1 for mediating thrombin signalling. In this regard, thrombin enhanced HIF-1α protein levels involving Rac1 in endothelial cells similar to the situation in thrombin-stimulated smooth muscle cells (25) where ROS have been shown to increase HIF-1α mRNA levels via nuclear factor κB-dependent transcription (32) or inhibition of prolyl hydroxylases thereby increasing HIF-1α stability (33). The importance of Rac1-dependent HIF-1α induction by thrombin is further supported by the findings that the HIF target gene PAI-1 is also upregulated and released in response to this pathway. In line, PAI-1 expression and promoter activity were shown in endothelial cells to be controlled in a redox-dependent manner in response to tumour necrosis factor-α or to hydrogen peroxide (H₂O₂) (34). Similarly, angiotensin II-stimulated PAI-1 expression was inhibited in rat endothelial cells by N-acetyl cysteine or the NADPH oxidase inhibitor DPI (35). In addition, Rac1 and HIF-1α have been shown to promote thrombin-induced PAI-1 expression in smooth muscle cells (25, 32, 33), further confirming the importance of redox-sensitive activation of Rac1 in thrombin-induced PAI-1 generation.

**Thrombin-induced angiogenesis is regulated by Rac1**

Thrombin-induced PAI-1 generation can promote a prothrombotic and profibrotic state (36). Our data show, that this pathway, activated by Rac1 and HIF-1α in endothelial cells, also induces an angiogenic response. While thrombin was described to be a potent angiogenic factor in vitro as well as in vivo (4, 7, 36), molecular mechanisms involved in thrombin-stimulated angiogenesis are not well documented. Thrombin reduced the ability of endothelial cells to attach to the basal membrane and increased dissolution of the basal membrane by activating metalloproteinases. It further increases the expression of VEGF receptors in endothelial cells and promotes αβ3-dependent endothelial cell survival, which is required for endothelial cell detachment from their anchorage site on the basement membrane and their migration to distal sites during angiogenesis (37). Here we now demonstrate that PAI-1 is also importantly involved in the angiogenic response towards thrombin. The role of PAI-1 in mediating angiogenesis is rather complex. As PAI-1 is an important inhibitor of plasminogen activation one would expect that PAI-1 would inhibit angiogenesis. However, both proangiogenic and angiostatic as well as pro- and antiproliferative capacities were associated with PAI-1 (36). Although the exact reasons for these apparently conflicting data are still not completely resolved, the overall effect of PAI-1 on angiogenesis seems to depend on the cellular and structural context and the vascular bed as well as on the concentration of PAI-1. At lower concentrations as can be found in our experimental settings, PAI-1 may limit the activities of proteases to degrade matrix proteins thereby promoting an angiogenic response (38).

In line with our findings that active Rac1 is able to enhance capillary formation, it has been shown that expression of active or inactive Rac1 modulates angiogenesis in a tube forming assay (39). Endothelial cell proliferation, migration and tube formation are essential processes during angiogenesis and Rac1 activity is believed to be important for these cellular processes in vitro (27). Interestingly, in the in vivo setting, controversial results have been observed regarding the involvement of endothelial Rac1 in angiogenesis which seem to depend on differences in the role of endothelial Rac1 in embryonic versus adult vessel formation, and in the role of...
Figure 5: Crosstalk between NO and ROS modulates capillary formation. A, B) Human microvascular endothelial cells (HMEC-1) were pre-incubated with N-omega-nitro-L-arginine (L-NNA, 100 μM), ascorbate (Asc, 100 μM) or both (B) for 30 min prior to thrombin stimulation (3 U/ml) for 2 h. ROS generation was visualised by DHE staining (A) or measured by DHE fluorescence in a multiplate reader (B). Data are presented as relative change to untreated cells set to 100% (n=3; *p<0.05 treated cells vs. untreated control cells, #p<0.05 ascorbate treated cells vs. thrombin treated cells). Representative experiment of three independent experiments shown. C) HMEC-1 cells were treated with L-NNA (100 μM), ascorbate (Asc, 100 μM) or both and the formation of capillary like structures was analysed after 4 h in a matrigel assay. Data are presented as relative change to untreated cells set to 100% (n=3; *p<0.05 treated cells vs. untreated control cells, #p<0.05 ascorbate and L-NNA treated cells vs. L-NNA treated cells). D) HMEC-1 cells were treated with L-NNA (100 μM) in the presence or absence of ascorbate (Asc, 100 μM) for 15 min and pulldown of active Rac1 was performed followed by Western blot analysis for active Rac1 (GTP-Rac1) or total Rac1. A representative blot from three independent experiments is presented. E) HMEC-1 cells were treated with different concentrations of the peroxynitrite donor 3-morpholinosydnonimine (SIN-1) for 4 h and formation of capillary like structures was analysed by in vitro matrigel assay. A representative blot from four independent experiments is presented. F) HMEC-1 cells were treated with the peroxynitrite scavenger uric acid (100 μM) and formation of capillary like structures was analysed after 4 h. Data are presented as relative change to untreated cells (Ctr) set to 100%. (n=3; *p<0.05 uric acid treated cells vs. control cells).
Rac1 in basal versus stimulated endothelial cells (40, 41). In line with our results, activated Rac1 positively affected angiogenesis while basal, unstimulated Rac1 did not seem to be required for an angiogenic response (41). Thus, one might speculate that in conditions where Rac1 is activated as in our setting, Rac1 can increase ROS generation and thereby promote pro-angiogenic signalling, while Rac1-dependent effects on the cytoskeletal structure alone may not be sufficiently required to enhance angiogenesis.

**NO formation limits Rac1-dependent ROS generation**

In addition to ROS generation, thrombin has been reported to activate eNOS to form NO in endothelial cells (4, 7) although the exact relevance of NO versus ROS generation in the response to thrombin is not completely clear.

In our setting, thrombin induced eNOS phosphorylation at serine 1177 which is important for NO production, and increased DAF2 fluorescence as an indicator of NO generation. Application of the eNOS inhibitor L-NNa reduced thrombin-induced DAF2 fluorescence, but not DHE fluorescence as indicator of ROS generation. Since L-NNa blocks coupled as well as uncoupled eNOS (29) these data argue against a major role of eNOS uncoupling in ROS generation by thrombin. Importantly, L-NNa was able to increase ROS levels under basal conditions, and this response was prevented by antioxidants suggesting that in our setting NO reduced ROS bioavailability.

Our data further suggest that NO prevents activation of endothelial cells by reducing ROS bioavailability under basal and thrombin stimulated conditions since L-NNa increased, similar to thrombin, Rac1 activity, and both responses were prevented by antioxidant treatment. In support, inhibition of eNOS was shown to increase membrane translocation of Rac1 in cerebral vessels concomitant with an increase in ROS levels (31), suggesting that NO formation prevents Rac1 activation thereby limiting the positive feedback loop initiated by thrombin and Rac1 stimulated ROS generation.

Our data also show that ROS are positively involved in activation of eNOS since antioxidants prevented phosphorylation of eNOS by thrombin. These data suggest, that ROS derived from Rac1 themselves promote fine tuning of the balance between NO and ROS in response to thrombin due to stimulation of a negative feedback by increasing NO levels. In line with our observations levels of phosphorylated eNOS and NO were decreased in endothelial cells treated with a Rac inhibitor or in vessels derived from endothelial-specific Rac1 haploinsufficient mice (EC-Rac1-/-) (40).

In this regard, it has been shown that Rac1 can interact with eNOS thus leading to enhanced NO formation concomitant to elevated levels of ROS (42). Although in our experimental setting we could not observe a physical interaction between Rac1 and eNOS, the observation that Rac1 can regulate phosphorylation of eNOS which should induce subsequent NO generation together with our observation that NO can limit Rac1 activation further support our model that Rac1 is a central key to fine tuning the redox-dependent response towards thrombin in endothelial cells.

**NO formation limits induction of capillary formation, HIF-1α and PAI-1**

Our notion that NO may act to limit activation of endothelial cells under basal conditions, but also in the response to thrombin by reducing ROS availability is further supported by our findings that application of L-NNa or L-NAME increased capillary formation in the matrigel tube formation assay as well as in the spheroid sprouting assay and that antioxidant treatment prevented this response. While the importance of NO in regulating angiogenic pathways has been well documented, the exact mechanisms are still unclear and controversial (for review see [43]). In fact, NO has been shown to induce various angiogenic responses in different model systems but also to exert anti-angiogenic effects (15, 44–46).

NO has been reported to mediate VEGF-controlled angiogenesis since VEGF is able to induce eNOS and NO generation in endothelial cells, whereby NO increases VEGF production in smooth muscle or other non-endothelial cells, which then stimulates angiogenesis in endothelial cells (43, 47–50). In accordance with these data, requiring the cross-talk between different organs and/or cell types, wound healing or ischaemia-induced angiogenesis were diminished in mouse models deficient in eNOS (51). However, it has also been reported that in the consequence of vascular injury, NO secreted by a restored endothelium functions as the negative feedback mechanism that downregulates VEGF expression to basal levels in smooth muscle cells (52) further supporting the concept that NO limits endothelial activation. While the role of NO in controlling angiogenesis in vivo or in multicellular

---

**Figure 6: Scheme of the interaction between NO and ROS under basal and thrombin stimulation.**

A) In endothelial cells, basal NO generation by the endothelial NO synthase (eNOS) limits ROS bioavailability due to formation of peroxynitrite thereby preventing an angiogenic response. B) Thrombin enhances, in addition to NO formation by eNOS, ROS generation due to activation of Rac1 thereby exceeding the inhibitory effect of NO resulting in induction and activation of the HIF pathway and PAI-1 dependent angiogenesis. This mechanism can be positively modulated by a further activation of Rac1 by ROS, but is limited by inactivation of Rac1 by NO and eNOS by Rac1 and ROS thereby preventing exceeding responses to thrombin.
The variability of endothelial angiogenic responses towards modulation of NO availability indicates that various factors including the model and context, concentration of inhibitors or donors, the nature of stimuli and the overall redox state contribute to the overall angiogenic response towards NO (60, 61). The latter notion is supported by our findings that L-NNA-induced capillary formation was reversed by application of antioxidants. Furthermore, the rapid interaction of NO with superoxide to form peroxynitrite affects redox state and the bioavailability of NO and ROS. In fact, thrombin further enhanced peroxynitrite levels as suggested by enhanced 3-nitrotyrosine levels, similar to the action of the NO donor (data not shown). In contrast, application of a peroxynitrite donor reduced capillary formation while application of the peroxynitrite scavenger uric acid enhanced capillary formation. Thus, under basal conditions, NO might decrease ROS bioavailability limiting capillary formation either directly or due to the action of peroxynitrite. Upon stimulation with thrombin, the increase in ROS and NO generation may lead to enhanced levels of peroxynitrite, although in summary the increase in ROS generation might overwhelm the limiting actions of peroxynitrite thus allowing capillary formation.

These findings suggest that the fine balance between NO and ROS bioavailability is important for the activation of endothelial cells and subsequent formation of capillaries. In support, the proangiogenic effect of uric acid has been reported previously (62), although other compounds which reduced peroxynitrite action had opposite effects (63, 64).

However, the “pro-angiogenic” effect of L-NNA in our setting was clearly supported by our findings that inhibition of eNOS increased basal and thrombin-stimulated levels of HIF-1α, which is known to promote angiogenesis in different model systems. In line with the notion that NO may limit HIF-1 activity in endothelial cells application of low concentrations of NO donors has been shown to limit HIF-1α induction and activity under hypoxic conditions (65), for example by increasing mitochondrial release of the prolyl hydroxylase (PHD) cofactors iron and 2-oxoglutarate (66). Interestingly, nitro-glycerine treatment has even been associated with reduced HIF-1α levels in patients with lung carcinoma (67).

In contrast, although dependent on the type of NO donor (68), high concentrations of NO donors were shown to induce HIF-1α levels and activity under normoxic and even under hypoxic conditions (65) and this response has been related to inhibition of HIF-1α hydroxylation or even to direct S-nitrosylation of this protein (69). While we have not specifically examined the role of NO donors on HIF-1α levels, our study that inhibition of eNOS increases HIF-1α levels was supported by findings in HUVEC where the eNOS inhibitor L-NNAME or eNOS silencing increased HIF-1α levels (67) as well as in a model of preeclampsia where L-NNAME treatment increased fetal and maternal HIF-1α levels in (70).

While it is not clear whether inhibition of eNOS can directly inhibit the pVHL/PHD system, or whether it leads, as has been reported previously (44), to mitochondrial dysfunction with decreased energy production thereby inducing a state of pseudohypoxia, which may result in HIF-1α stabilisation, our data show that inhibition of eNOS disrupts the balance between NO and ROS even under basal physiological conditions. This not only leads to a relative increase in ROS levels but even favours ROS generation by promoting Rac1 activation similar to the situation with thrombin. As ROS have been previously shown to interfere with the pVHL/PHD system thereby stabilising HIF-1α levels (33) and to induce HIF-1α at the transcriptional level (32, 71) these mechanisms may also underlie the increase in HIF-1α levels by L-NNAME in our study.

Consistently, we showed that inhibition of eNOS increased the levels of the pro-angiogenic HIF target PAI-1 under control and thrombin-stimulated conditions. Increased PAI-1 immunoreactivity was reported in the endothelium and the media of aorta and coronary arteries of rats after treatment with L-NNAME and has been related to the progression of vascular remodelling (72), and NO was shown to inhibit angiostatin–II induced PAI-1 expression in smooth muscle cells (73).

Taken together these findings suggest a model whereby under basal conditions, the balance between NO and ROS limits endothelial activation, proliferation and angiogenesis, possibly by the formation of peroxynitrite. However, upon stimulation with thrombin, for example at sites of vascular injury or in cardiovascular diseases such as atherosclerosis or pulmonary hypertension, activation of Rac1 results in increased ROS generation, which promotes activation of the HIF signalling cascade and its target genes such as PAI-1, resulting in endothelial proliferation and an angiogenic response. The concomitant activation of eNOS, also promoted by ROS derived from Rac1 activation, limits ROS bioavailability not only by formation of peroxynitrite, but also by inhibition of Rac1 activation, subsequently diminishing ROS generation, HIF activation and the angiogenic response to PAI-1. Thus, redox sensitivity of Rac1 activation plays a pivotal role in fine tuning the angiogenic response towards thrombin and may modulate regenerative and proliferative responses in various cardiovascular disorders.
Limitations of this study include the lack of in vivo angiogenesis models, the use of a chemical inhibitor for coupled and uncoupled eNOS, and the determination of DAF2 fluorescence as indicator of NO generation. Thus, further studies will be required to more directly assess the role of NO in the interplay with superoxide and other ROS in controlling the different steps of the angiogenic process. However, our data point towards an important role of ROS bioavailability in controlling the formation of capillaries under basal and thrombin-stimulated conditions in endothelial cells in vitro.

Acknowledgements
This work was supported by DFG GO709/4–5, EU 7th FP 222471 (Metoxia) and Fondation Leducq. AG is member of the Munich Heart Alliance.

Conflicts of interest
None declared.

References

Nothing declared.