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 oxidases 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 knockout 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 b558. 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, vena 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 the 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).

**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 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 peptide 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

**Fig. 4. HIF-1α mediates U-II-induced NOX2 expression and activity.** (A) HMEC-1 cells were stimulated with U-II (100 nM) for different times. Western blot analyses were performed using an antibody against HIF-1α. Actin served as a loading control. (B, C) HMEC-1 cells were transfected with vectors encoding HIF-1α (HIF1α), control shRNA (shCtr) or shRNA against HIF-1α (shHIF1) and stimulated with U-II for 4 hours. (B) Northern blots were performed using specific probes for NOX2 or HIF1α. Ethidium bromide staining for 18S RNA served as a loading control. (C) Western blot analyses were performed using antibodies against NOX2 or HIF-1α. Actin was used as loading control. All blots are representative of three independent experiments. (D) Lung tissue and venae cavae were isolated from endothelial-cell-specific HIF-1α knockout (Hif1α EC, −/−) or control mice (Hif1α EC, +/+). Tissues were stimulated ex vivo with U-II for 24 hours or left untreated, and protein was isolated. Western blot analyses were performed with antibodies against NOX2, HIF-1α or actin. Blots are representative of three independent experiments. (E) HMEC-1 cells were stimulated with U-II for 4 hours and chromatin immunoprecipitation was performed with an antibody against HIF-1α. qPCR was performed using specific primers to amplify a fragment of the NOX2 promoter containing a putative HIF binding site. Quantification is shown in promille to chromatin input after background IgG subtraction (n=3, *P<0.05 versus Ctr). (F) HMEC-1 cells were cotransfected with vectors coding for HIF-1α (HIF1α) or control vector and vectors coding for shRNA against NOX2 (shNOX2) or control shRNA (shCtr). ROS levels were evaluated by DHE fluorescence. ROS levels of cells transfected with control vectors were set to 100% (n=3; *P<0.05 versus control or #P<0.05 versus cells overexpressing HIF-1α). (G) Human umbilical vein endothelial cells (HUVEC) were transfected with vectors encoding shRNA against HIF-1α (shHIF1α) or control shRNA (shCtr) and plated on Matrigel. Tube formation was assessed 4 hours after stimulation with U-II (100 nM). The images are representative of three independent experiments.
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 non-hypoxic 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-α levels in response to thrombin (Gorlach et al., 2001) and several other stimuli (BelAlba 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-α levels and provides functional proof of the importance of NADPH-oxidase-derived ROS for maintaining HIF-α levels under non-hypoxic conditions, which occurs by either stabilizing HIF-α by impairing interaction with the ubiquitin 3 ligase pVHL, or by transcriptional upregulation of HIF-1α by an NFkB-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
Angiogenesis

Fig. 6. Scheme. The scheme summarizes the pathway described: urotensin-II binds to its receptor GPR14, leading to ROS generation from a NOX2-containing NADPH oxidase. This results in upregulation of the hypoxia-inducible transcription factor HIF-1α. Active HIF-1 binds to the NOX2 promoter resulting in increased NOX2 expression. Enhanced NOX2 levels result in increased ROS generation, which fuels this feed-forward loop leading to increased angiogenesis.

NOX2 elevates HIF-1α levels leading to HIF-1α binding to the NOX2 promoter and enhanced transcription of NOX2. Higher levels of ROS increase the angiogenic response towards U-II and also help to keep HIF-1α levels elevated for a more prolonged time period (Fig. 6). One might speculate that this positive-feedback loop is limited by a mechanism similar to the situation under hypoxia (del Peso et al., 2003): increased HIF-1α levels would over time upregulate the levels of prolyl hydroxylases known to be HIF target genes. This would then in turn limit further increases in HIF-1α and subsequently in NOX2.

Our results provide support for the importance of the crosstalk between NADPH oxidases and HIF proteins in a variety of conditions associated with enhanced angiogenesis under non-hypoxic conditions.

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 Lonza (Wuppertal, Germany). Human umbilical endothelial cells (HUVEC) were from Lonza (Wuppertal, Germany). Human umbilical endothelial cells (HUVEC) were from Lonza (Wuppertal, Germany).

Plasmids and transfections

The vectors encoding shRNA against HIF-1α, NOX2, NOX4 or a nonspecific random sequence (shCtrl) 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'-GTCGCGTGGCCCTCATTGA-3') against GPR14 (siGPR14) was created using the siRNA Design Template and the manufacturer's protocols. Hairpin Cloning System (Promega, Mannheim, Germany). All constructs were confirmed by DNA sequencing. The plasmid encoding V5-tagged HIF-1α was kindly provided by Thomas Kietzmann, Oulu, Finland (Klein et al., 2008). The construct pE10POHRE-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) as described (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 μg) 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 CDPStar (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 μg 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-1α (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 luminol-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-1α antibody (Abcam) overnight at 4°C. qPCR was performed with primers for the NOX2 promoter (forward: 5'-CCGTTGTGTTGAAGAGATTGTAGGA-3'; reverse: 5'-TCTCTGAGAATGCCCACACTGAGA-3') flanking the potential HIF-1 binding site (antisense sequence: aactata-CTGTTg, HRE at −5767 to −5771 bp) using a Rotorgene 6000 (Corbett, Wackersee, Germany). As a negative control for nonspecific binding and precipitation, qPCR using primers amplifying a region of the third intron of β-actin (gene ID 60) without a putative HRE (5'-ACGTCG-3') was performed (forward: 5'-AACCAGTGCTCTGTTGGCAAC-3'; reverse: 5'-AAAGTGGCAGAAAAAA-3').
GGCT-3'). As a background control, chromatin immunoprecipitation was performed using standard IgG (Santa Cruz). Statistical analysis was performed using a standard curve of the input, and increased HIF-1α binding to chromatin is presented after background (IgG) subtraction as relative amount of the input used. In addition, a non-quantitative PCR was performed in a standard Thermocycler (Applied Biosystems, Invitrogen, Germany).

Measurement of ROS production
ROS generation was detected using the fluorochrome dihydroethidium (DHE, Invitrogen, Karlsruhe, Germany) in a microplate reader (Tecan, Crailsheim, Germany) as described previously (Diebold et al., 2009).

Ex vivo Matrigel assay
Endothelial cells were seeded at a density of 25,000 cells per well on a 96-well plate mounted with growth-factor-reduced Matrigel (BD Biosciences, Heidelberg, Germany). Cells were then incubated for 24 hours at 37°C and stained using Diff-Quick (Baxter Diagnostics, Duedineng, Switzerland). The formation of closed 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 ImageJ software (Wright Cell Imaging Facility, Toronto, Canada) as described previously (Diebold et al., 2009).

Matrigel plug assay
Growth-factor-reduced Matrigel (BD Biosciences) was mixed with U-1 (100 nM) and/or uridine (100 nM). The Matrigel mixture was subcutaneously injected into 6-week-old male mice (C57BL/6, or NOD2−/− mice) were killed and the vena cava was excised. The vena cava was transferred into a dish with sterile PBS. After removing the fibroblast tissue, vessels were sectioned into 1–1.5 mm long cross sections, placed on Matrigel-coated wells (BD Bioscience; 100 μl/well) and incubated at 37°C for 20 minutes. Thereafter the rings were incubated with 500 μl DMEM medium. The cultures were kept at 37°C in a humidified environment for 4 days. Every second day vessel sprouting was assessed by light microscopy (Olympus) using the Openlab Modular Software for Scientific Imaging (Improvision) and was quantified using ImageJ software (Wright Cell Imaging Facility). All animal experiments were approved by Regierung von Oberbayern.

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References
In addition, a non-quantitative PCR was performed in a standard Thermocycler using a standard curve of the input, and increased HIF-1α levels were determined by staining with an antibody against CD31 (DAKO, Glostrup, DK) in a 1:50 dilution. HIF-1α levels were determined by staining with an antibody against HIF-1α (Abcam) in a 1:100 dilution. 1% BSA was used as negative control. All animal experiments were approved by Regierung von Oberbayern.

Statistical analysis
Values are presented as means ± standard deviation (s.d.). Results were compared by ANOVA for repeated measurements followed by Student–Newman–Keuls t-test. P<0.05 was considered statistically significant.

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