Phosphodiesterase 2 Mediates Redox-Sensitive Endothelial Cell Proliferation and Angiogenesis by Thrombin via Rac1 and NADPH Oxidase 2

Isabel Diebold, Talija Djordjevic, Andreas Petry, Armin Hatzelmann, Hermann Tenor, John Hess, Agnes Görlach

Abstract—Cyclic nucleotide phosphodiesterases (PDEs) control the levels of the second messengers cAMP and cGMP in many cell types including endothelial cells. Although PDE2 has the unique property to be activated by cGMP but to hydrolyze cAMP, its role in endothelial function is only poorly understood. Reactive oxygen species (ROS) have been associated with important vascular functions including regulation of the vascular tone, proliferation, and apoptosis.1

The levels of cyclic nucleotides are regulated by a dynamic balance between synthesis by adenylyl and guanylyl cyclases and hydrolysis by cyclic nucleotide phosphodiesterases (PDEs).2 Although PDEs have been implicated in an increasing number of vascular functions,3,4 the molecular mechanisms that are regulated by PDEs are only incompletely understood.

PDEs are encoded by at least 21 different genes that are grouped, based on sequence similarity, mode of regulation, and preference for cAMP or cGMP as substrate, into 11 gene families. Transcription from different initiation sites in these genes and differential mRNA splicing results in the generation of close to hundred isoforms, many of which vary with respect to tissue distribution, intracellular localization, and crosstalk with other signaling cascades.7,5 PDEs that have been described in the vasculature are the cAMP-hydrolyzing PDE4 and the cGMP-specific PDE5, as well as the dual-specificity enzymes PDE1, PDE2, and PDE3, which can hydrolyze both cAMP and cGMP. Of the latter group of enzymes, PDE2 is unique in being potently stimulated by cGMP, but primarily hydrolyzing cAMP, thus mediating a negative “crosstalk” between cAMP and cGMP signaling.4 Although initial data suggest an involvement of PDE2 in endothelial barrier function and migration,6,7 the specific role of PDE2 in controlling endothelial function and signaling is not completely clarified.

In addition to cyclic nucleotides, reactive oxygen species (ROS) have been associated with important vascular functions including regulation of the vascular tone, proliferation of vascular cells, angiogenesis, and endothelial permeability.8–10 NADPH oxidases have been considered as important...
sources of ROS in endothelial cells. These multiprotein enzyme complexes contain cytoplasmic regulatory subunits as well as membrane-bound proteins such as p22phox and the catalytic NADPH oxidase subunits. To date, 5 different NOX proteins have been described. Originally, a NOX2 (formerly gp91phox)-containing NADPH oxidase was described to be responsible for the respiratory burst in neutrophils. A NOX2-containing enzyme was also found to be active in endothelial cells. Subsequently, other NOX proteins, including NOX4 and NOX5, have been found in these cells. Full activation of some NOXs, including NOX1, NOX2, and NOX3, require activation of the GTPase Rac1. Active Rac1 also binds to p21-activated kinase (PAK1), which regulates cytoskeletal functions required for migration and proliferation. We thus investigated whether PDE2 would link to ROS generation by NOXs and endothelial proliferative responses. We show that PDE2 mediates ROS production, endothelial proliferation, and angiogenesis involving Rac1 and NOX2.

Materials and Methods

Cell culture, plasmids, short hairpin (sh)RNA, transfection, luciferase assays, ROS measurements, Western blot, Rac activity assays, cAMP and cGMP assays, proliferation assays, in vitro angiogenesis assays, ex vivo mouse aortic explant assays, in vivo mouse Matrigel plug assays, and statistics are described in the expanded Materials and Methods section in the online data supplement, available at http://circres.ahajournals.org.

Results

PDE2 Mediates ROS Formation

Thrombin has been previously described to increase ROS levels in vascular cells. In fact, exposure of human umbilical vein endothelial cells (HUVECs) to thrombin (3 U/mL) rapidly increased intracellular ROS levels measured by dichlorofluorescein (DCF) (Figure 1A) and dihydroethidium (DHE) fluorescence (Figure 1B) and extracellular ROS levels determined by MCLA chemiluminescence (Figure 1C). We thus determined the involvement of PDE2 in ROS generation by thrombin. Treatment of HUVECs with an inhibitor of PDE2, PDP, which has been shown previously to completely and selectively inhibit PDE2 activity, diminished thrombin-induced ROS generation (Figure 1). To avoid the possibility that PDP could quench radicals, we targeted PDE2 by shRNA, leading to downregulation of PDE2 protein levels by ≈35% (Figure 1D). Similar to the effect of the PDE2 inhibitor, thrombin-stimulated ROS production was abrogated in PDE2-depleted HUVECs (Figure 1). PDE2 depletion also decreased thrombin-stimulated ROS production in pulmonary artery smooth muscle cells (Figure I in the online data supplement), which expressed PDE2 to a similar extent than HUVECs, suggesting that PDE2 may be important for controlling ROS levels also in other vascular cells. This assumption was further confirmed in PDE2-overexpressing HUVECs, which contained ≈50% more PDE2 protein than controls (Figure 2A) and showed significantly increased ROS levels, as determined by MCLA chemiluminescence or DHE fluorescence (Figure 2B and 2C). Stimulation with thrombin further increased ROS levels in PDE2-overexpressing HUVECs, confirming that overexpressed PDE2 is functionally active in our experimental system. In support of this finding, addition of the antioxidants N-acetylcysteine (NAC) or vitamin C abrogated PDE2-mediated ROS generation (Figure 2D).

PDE2 Mediates Activation of Rac1 and PAK1

We next explored the mechanisms underlying PDE2-mediated ROS generation. NADPH oxidases are important enzymatic sources of ROS in endothelial cells, which are activated by the GTPase Rac1. Indeed, expression of constitutively active Rac1V12 increased ROS production (Figure
Subsequently, thrombin rapidly activated Rac1 after 15 seconds of stimulation, and this response remained elevated for 5 minutes (Figure 3A), whereas inhibition of PDE2 by PDP or PDE2 depletion by shRNA diminished thrombin-induced activation of this GTPase (Figure 3B). Rac1 was

Figure 2. PDE2-induced ROS production is dependent on Rac1 and NOX2. HUVECs were transfected with a plasmid encoding PDE2 or control vector (Ctr). A, Western blot analyses using antibodies against PDE2, c-myc, and actin were performed to test transfection efficiency. PDE2 protein levels are presented as relative changes to control vector (100%) (n=3; *P<0.05). B, Cells were stimulated with thrombin (Thr) (3 U/mL) for 5 minutes. ROS production was determined by MCLA-derived chemiluminescence. Data are presented as relative changes to untreated control vector (100%) (n=3; *P<0.05 vs Ctr, #P<0.05 vs PDE2-overexpressing Ctr). C, HUVECs were transfected with vectors encoding PDE2 or active RacG12V (RacV12) and stimulated with thrombin (Thr). ROS production was measured by DHE fluorescence. Data are presented as relative changes to untreated control vector (100%) (n=3; *P<0.05 vs untreated Ctr, #P<0.05 vs PDE2-overexpressing cells). D, HUVECs overexpressing PDE2 were treated for 30 minutes with vitamin C.
activated in PDE2-overexpressing HUVECs, and Rac1 activation was further enhanced by thrombin (Figure 3A), indicating that PDE2 activation by thrombin is essentially involved in Rac1 activation.

Because PAK1 is a substrate of Rac1, we determined whether this kinase could also be activated in a PDE2-dependent manner. Thrombin stimulated PAK1 phosphorylation within 15 seconds, whereas treatment with PDP abolished these responses (Figure 3C), confirming the relevance of PDE2 in activation of Rac1-dependent pathways.

Because tumor necrosis factor (TNF)-α has been described to activate PDE2,7 we determined the levels of activated Rac1 and ROS in TNF-α-treated HUVECs. Similar to thrombin, TNF-α increased Rac1 activation and ROS production (Online Figure II), whereas depletion of PDE2 abrogated these responses, confirming the importance of PDE2 in Rac1 activation and ROS formation.

PDE2 Mediates ROS Production by Rac1 and NOX2

Next, we determined whether Rac1-dependent NOX2 contributes to ROS production in the presence of PDE2. PDE2-overexpressing HUVECs were transfected with dominant-negative RacT17N, resulting in significantly decreased ROS production compared to PDE2-overexpressing controls (Figure 2D). Subsequently, depletion of NOX2 by shRNA reduced ROS generation of PDE2-overexpressing HUVECs (Figure 2D). Interestingly, NOX2 protein levels were upregulated in PDE2-overexpressing cells but were diminished in NOX2-depleted cells (Online Figure III), suggesting that PDE2 is not only involved in rapid stimulation of ROS production by activation of Rac1-dependent NOXs but may also contribute to more prolonged ROS formation because of upregulation of NOX2.

Decreased cAMP Levels Permit Rac1 Activation and ROS Production by Thrombin

Because PDE2 can hydrolyze cAMP, we investigated the role of cAMP in the regulation of Rac1 activity and ROS generation in our cellular system.

Similar to PDE2 overexpression, thrombin decreased intracellular cAMP levels in control cells and even further in PDE2-overexpressing cells (Figure 4A). These data suggest that decreased cAMP levels resulting from PDE2 activation may allow Rac1 activation and ROS generation, whereas inhibition of PDE2 would elevate cAMP levels and thus prevent ROS production. To test this assumption, HUVECs were treated with the activator of adenylyl cyclase, forskolin, or the cell-permeable cAMP analog db-cAMP, or the PDE2 inhibitor. All substances restored cAMP levels in the presence of thrombin (Figure 4B). Similar to PDE2 inhibition or depletion, forskolin or db-cAMP decreased ROS production and prevented Rac1 activation by thrombin (Figure 4C and 4D). Inhibition of the cAMP-hydrolyzing enzymes PDE3 and PDE4 by motapizone and piclamilast also decreased thrombin-induced ROS production and Rac1 activation (Online Figure IV) further supporting a role of cAMP as a regulatory element in controlling ROS production by PDEs.

cGMP Mediates Rac1 Activation and ROS Production

Because PDE2 can be activated by cGMP, but has also been described to hydrolyze cGMP under certain conditions, we evaluated the role of cGMP in mediating endothelial ROS
Thrombin significantly increased cGMP levels within 5 minutes of application (Figure 5A), but this response was not affected by depletion or overexpression of PDE2 suggesting that PDE2 cGMP-hydrolyzing activity was not prominent in our system. Subsequently, treatment with the cell-permeable cGMP analogue 8-Br-cGMP, or atrial natriuretic peptide (ANP), as a direct activator of transmembrane guanylyl cyclases, activated Rac1 (Figure 5B). On PDE2 inhibition or downregulation, ANP-mediated Rac1 activation was diminished. In line, 8-Br-cGMP or ANP enhanced ROS levels, and this response was also dependent on PDE2 (Figure 5C), suggesting that under conditions of elevated cGMP levels, including thrombin and ANP treatment, PDE2 is involved in Rac1 activation and ROS formation.

Interestingly, expression of constitutively active RacG12V or PAKT423E increased intracellular cGMP levels (Figure 5D). These findings suggest that Rac1 and PAK1 are also involved in the control of cGMP generation and that a positive-feedback loop may exist where cGMP-stimulated PDE2 activates Rac1 and PAK1, which then elevate cGMP levels. Further studies, which are beyond the scope of this study, will be required to delineate the exact mechanisms underlying this observation.

PDE2 Mediates Endothelial Cell Proliferation and the Angiogenic Response

We next investigated the effect of PDE2 on long-term endothelial responses, such as proliferation and angiogenesis. We first determined whether thrombin is able to modulate PDE2 protein levels using Western blot analyses. Thrombin increased PDE2 protein levels, peaking at 4 hours (Online Figure V). We next investigated the effect of PDE2 on endothelial proliferation. Exposure of HUVECs to thrombin resulted in increased DNA synthesis, as was monitored by 5-bromodeoxyuridine (BrdUrd) incorporation, whereas addition of PDP or depletion of PDE2 by shRNA completely abolished this proliferative response (Figure 6A) without signs of cell death. Similarly, ANP increased endothelial proliferation, and this effect was not observed in PDE2-depleted cells. On the other hand, BrdUrd incorporation was significantly increased in PDE2-overexpressing HUVECs (Figure 6A), whereas this effect was diminished on treatment with the antioxidant NAC, depletion of NOX2, or overexpression of RacT17N (Figure 6B), indicating that PDE2 is upstream of NOXs and ROS generation in mediating proliferation of endothelial cells.

Because proliferation is a prerequisite for angiogenesis, we evaluated whether PDE2 plays a role in the formation of capillary-like structures using in vitro Matrigel assays. Thrombin induced the formation of capillary-like structures, whereas depletion of PDE2 (Figure 6C) or treatment with the PDE2 inhibitor (Online Figure V) prevented this response. ANP also stimulated the formation of new capillaries in a PDE2-dependent manner (Figure 6C), further indicating that PDE2 is upstream of NOXs and ROS generation in mediating proliferation of endothelial cells.

To test whether ROS play a role in PDE2-stimulated proliferation or angiogenesis, HUVECs overexpressing PDE2...
were treated with NAC or were transfected with RacT17N or NOX2 shRNA (Figure 6D). In all cases, PDE2-induced formation of capillary-like structures was abrogated, confirming again that ROS are downstream mediators of PDE2 signaling controlling endothelial proliferative responses.

In a next step, mouse aortic rings were embedded in a collagen matrix in an ex vivo sprouting assay and transduced with lentiviral constructs expressing shRNA against PDE2, resulting in depletion of PDE2 and subsequently diminished vessel sprouting compared to controls (Figure 7A and 7B). Similarly, treatment with the PDE2 inhibitor prevented vessel outgrowth (Online Figure V).

Finally, we used an in vivo model to evaluate the involvement of PDE2 in angiogenesis. HUVECs were embedded in Matrigel plugs in the presence or absence of PDP. Plugs were treated with NAC or were transfected with RacT17N or NOX2 shRNA (Figure 6D). In all cases, PDE2-induced formation of capillary-like structures was abrogated, confirming again that ROS are downstream mediators of PDE2 signaling controlling endothelial proliferative responses.

In a next step, mouse aortic rings were embedded in a collagen matrix in an ex vivo sprouting assay and transduced with lentiviral constructs expressing shRNA against PDE2, resulting in depletion of PDE2 and subsequently diminished vessel sprouting compared to controls (Figure 7A and 7B). Similarly, treatment with the PDE2 inhibitor prevented vessel outgrowth (Online Figure V).

Finally, we used an in vivo model to evaluate the involvement of PDE2 in angiogenesis. HUVECs were embedded in Matrigel plugs in the presence or absence of PDP. Plugs were
injected subcutaneously into mice, and vessel formation was assessed after 7 days (Figure 7C). Compared to control plugs, the formation of new vascular structures was significantly inhibited in plugs containing the PDE2 inhibitor, further confirming that PDE2 plays an important role in the control of angiogenesis.

**Discussion**

In this study, we show for the first time that PDE2 is actively involved in thrombin stimulation of ROS production in HUVECs, by activating Rac1 and a NOX2-dependent NADPH oxidase. We further provide evidence that PDE2-mediated activation of ROS production by NOX2 promotes endothelial cell proliferation and angiogenesis in vitro and in vivo.

**PDE2 Stimulates Formation of ROS via Rac1 and NOX2**

Our findings clearly highlight a role of PDE2 in the regulation of ROS levels in HUVECs because thrombin-stimulated intracellular and extracellular ROS generation was diminished by the selective PDE2 inhibitor PDP or PDE2 depletion.

Importantly, our data provide evidence that NADPH oxidases are a source of ROS controlled by PDE2 and thrombin, because PDE2 overexpression activated the GTPase Rac1, whereas PDE2 inhibition prevented thrombin-induced Rac1 activation. Rac1 is a known activator of NOX2-dependent NADPH oxidases, which are importantly involved in endothelial ROS generation in response to many stimuli including thrombin. Indeed, expression of constitutively active Rac1 increased ROS levels. In contrast, dominant-negative Rac1 or depletion of NOX2 by shRNA decreased ROS production by thrombin (data not shown). Importantly, PDE2 overexpression increased ROS levels and Rac1 activation, and these effects were enhanced by thrombin, indicating that overexpressed PDE2 is functionally active, also without cGMP addition, similar to the situation described in HUVECs and COS1 cells. In line with this, PDE2-stimulated ROS production was abrogated in the presence of Rac1 or in the absence of NOX2, confirming that PDE2 mediates ROS production by activating Rac1 and NOXs in endothelial cells. Interestingly, in addition to this rapid response, PDE2 overexpression was associated with enhanced levels of NOX2, suggesting that PDE2 may be also involved in prolonged ROS production in endothelial cells associated with upregulation of NOXs and endothelial dysfunction.

Our data further suggest that thrombin, similar to ANP, enhances cGMP levels, thus activating PDE2 to primarily hydrolyze cAMP. In fact, inhibition of PDE2 restored cAMP levels in thrombin-stimulated cells, whereas depletion of PDE2 did not significantly affect thrombin-stimulated cGMP levels. In line with this, cAMP hydrolysis was important for ROS generation and Rac1 activation in response to thrombin or ANP, because elevation of cAMP levels by forskolin or db-cAMP prevented thrombin-induced Rac1 activation and ROS production.

A crosstalk between the cGMP and cAMP pathways has been reported in other cellular settings. In platelets or adrenal glomerulosa cells, increased cGMP levels resulting from NO or ANP, activated PDE2, and reduced cAMP. In line with this, previous studies have shown that increased cAMP levels blocked superoxide formation in neutrophils and that the nonhydrolyzable S(p)-cAMPS decreased the levels of activated Rac1 by inhibition of the Rac1-specific guanine nucleotide exchange factor p-Rex1. However, inhibition of other cAMP-hydrolyzing PDEs, namely PDE3 or PDE4, decreased ROS generation in isolated rat glomeruli or in mesangial cells. Of note, in our cellular system, inhibition of PDE3 and PDE4 also diminished thrombin-stimulated ROS production and Rac1 activity in endothelial cells (Online Figure IV).

In addition to thrombin and ANP, PDE2 inhibition prevented Rac1 activation and ROS production by TNF-α in HUVECs. TNF-α has been previously shown to stimulate PDE2 in HUVEC, indicating that the involvement of PDE2 activation in the control of ROS levels is a more general response. In support of this, PDE2 also contributed to ROS formation by thrombin in pulmonary artery smooth muscle cells, which expressed PDE2 protein similar to HUVECs.

Previous studies have shown that cAMP and protein kinase A inhibited PAK1 in vivo, whereas cGMP stimulated PAK1.
in endothelial cells. Concomitantly, inhibition of PDE2, which restored cAMP levels, decreased PAK1 phosphorylation by thrombin. Our data also showed that active Rac1 or PAK1 increased cGMP levels in HUVECs, indicating that cGMP is not only upstream of the Rac1-PAK1 pathway but could be also downstream. In this regard, it was demonstrated that Rac1 uses PAK1 to directly activate transmembrane guanylyl cyclases, leading to increased cellular cGMP levels. Thus, PDE2-mediated Rac1 and PAK1 activation and the subsequent increase in cGMP may form a positive-feedback loop to maintain PDE2 activity, which could initiate a vicious circle promoting sustained ROS generation (Figure 8). Sustained elevation of ROS levels has been associated with deterioration of endothelial function because of decreased NO bioavailability. Thus, our study suggests that inhibition of PDE2 may have antioxidant effects by preventing activation of NOXs and decreasing ROS levels, and may have additional protective effects because of increased bioavailability of NO in situations of endothelial dysfunction.

PDE2 Enhances Endothelial Proliferation, Tube Formation, and Angiogenesis

Our results further provide strong evidence in vitro and in vivo that PDE2 is an important regulator of the angiogenic response, because overexpression of PDE2 enhanced endothelial proliferation and in vitro tube formation, whereas inhibition or depletion of PDE2 prevented thrombin-stimulated endothelial proliferation and formation of tube-like structures. Importantly, vessel sprouting was abrogated in mouse aortic rings depleted of PDE2, and the formation of new vessels was also prevented in vivo by inhibition of PDE2 as assessed by a murine angiogenesis model. Our findings that thrombin increases PDE2 levels in HUVECs and that PDE2 overexpression potently enhances endothelial proliferation and formation of capillary structures confirms the importance of PDE2 in controlling endothelial proliferation and angiogenesis and supports the notion that PDE2 levels may be limiting.

In contrast, a previous study showed that PDE2 inhibition by EHNA alone was not sufficient to prevent vascular endothelial growth factor (VEGF)-induced endothelial proliferation but additional application of a PDE4 inhibitor was required to reduce the proliferative responses. Although the reasons for these conflicting data are not clear, they may relate to different efficiency and specificity of the PDE2 inhibitors used and/or the use of different stimuli. In support of our study, the PDE2 inhibitor BAY31-9742 decreased endothelial migration in response to VEGF similar to inhibition of PDE3 and PDE4 and our results demonstrating that inhibition of PDE3 and PDE4 decreases thrombin-induced tube formation. However, our study is the first to show that enhanced levels of PDE2 are sufficient to increase endothelial cell proliferation and formation of tube-like structures.

Upregulation of PDE2 by TNF-α was previously shown to regulate endothelial permeability by thrombin and increased PDE2 expression in response to VEGF was a prerequisite for migration of HUVECs.

Most importantly, the angiogenic response mediated by PDE2 was critically dependent on Rac1 and NOX2, thus providing, for the first time, evidence that PDE2 regulates the angiogenic response by controlling activation of NOXs and ROS levels. In line with this, NOX2, Rac1, and PAK1 are well-known regulators of angiogenesis, and the NOX components p22phox and NOX5 have been shown to contribute to thrombin-stimulated endothelial proliferation and formation of capillaries. Thus, PDE2-dependent activation of NOX-derived ROS and of PAK1 may be crucially involved in endothelial proliferation in response to thrombin.

Taken together, this study demonstrated that PDE2 increased NOX-dependent ROS generation in endothelial cells, involving Rac1 activation and NOX2 upregulation, and that this pathway promotes endothelial proliferation and angiogenic responses. Elevation of cGMP levels by thrombin may thereby stimulate PDE2 to lower cAMP levels, thus allowing activation of Rac1, PAK1, and NOXs. Because active Rac1 and PAK1 also enhanced cGMP levels, which may then further activate this pathway, PDE2 inhibition may provide a new therapeutic strategy applicable to cardiovascular disorders associated with endothelial dysfunction and oxidative stress, vascular proliferation, and remodeling or angiogenesis.

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Disclosures
A.H. and H.T. are full-time employees at NYCOMED GmbH, Konstanz, Germany.

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Diebold et al. PDE2 Increases ROS-Dependent Angiogenesis


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EXPANDED MATERIAL AND METHODS

Reagents
The PDE2 inhibitor 9-(6-Phenyl-2-oxohex-3-yl)-2-(3,4-dimethoxybenzyl)-purin-6-one (PDP) was provided by NYCOMED GmbH. PDP inhibits PDE2 with an IC50 of 0.6 nmol/L and with a >5000-fold selectivity over other PDE isoenzymes. PDP was dissolved in DMSO (0.1% final concentration) and used at 100 nmol/L assuring complete and selective inhibition of PDE2. N-ω-nitro-L-arginine (L-NNA) was from Calbiochem (Darmstadt, Germany). Human α-thrombin was from Haemochrom Diagnostika (Essen, Germany). All other chemicals were from Sigma (Taufkirchen, Germany).

Cell culture
Human umbilical vein endothelial cells (HUVEC) were from Lonza (Wuppertal, Germany) and cultured as recommended in EGM-2 medium. Cells were used up to passage 5 and serum-starved for 16 h before stimulation. Pulmonary artery smooth muscle cells were from Lonza and cultured in medium provided as recommended. Cells were used up to passage 10 and serum starved 16 h before stimulation.

Plasmids
The expression vector encoding c-myc-tagged PDE2 (pcDNA3.1PDE2A) was kindly provided by Dr Gianluca Quintini (NYCOMED GmbH). Vectors encoding c-myc-tagged RacG12V or PAKT423E have been described previously1-4. Specific short hairpin RNA encoding for 19mer siRNA against PDE2 (5′GGA GCT GAT CTA CAA AGA A 3′) was designed using the siRNA Target Designer and created using the siSTRIKE U6 Hairpin Cloning System (both Promega, Mannheim, Germany). Control shRNA has been described previously5.
For silencing of human and mouse PDE2 in primary cells and tissues, the BLOCK-iT Inducible H1 Lentiviral RNAi System\textsuperscript{TM} (Invitrogen, Karlsruhe, Germany) was used to generate two viruses coding for two different shRNAs against mouse and human PDE2. Due to sequence homology, both shRNAs were specific in both species. Gene-specific inserts were cloned into pLenti4 according to the manufacturer’s instructions. The lentivirus was produced in HEK 293FT cells, and the virus-containing media was harvested for infection of mouse aortic rings in accordance to the manufacturer’s manual. The following insert sequences for the shRNA against PDE2 were used:

shPDE2a:
\[
5'-\text{CACCGCAGCTCAAAGTGCTCCAATACGAATATTGGAGCACTTTGAGCTGC-3'};
\]

shPDE2b:
\[
5'-\text{CACCGCTGGAAGACCACCAGAAAGACGAATCTTTCTGGTGGTCTTCCAGC-3'}
\]

**Transfections and luciferase assay**

HUVEC were plated one day before transfection. Cells were transfected with SuperFect (QIAGEN, Hilden, Germany) according to the manufacturer’s protocol using a ratio of 12 µg DNA to 60 µL SuperFect per 10 cm dish. PASMC were plated one day before transfection. Cells were transfected with FuGene reagent (Roche, Mannheim, Germany) according to the manufacturer’s protocol using 6 µg DNA to 36 µL FuGene per 10 cm dish.

**Measurements of ROS production**

ROS generation was detected using DCF (CM-H\textsubscript{2}DCFDA, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester, Invitrogen), dihydroethidium (DHE, Invitrogen) or 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3-one hydrochloride (MCLA, Invitrogen).

For DCF fluorescence measurements, HUVEC (10\textsuperscript{6} cells/mL) were plated in 96 well plates to achieve around 80 % confluency on the next day. The day after, cells were starved in serum-free EGM-2 (without any additives) for 16 h. Cells were washed with Hank’s balanced salt
solution (HBSS) and incubated with the PDE2 inhibitor in HBSS for 30 min in the presence of the NO synthase inhibitor N-ω-nitro-L-arginine (L-NNA, 10 µmol/L) to prevent NO formation prior to stimulation. This was required to prevent peroxynitrite formation which may perturb the DCF signal. DCF was added to a final concentration of 8.5 µmol/L for 5 min. Cells were washed with HBSS and stimulated with 3 U/mL thrombin for 5 min in the presence of inhibitors. Fluorescence was measured in a microplate reader (Tecan, Crailsheim, Germany). DCF fluorescence was standardized to the number of viable cells using the alamarBlue test as described (Biosource, Nivelles, Belgium).

To detect ROS by chemiluminescence, HUVEC were detached by trypsinisation and 0.5 x 10⁶ cells were resuspended in HBSS containing 10 µmol/L MCLA with or without the PDE2 inhibitor. Thereafter, 3 U/mL thrombin was added and luminescence was detected after 5 min in a luminometer (Berthold Technologies, Bad Wildbad, Germany).

Western blot analysis

Western blot analysis was performed as described using primary antibodies against PDE2A (Abcam, Cambridge, UK), Rac1 (BD Biosciences, Heidelberg, Germany), PAK1 and pPAK1 (Cell Signaling Technology, Danvers, MA). Loading of equal amounts of proteins was confirmed by reprobing the membranes with an actin antibody (Santa Cruz).

Rac1 activity assay

Rac1 activity was evaluated by an affinity precipitation assay using the PAK1-PBD conjugated glutathione agarose beads according to the manufacturer’s instructions as described (Millipore, Schwalbach, Germany).

Proliferation assays

HUVECs were seeded in 96 well plates to achieve 50% of confluency. Cells were transfected on the following day with corresponding vectors, and left in 2% serum overnight. On the next
day, cells were exposed to stimuli as described in the respective legends. DNA synthesis was assessed by 5-bromo-2'‐deoxyuridine (BrdU) labeling (Roche) as described previously.

**Angiogenesis assays**

HUVEC were seeded in a density of 25000 cells per well on a 96-well plate mounted with growth factor reduced Matrigel (BD Biosciences). Cells were then incubated for 24 h at 37°C and stained using Diff-Quick (Baxter Diagnostics, Duedingen, Switzerland). The formation of capillary-like structures was assessed by light microscopy (Olympus, Hamburg, Germany) using the Openlab Modular Software for Scientific Imaging (Improvision, Heidelberg, Germany) and was quantified using Image J software (Wright Cell Imaging Facility, Toronto, Canada).

**In vivo angiogenesis assay**

Growth factor reduced Matrigel (BD Biosciences) was mixed with HUVEC (150 000 cells/plug) and the PDE2 inhibitor or vehicle (0.1% DMSO). The matrigel mixture was subcutaneously injected into mice (C5BL6, 6 weeks, male). 1-2 weeks after injection, matrigel plugs were excised, formalin-fixed and paraffin embedded to prepare sections for histochemical staining. The formation of capillary-like structures from HUVEC was assessed by staining with an antibody against CD31 (DAKO, Glostrup, DK) in a 1:50 dilution.

**Ex vivo aortic ring sprouting assay**

6 week old mice (C5BL6, male) were sacrificed and the thoracic aorta was excised. The aorta was transferred into a dish with sterile PBS buffer. After removing the fibroadipose tissue, arteries were sectioned into 1-1.5 mm long cross sections and placed on matrigel-coated wells (BD Biosciences; 100 µL/well) and incubated at 37°C for 20 min. Thereafter the rings were incubated with 500 µL DMEM medium. The cultures were kept at 37°C in a humidified environment for one week. Every second day vessel sprouting was assessed by
light microscopy (Olympus) using the Openlab Modular Software for Scientific Imaging (Improvision) and was quantified using Image J software (Wright Cell Imaging Facility).

**Statistical analysis**

Values presented are means±standard deviation (SD). Results were compared by ANOVA for repeated measurements followed by Student-Newman-Keuls t-test. $P<0.05$ was considered statistically significant.
LEGENDS

**Online Figure I: Phosphodiesterase-2 mediates ROS production by thrombin in smooth muscle cells**

(A) Phosphodiesterase-2 (PDE2) protein levels were determined by Western blot analysis in human umbilical vein endothelial cells (EC) and pulmonary artery smooth muscle cells (SMC) using an antibody against PDE2. Actin was used as loading control. A representative blot is shown (n=3). (B) SMC were transfected with a vector encoding shRNA against PDE2 (siP2) or control shRNA (siCtr) and stimulated with thrombin (3 U/mL) for 5 min or left untreated. ROS production was determined using the fluorophore dihydroethidium (DHE). Data are presented as relative change to control (100%). (n=3, *p<0.05 versus unstimulated cells, #p<0.05 versus thrombin-stimulated control (siCtr)). (C) PASMC were transfected with plasmids encoding an shRNA against PDE2 (siP2) or control shRNA (siCtr). Western blot analyses using antibodies against PDE2 or actin were performed. A representative blot is shown (n=3).

**Online Figure II: Phosphodiesterase-2 modulates ROS production and Rac1 activity by TNF-α**

(A) Human umbilical vein endothelial cells (HUVEC) were transfected with vectors encoding shRNA against PDE2 (siP2) or control shRNA (siCtr) and exposed to TNF-α (10 µmol/L) for 5 min. ROS production was determined using the fluorophore dihydroethidium (DHE). Data are presented as relative change to control (100%) (n=3, *p<0.05 versus control, #p<0.05 versus thrombin-stimulated control (siCtr)). (B) HUVEC were transfected with vectors encoding shRNA against PDE2 (siP2) or control shRNA (siCtr) and then stimulated with TNF-α (10 µmol/L) for 15 sec. Rac1 activity was determined by a pull down assay. A representative blot is shown (n=3).
Online Figure III: Phosphodiesterase-2 modulates the levels of NOX2
HUVEC were transfected with a plasmid encoding PDE2 (PDE2) or control vector (Ctr) and cotransfected with vectors encoding shRNA against NOX2 (siNOX2) or control shRNA. Western blot analyses using antibodies against NOX2 or actin were performed. NOX2 protein levels were quantified from immunoblots by densitometry and presented as relative change to control (100%) (n=3, *p<0.05 versus control, #p<0.05 versus cells overexpressing PDE2).

Online Figure IV: Inhibition of phosphodiesterases-3 and -4 modulates ROS production, Rac1 activity and tube formation by thrombin
(A) Human umbilical vein endothelial cells (HUVEC) were preincubated for 30 min with the PDE3 inhibitor motapizone (10 µmol/L) and the PDE4 inhibitor piclamilast (1 µmol/L) (M+P) or with vehicle (0.1% DMSO) (Ctr), loaded for 5 min with DCF, washed and stimulated with thrombin (3 U/mL) for 5 min. DCF fluorescence was measured in a microplate reader. Data are presented as relative change to unstimulated cells (Ctr) set to 100% (n=3, *p<0.05 versus Ctr, #p<0.05 versus thrombin-stimulated Ctr). (B) HUVEC were preincubated for 30 min with motapizone and piclamilast (M+P) or vehicle (Ctr) and then stimulated with thrombin for 15 sec. Rac1 activity was determined by a pull down assay. A representative blot is shown (n=3). (C) HUVEC were plated on growth factor-reduced matrigel, preincubated with motapizone and piclalimast (M+P) or vehicle (Ctr) for 1 hour and thereafter stimulated with thrombin. Formation of capillary-like structures was allowed for 24 h at 37°C. A representative assay is shown (n=3).

Online Figure V: Phosphodiesterase-2 inhibition abolishes thrombin-induced proliferation and angiogenesis in endothelial cells
(A) Human umbilical vein endothelial cells (HUVEC) were exposed to thrombin (3 U/mL) for different time points and PDE2 levels were evaluated by Western blot. Actin served as loading control. Representative blots are shown (n=3). (B) HUVEC were plated on growth
factor-reduced matrigel, preincubated with the PDE2 inhibitor PDP (100 nmol/L) or vehicle (Ctr) for 30 min and thereafter stimulated with thrombin. Formation of capillary-like structures was allowed for 24 h at 37°C. Tube-forming structures were quantified using Image J software. Data are presented as relative change to control (100%) (n=3, *p<0.05 versus Ctr, #p<0.05 versus PDE2-overexpressing cells or thrombin-stimulated control (Ctr)). (C) Mouse aortic rings were cultured in collagen gels and treated with the PDE2 inhibitor PDP or vehicle (Ctr). Vessel sprouting was allowed for 4 days. Representative pictures are shown (n=3).

References


ONLINE Figure 1

A

B

C

PDE2
Actin

EC  SMC

Thrombin

siCtr  siP2  siCtr  siP2

PDE2
Actin

siCtr  siP2
ONLINE Figure II

A

![Graph showing Relative DHE Fluorescence](image)

B

![Images showing Active and Total Rac1](image)
ONLINE Figure III
ONLINE Figure IV

A

B

C

Diebold et al
ONLINE Figure V

A

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B

Ctr  PDP  Ctr  PDP

Rel. formation of capillary-like structures [% control]

C

Ctr  PDP  Ctr  PDP