The HIF1 target gene NOX2 promotes angiogenesis through urotensin-II

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Summary
Urotensin-II (U-II) has been considered as one of the most potent vasoactive peptides, although its physiological and pathophysiological role is still not finally resolved. Recent evidence suggests that it promotes angiogenic responses in endothelial cells, although the underlying signalling mechanisms are unclear. Reactive oxygen species derived from NADPH oxides are major signalling molecules in the vasculature. Because NOX2 is functional in endothelial cells, we investigated the role of the NOX2-containing NADPH oxidase in U-II-induced angiogenesis and elucidated a possible contribution of hypoxia-inducible factor-1 (HIF-1), the master regulator of hypoxic angiogenesis, in the response to U-II. We found that U-II increases angiogenesis in vitro and in vivo, and these responses were prevented by antioxidants, NOX2 knockdown and in Nox2+/− mice. In addition, U-II-induced angiogenesis was dependent on HIF-1. Interestingly, U-II increased NOX2 transcription involving HIF-1, and chromatin immunoprecipitation confirmed NOX2 as a target gene of HIF-1. In support, NOX2 levels were greatly diminished in U-II-stimulated isolated vessels derived from mice deficient in endothelial HIF-1. Conversely, reactive oxygen species derived from NOX2 were required for U-II activation of HIF and upregulation of HIF-1. In line with this, U-II-induced upregulation of HIF-1 was absent in Nox2−/− vessels. Collectively, these findings identified HIF-1 and NOX2 as partners acting in concert to promote angiogenesis in response to U-II. Because U-II has been found to be elevated in cardiovascular disorders and in tumour tissues, this feed-forward mechanism could be an interesting anti-angiogenic therapeutic option in these disorders.

Key words: Angiogenesis, Reactive oxygen species, NADPH oxidase, NOX2, Urotensin-II, HIF-1, Endothelial

Introduction
Angiogenesis plays a major physiological role during embryonic development and later, in adult life, in several physiological and pathophysiological conditions, including cancer, inflammation and various cardiovascular diseases. The angiogenic process includes the formation of capillaries from pre-existing vessels, i.e. capillary and postcapillary venules that develop as a result of endothelial sprouting or intussusceptive microvascular growth (Folkman, 2003). A major stimulus of angiogenesis, particularly in cancer and inflammation, is hypoxia. Within this setting the family of hypoxia-inducible transcription factors (HIFs) plays a pivotal role (Coulon et al., 2010). These heterodimers consist of an oxygen-sensitive α-subunit, and a barely regulated β-subunit. HIF-1α is the most abundant of the regulated subunits of this family. It is unstable under normoxia because of its interaction with the E3 ubiquitin ligase pVHL, which primes it for proteasomal degradation (Semenza, 2001). Under hypoxia, this interaction ceases thus stabilizing HIF-1α and allowing its transcriptional activity (Ratcliffe et al., 1998; Semenza, 2000; Wenger et al., 2005).

In recent years, it has been shown that HIF-1α is also responsive to a variety of growth factors, procoagulant factors, hormones and cytokines even under normoxic conditions (Dery et al., 2005; Gorlach and Kietzmann, 2007). Because several of these factors can also promote angiogenesis, activation of HIF by these factors could contribute to the proangiogenic response not only under hypoxic but also under normoxic conditions.

Recent evidence suggests that in addition to proangiogenic growth factors such as VEGF and PDGF, several peptide hormones that have known functions in controlling vascular tone, including angiotensin-II and endothelin-1, are able to promote endothelial proliferative responses (Ribatti et al., 2007).

One of the most potent vasoactive peptides is urotensin-II (U-II; also known as urotensin-2), which was originally isolated from the fish urophysis. Human U-II is a cyclic 11-amino-acid peptide that has been identified as an endogenous ligand of the orphan receptor GPR14, also named urotensin-II receptor (Ames et al., 1999; Maguire and Davenport, 2002). U-II and GPR14 are expressed in many organs and in different vascular beds in endothelial and smooth muscle cells. They have been associated with several cardiovascular pathologies including atherosclerosis and pulmonary vascular remodelling (Djordjevic and Gorlach, 2007; Watanabe et al., 2009). Interestingly, U-II and its receptor have also been found in several tumour cell lines (Yoshimoto et al., 2004). Although its importance is yet unclear in the tumour setting, U-II has been shown to exert a marked mitogenic and proliferative action on vascular cells (Djordjevic et al., 2005; Spinazzi et al., 2006) so that it might also play an important role in controlling tumor adaptation to the microenvironment and survival by contributing to angiogenesis. However, the molecular
mechanisms underlying activation of endothelial cells by U-II are not clear.

Reactive oxygen species (ROS) have been identified as important signalling molecules in many cell types including endothelial cells, and have been associated with endothelial cell proliferation and new vessel formation in response to growth factors including VEGF and PDGF (Maulik, 2002). A major source of vascular ROS are NADPH oxidases (Lassegue and Griendling, 2010). This family of multiprotein enzymes contains one of five membrane-bound catalytic NOX proteins (termed NOX1 to NOX5) and the p22phox subunit comprising the cytochrome b 558. The originally identified NOX2-containing enzyme (formerly termed gp91phox), as well as some other family members, include additional cytosolic regulatory subunits, among them the GTPase Rac. These subunits are required for activation of the leukocyte enzyme to generate superoxide and consecutive ROS in the innate immune response (El-Benna et al., 2008). Interestingly, NOX2 has also been identified as a major source of ROS in endothelial cells and has been related to the control of vascular tone and angiogenesis (Gorlach et al., 2000; Ushio-Fukai et al., 2002). Subsequently, other NOX enzymes, including NOX4 and NOX5 have also been found to contribute to ROS generation in these cells (Petry et al., 2006; BelAiba et al., 2007) although the exact importance of this apparent redundancy remains unclear.

Given the importance of NOX2 in endothelial function we investigated the role of this enzyme in the angiogenic response towards U-II, and identified HIF-1α as a central element promoting a feed-forward mechanism of ROS-mediated angiogenesis by U-II.

**Results**

**Urotensin-II stimulates angiogenesis through GPR14**

In a first step we investigated the role of U-II in the angiogenic response of human endothelial cells using a Matrigel tube formation assay. Exposure to U-II significantly induced the formation of capillary-like structures by 1.5±0.1 fold \((P<0.05)\). However, treatment with the GPR14 antagonist urantide completely prevented this response (Fig. 1A). Similarly, depletion of GPR14 by shRNA significantly decreased U-II-induced endothelial tube formation (Fig. 1B) from 1.7±0.04 to 1.2±0.1 fold \((P<0.05)\) indicating that U-II acts through its receptor to promote an angiogenic response.

Because angiogenesis in vivo typically initiates from the venous side of the vascular bed (Folkman, 2003) we cultured mouse vena cava explants in a collagen matrix and monitored angiogenic outgrowth. Exposure to U-II increased vessel sprouting, whereas pretreatment with urantide reduced this response (Fig. 1C). Subsequently, we used an in vivo model to evaluate the involvement of U-II and GPR14 in angiogenesis. To this end, Matrigel plugs containing U-II in the presence or absence of urantide were injected subcutaneously into mice and invasion of vessels into the plug was assessed after 7 days by immunohistochemistry using an antibody against CD31 (Fig. 1D). Compared with control plugs, vessel invasion was markedly increased into U-II-containing plugs. However, this response was not observed with Matrigel plugs also containing urantide. Because U-II also stimulated endothelial cell proliferation, as measured by BrdU incorporation, and urantide diminished this response (data not shown), our data clearly show...
that U-II stimulates angiogenesis in vitro and in vivo by acting through its receptor GPR14.

**NOX2-derived ROS are involved in angiogenesis**

Because ROS have been previously described to promote angiogenesis, we determined their contribution to U-II-induced angiogenesis. First, we measured ROS levels in the presence of U-II in endothelial cells by dihydroethidium (DHE) fluorescence (Fig. 2A,B). U-II was able to induce ROS formation, and this response was diminished by urantide or depletion of GPR14, indicating that U-II increases ROS levels in endothelial cells by acting through its receptor. Next, endothelial cells submitted to a Matrigel assay were treated with the antioxidant N-acetylcysteine (NAC) and then exposed to U-II. Compared with controls NAC completely prevented U-II-induced tube formation, suggesting the involvement of ROS in this response (Fig. 2C).

The NOX2-containing NADPH oxidase has been identified as an important source of ROS in endothelial cells, so we tested the involvement of NOX2 in U-II-induced angiogenesis. To this end, NOX2 was depleted by shRNA in endothelial cells. Compared with cells transfected with control shRNA, U-II-stimulated tube formation in the Matrigel assay was completely abolished in NOX2-depleted cells (Fig. 2D). In line with this, U-II was not able to increase ROS formation in NOX2-depleted endothelial cells (Fig. 2B).

To further test the functional relevance of NOX2 in the angiogenic response, we monitored vascular outgrowth from vena cava explants derived from Nox2 knockout (Nox2−/−) and wild-type mice. Upon stimulation with U-II, vessel sprouting was decreased in Nox2−/− explants (Fig. 2E). Subsequently, invasion of new vessels into Matrigel plugs containing U-II was diminished in Nox2−/− mice compared with wild-type mice (Fig. 2F), confirming that U-II promotes angiogenesis through NOX2 also in vivo.

**HIF-1α mediates U-II-induced NOX2 expression and activity**

We then investigated, whether NOX2 expression is regulated by U-II in endothelial cells. Indeed, NOX2 mRNA levels were rapidly elevated 2 hours after U-II stimulation (Fig. 3A) followed by increased NOX2 protein levels. Treatment with actinomycin D before exposure to U-II prevented U-II induction of NOX2 (Fig. 3B), suggesting that a transcriptional mechanism contributes to NOX2 upregulation by U-II.

Because the transcription factor HIF-1 plays an important role in controlling angiogenesis under hypoxia, we determined its involvement in the endothelial response to U-II. Interestingly,
U-II rapidly increased the levels of HIF-1α within 1 hour of stimulation (Fig. 4A). Subsequently, depletion of HIF-1α by shRNA diminished U-II-induced NOX2 mRNA and protein levels, whereas overexpression of HIF-1α increased NOX2 expression, indicating a tight link between HIF-1α and NOX2 (Fig. 4B,C). To determine whether HIF-1α upregulates NOX2 also in vivo, venae cavae and lungs were isolated from mice with a specific deletion of HIF-1α in endothelial cells (Hif1α EC, −/−, in Fig. 4D and supplementary material Fig. S1) or from control mice (Hif1α EC, +/+ ) and exposed to U-II for 1 day or left untreated (Fig. 4D). Although U-II stimulation increased NOX2 protein levels in wild-type tissues, NOX2 levels were reduced in tissues deficient in endothelial HIF-1α even in the presence of U-II, indicating that HIF-1α mediates NOX2 expression also in vivo. In fact, bioinformatic analysis of the NOX2 promoter (MatInspector, Genomatix, Munich, Germany) revealed the presence of a putative hypoxia-responsive element (HRE) at −5767 to −5771 bp, which is known to bind HIFs. We thus performed chromatin immunoprecipitation using an antibody against HIF-1α and analysed the precipitates by real-time PCR with primers amplifying the region of the NOX2 promoter containing the HRE or an intronic sequence of actin not containing an HRE. Compared with control conditions U-II enhanced binding of HIF-1α to the NOX2 promoter, whereas this was not the case for the actin gene (Fig. 4E, supplementary material Fig. S2). Finally, to evaluate the functional consequences of HIF-1α regulation of NOX2, we overexpressed HIF-1α and determined ROS levels. Compared with control cells, ROS levels were enhanced in HIF-1α-overexpressing endothelial cells (Fig. 4F). However, depletion of NOX2 in HIF-1α-overexpressing cells abrogated ROS generation, indicating that HIF-1α increases ROS production through its target gene NOX2. In line with this, depletion of HIF-1α completely prevented U-II-induced tube formation (Fig. 4G).

**NOX2 is involved in the upregulation and activation of HIF-1α**

Because ROS have previously been shown to be part of the signalling cascades regulating HIF-1α under non-hypoxic conditions, we investigated whether regulation of HIF-1α by U-II is also mediated by ROS. When endothelial cells were treated with NAC, HIF-1α induction by U-II was diminished (Fig. 5A). Subsequently, depletion of NOX2 prevented HIF-1α protein upregulation and HIF activation by U-II (Fig. 5B,C), indicating that NOX2 is not only a HIF-1 target gene, but NOX2-dependent ROS are also required for controlling HIF-1α levels by U-II. These findings suggested a positive feedback loop whereby NOX2 induces HIF-1α and vice versa. In line with this proposal, HIF-1α was absent in lung tissue and venae cavae isolated from Nox2−/− mice even in the presence of U-II (Fig. 5D). Additionally, immunohistochemical analysis of the Matrigel plugs implanted into wild-type or Nox2−/− mice detected increased HIF-1α staining in U-II-stimulated control plugs, but not in plugs from Nox2−/− mice (Fig. 5E).

**Discussion**

In this study we demonstrated that U-II is a potent stimulus of angiogenesis in vitro and in vivo and identified HIF-1α and its target NOX2 as crucial elements mediating this ROS-dependent response, because (1) U-II induced angiogenesis that was dependent on ROS, NOX2 and HIF-1α; (2) U-II increased NOX2 transcription that was dependent on HIF-1α in vitro and in vivo; (3) U-II upregulated HIF-1α that was dependent on ROS and NOX2; and (4) HIF-1α induced ROS generation through NOX2.

Recent evidence suggested that several peptidic hormones including angiotensin-II and endothelin-1 not only have vasoactive functions, but can also modulate proliferation and synthesis properties of vascular cells. Here we clearly demonstrated that the vasoactive peptide U-II is a strong activator of angiogenesis not only in vitro as has been indicated previously (Spinazzi et al., 2006) but also in vivo, and provided evidence that U-II acts through its receptor GPR14. Our study identified for the first time a ROS-dependent mechanism underlying the angiogenic response towards U-II and its receptor. We identified a NOX2-containing NADPH oxidase as a source of ROS: NOX2 depletion not only diminished U-II-induced ROS formation, but also prevented the angiogenic response towards U-II. This response was confirmed ex vivo in vena cava explants derived from Nox2−/− mice that were found to be resistant to the proangiogenic action of U-II, as well as in vivo,
in Nox2−/− mice in which the angiogenic response towards U-II was blunted. Although this is the first report demonstrating an involvement of NOX2 in the angiogenic response to U-II, our study is supported by previous reports indicating that NOX2 is important in the regulation of angiogenesis in response to VEGF, to hindlimb ischemia (Ushio-Fukai et al., 2002; Tojo et al., 2005) or to stimulation with thrombin under non-ischemic conditions (Diebold et al., 2009).

Although ROS-dependent angiogenesis by growth factors has been mainly attributed to activation of NOX2 through Rac1 (Ushio-Fukai et al., 2002), our study shows that in addition to rapid stimulation of ROS generation by U-II-induced Rac1 activation (data not shown), U-II also acts on a prolonged basis by inducing NOX2 expression by a transcriptional mechanism.

Importantly, this response was mediated by the transcription factor HIF-1α, the major regulatory element in the control of angiogenesis under hypoxic conditions. In fact, induction of NOX2 was completely absent in HIF-1α-depleted human endothelial cells as well as in vascular and lung tissue derived from mice deficient in endothelial HIF-1α. Importantly, using chromatin immunoprecipitation we could demonstrate direct binding of HIF-1α to the distal NOX2 promoter, identifying NOX2 as a true HIF-1 target gene. Of note, our previous data from smooth muscle cells showed that NOX4 also is a direct
target gene of HIF-1 under hypoxic conditions (Diebold et al., 2010a) and is also upregulated by U-II (Djordjevic et al., 2005). Similarly, U-II increased NOX4 levels in endothelial cells (supplementary material Fig. S3). In line with our previous reports that NOX4 contributes to endothelial proliferation (Petry et al., 2006), we also observed a contribution of NOX4 to U-II-induced angiogenesis (supplementary material Fig. S4) and ROS production (supplementary material Fig. S5). However, depletion of NOX4 was less effective in reducing ROS levels by U-II than depletion of NOX2, and knockdown of NOX2 and NOX4 did not further decrease ROS levels compared with NOX2 knockdown alone (supplementary material Fig. S5). This study, thus, clearly supports the importance of NOX2 in ROS generation and the angiogenic response towards U-II.

Together with our finding that the activator of NOX2, Rac1, is also transcriptionally regulated by HIF-1 (Diebold et al., 2010c), we consider there to be a close interaction between the HIF pathway and NADPH oxidases in different vascular cells. In fact, overexpression of HIF-1α was sufficient to induce ROS generation in endothelial cells and also in smooth muscle cells (Diebold et al., 2010a), but this response was prevented by depletion of NOX2 or NOX4, respectively. Because upregulation of NOX subunits is supposed to maintain elevated levels of ROS over a prolonged period of time, our findings suggest that HIF-1α takes centre stage in regulating NOX subunits not only under hypoxic, but also under normoxia conditions associated with increased levels of pathogenetically relevant factors such as U-II. Interestingly, U-II has been also found in tumour cells (Takahashi et al., 2003), and our own findings showed that tumour cells exposed to hypoxia are able to secrete U-II (data not shown). Because U-II induced HIF-1α- and NOX2-dependent angiogenesis, our findings could provide a new mechanism of regulating tumour angiogenesis.

Interestingly, within this setting, we could show that HIF activity and HIF-1α protein levels are rapidly upregulated by U-II, and this response was dependent on ROS. Consequently, HIF-1α could not be upregulated by U-II in NOX2-depleted endothelial cells or in vascular or lung tissue from Nox2−/− mice, supporting the notion that NOX2 is of primary importance for induction and maintenance of endothelial HIF-1α protein, which in turn promotes NOX2 transcriptional upregulation. In addition to earlier studies that showed that p22phox and Rac1, which are important for NOX2 function, regulate HIF-1α levels in response to thrombin (Gorlach et al., 2001) and several other stimuli (BelAiba et al., 2004; Dery et al., 2005; Gorlach and Kietzmann, 2007), more recent studies provided evidence that NOX4-dependent ROS generation enhances HIF-1α and HIF-2α levels (Bonello et al., 2007; Diebold et al., 2010b). Our study now adds NOX2 to the sources of ROS regulating HIF-2α levels and provides functional proof of the importance of NADPH-oxidase-derived ROS for maintaining HIF-2α levels under non-hypoxic conditions, which occurs by either stabilizing HIF-2α by impairing interaction with the ubiquitin 3 ligase pVHL, or by transcriptional upregulation of HIF-1α by an NFκB-dependent pathway (Bonello et al., 2007; Diebold et al., 2010b).

Collectively, our data suggest a model in which a rapid increase in ROS generation as a result of U-II activation of NADPH oxidases leads to increased HIF-1α levels.
**Angiogenesis**

The scheme summarizes the pathway described: urotensin-II binds to its receptor GPR14, leading to ROS generation from a NOX2 promoter and enhanced transcription of NOX2. Higher levels of ROS increase the angiogenic response towards U-II and subsequently in NOX2.

**Materials and Methods**

**Materials**

Urotensin-II was from Bachem (Weil am Rhein, Germany), urantide was from International peptides (Louisville, KY). All other reagents were from Sigma (Munich, Germany) unless otherwise stated.

**Cell culture**

Human umbilical endothelial cells (HUVEC) were from Lonza (Wuppertal, Germany), cultured in the medium provided as recommended, and used up to passage 4. Human microvascular endothelial cells (HMEC-1) were purchased from CDC (Atlanta, GA) and grown in MCDB 131 medium (Gibco, Karlsruhe, Germany) with 10 mM Mg\(^{2+}\) as recommended, containing 10% fetal calf serum (PAN Biotech, Aidenbach, Germany), 100 IU/ml penicillin (Gibco), 100 \(\mu\)g/ml streptomycin (Gibco), 1 \(\mu\)g/ml hydrocortisone, 2 mM L-glutamine (Gibco) and 1 g/l glucose, 10% fetal calf serum and 100 \(\mu\)g/ml streptomycin. All cells were grown at 37°C in 5% CO\(_2\) and passaged twice a week.

**Animals**

NOX2-deficient mice (Nox2\(^{-/-}\)) were obtained from Jackson Laboratories, Bar Harbor, ME (B6.129S6-Cbybm\(^{1}\)Dnu/J) (Pollock et al., 1995). Mice with endothelial cell-specific inactivation of HIF-1\(\alpha\) were generated by cross-breeding Tie2-Cre transgenic mice (B6.Cg-T(cre)12Fv/J; Jackson Laboratories) with Hif1a\(^{+/1}\) mice homozygous for the Hif1a allele with exon 2 flanked by loxp sites (B6.129-Hif1a\(^{cre}\); Jackson Laboratories) (Ryan et al., 2000). All mice were of a genetic C57BL/6 background. In all experiments, littermates from the same breeding pair were used as controls. For genotyping, genomic DNA was isolated from the tail, an intact aorta or an aorta with manually removed endothelium, using phenol–chloroform extraction according to standard protocols. Hif1a 1-lox and 2-lox alleles were detected with the following primers: forward 1: 5'- TTGGGGATGAAAACATCCTG3'; forward 2: 5'-GACGTTTAAAGACACTAGTGG3'; and reverse: 5'-GGAGCCTCTCTGCTAGACC3'. The 2-lox allele (flanked allele) was identified as a 260 bp band and the 1-lox allele (Hif1a deletion) as a 270 bp band. Tie2-Cre\(^{cre}\) was detected using primers within the Tie2 promoter and Cre-coding region (forward: 5'-CCCTTGCTCAAGCATGAATA3'; reverse: 5'-GCGATACACCTGGAACACACTTGG3'). All animal experiments were approved by the Regierung von Oberbayern.

**Plasmids and transfections**

The vectors encoding siRNA against HIF-1\(\alpha\), NOX2, NOX4 or a nonspecific random sequence (shCtr) have been previously described (Petry et al., 2006; Bonello et al., 2007; Diebold et al., 2010a). Specific short hairpin RNA encoding for a 19mer siRNA (5'-GGCGGTGGGCTCCATG3') against GPR14 (siGPR14) was created using the siRNA AttTarget Designer and the siREx U6 Hairpin Cloning System (Promega, Mannheim, Germany). All constructs were confirmed by DNA sequencing. The plasmid encoding V5-tagged HIF-1\(\alpha\) was kindly provided by Thomas Kietzmann, Oulu, Finland (Klein et al., 2008). The construct pGI53/EPOHRE-Luc containing three HIF-binding sites in front of the SV40 promoter has been described previously (Kietzmann et al., 2001). Cells were plated to a density of 70% and cultured for 24 hours. Transfections were performed using FuGene reagent (Roche, Mannheim, Germany) or NOX4 (Diebold et al., 2009). Transfection efficiency was on average 40%.

**Northern blot analysis**

Total RNA from endothelial cells was isolated using the RNasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA (10–15 \(\mu\)g) was separated on a 1.3% agarose gels, transferred to nylon membranes, and cross-linked by UV irradiation. Northern hybridizations were carried out with digoxigenin-labelled antisense RNA probes for NOX2 and HIF1A. Detection was performed after incubation with a digoxigenin antibody conjugated to alkaline phosphatase using the chemiluminescent substrate CDPScreen (Roche). Loading of equal amounts of RNA was confirmed by ethidium bromide staining of 18S RNA.

**Western blot analysis**

Western blot analysis was performed as described previously (Petry et al., 2006). Briefly, 50 \(\mu\)g of isolated proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. After blocking for 1 hour in TBS (Tris-buffered saline) containing 5% milk and 0.3% Tween 20 (TBS-T), membranes were incubated overnight at 4°C with a monoclonal antibody against NOX2 (mAb 48, kindly provided by Dirk Roos, Amsterdam), monoclonal or polyclonal antibodies against HIF-1\(\alpha\) (Transduction Laboratories, Heidelberg, Germany or Abcam, Cambridge, UK), or polyclonal antibodies against GPR14 (Santa Cruz, Heidelberg, Germany) or NOX4 (Diebold et al., 2010a). After incubation with a horseradish-peroxidase-conjugated secondary antibody (Calbiochem, Darmstadt, Germany) for 1 hour, proteins were visualized by lumino-enhanced chemiluminescence. Loading of equal amounts of proteins was confirmed by reprobing the membranes with an actin antibody (Santa Cruz, Heidelberg, Germany). Blots were scanned and analysed using GelDoc software (Bio-Rad, Munich, Germany).

**Chromatin Immunoprecipitation**

Confluent endothelial cells were serum-starved for 16 hours, and exposed to U-II for 4 hours. Cells were fixed with formaldehyde, lysed and sonicated to obtain DNA fragments of approximately 500–1000 base pairs. Chromatin was then precipitated with an HIF-1\(\alpha\) antibody (Abcam) overnight at 4°C. qPCR was performed with primers for the NOX2 promoter (forward: 5'-CGGCGGTTG- GGAGGAGAGTCGACACAG3'; reverse: 5'-TCTCGAGAAATGCGCCACACTGA3') flanking the potential HIF-1 binding site (antisense sequence: aacactA- CGTGgc, HRE at –5767 to –5771 bp) using a Rotorgene 6000 (Corbett, Sydney, Australia). As a negative control for nonspecific binding and precipitation, qPCR using primers amplifying a region of the third intron of \(\beta\)-actin (gene ID: 60) without a putative HRE (5'-ACGGT-3') was performed (forward: 5'-AAACTGGTTGCTCTGTGAACA3'; reverse: 5'-AAAGTGCAAAAGACAC-


Fig. S1: Genotyping of endothelial-specific Hif1α knock-out.
Genomic DNA was isolated from tail or from aortae from wildtype (ECHif1α+/+) or HIF-1α-deficient (ECHif1α-/−) mice with (+EC) or without (-EC) endothelium. PCR was performed corresponding to the floxed Hif1α allele (2-Lox), the exon 2 deleted Hif1α allele (1-Lox) or the Tie2Cre transgene (Tie2cre).
Fig. S2: HIF-1α binds to the NOX2 gene in response to Urotensin-II.

A. Human microvascular endothelial cells were stimulated with urotensin-II (U-II, 100 nM) for 4 h. Chromatin immunoprecipitation was performed using an antibody against HIF-1α or unspecific IgG followed by a semiquantitative PCR using primers amplifying a region flanking the predicted hypoxia-responsive element (HRE) within the NOX2 promoter (NOX2) or a region in the first intron of β-actin not containing an HRE. Template was omitted for negative control (H2O). B. Compilation of DNA sequences containing HREs from known HIF1 target genes and the predicted NOX2 HRE. VEGF-R1: vascular endothelial growth factor receptor-1; PAI: plasminogen activator inhibitor.
Figure S3: Urotensin-II induces NOX4 expression in endothelial cells.
Human microvascular endothelial cells were stimulated with urotensin-II (100 nM) for the indicated time points. Western blot analysis was performed using an antibody against NOX4. Ponceau S staining (Ponc) of the membrane served as loading control.
Fig. S4: NOX4 contributes to Urotensin-II-mediated angiogenesis.
Human microvascular endothelial cells were transfected with plasmids coding for NOX4 shRNA (shNOX4) or for control shRNA (shCtr). Cells were plated on growth factor reduced matrigel and were either stimulated with urotensin-II (100 nM) or left untreated. Formation of capillary-like structures was monitored after 6 h.
**Fig. S5: NOX4 contributes to Urotensin-II-induced ROS generation.**

Human microvascular endothelial cells were transfected with plasmids encoding for shRNA against NOX2 (shNOX2), NOX4 (shNOX4) or control shRNA (shCtr) as indicated. Cells were stimulated with 100 nM urotensin-II for 4 h and ROS generation was determined using dihydroethidine (DHE) fluorescence in a microplate reader. ROS generation is represented as relative change to unstimulated control (shCtr) (n=3, *p<0.05 versus shCtr; #p<0.05 versus U-II stimulated shCtr). NOX4 knockdown efficiency was determined by Western blot analysis using an antibody against NOX4. Ponceau S staining (Ponc) served as loading control.