Hypoxia-Induced Signaling in Angiogenesis

Role of mTOR, HIF and Angiotensin II

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1. Summary

This thesis includes the work of four different projects I have been following during my time as a PhD student; (1) the characterization of mTOR-associated signaling and endothelial cell proliferation in response to hypoxia, and (2) identification of signaling pathways responsible for HIF stabilization during hypoxia. A side project aimed at (3) elucidating mechanisms of angiotensin II-induced angiogenesis. Furthermore, I have contributed to a review about antihypertensive drugs and microvascular rarefaction.

Hypoxia is the main stimulus for angiogenesis, the formation of new microvessels from pre-existing ones. To maintain adequate metabolism and supply of energy, eukaryotic cells adapt when oxygen levels drop. β-Oxidation is switched off while enzymes for glycolysis are induced. In most cells, cell cycle is arrested to reduce the number of oxygen consuming cells.

When oxygen levels are low for a longer period, erythropoiesis and angiogenesis are induced to increase tissue oxygenation. Specialized cells such as vascular endothelial cells (EC) and smooth muscle cells (SMC) are activated and increase proliferation and gene expression in response to hypoxia. EC proliferation and angiogenesis in response to hypoxia is, amongst others, rapamycin-sensitive. Thus, we hypothesized that mammalian target of rapamycin (mTOR) is involved in the response to hypoxia in endothelial cells. mTOR is central in regulating cell growth and proliferation, and integrates signals from nutrients, growth factors, energy status and stress such as hypoxia. Recent studies have identified two structurally distinct mTOR multi protein complexes (mTORC1 containing raptor and mTORC2 containing rictor) with individual downstream targets.

Study 1: In the first project, we have investigated mTOR-associated signaling components under hypoxia and their role in cell proliferation in rat aortic endothelial cells (RAECs). By analyzing mTOR and the distinct downstream targets of mTORC1 (S6 kinase) and mTORC2 (PKB/AKT), we found that hypoxia activates mTOR signaling in a timed program, leading to early activation and late inhibition of mTORC1 and a delayed but sustained activation of mTORC2. Raptor and rictor knock down demonstrated that rictor (mTORC2) is essential for hypoxia-induced endothelial proliferation, whereas raptor knock down only partially inhibited increased proliferation.

When studying the pathways directing the hypoxic stimulus to mTOR, we found that hypoxia-induced cell proliferation is independent of regulation by TSC (tuberous sclerosis complex). TSC is upstream of mTORC1 and directs growth factor signals and energy and nutrient status into this signaling pathway. Thus, hypoxia impinges on mTOR TSC-independently; rapid mTOR phosphorylation under hypoxia rather suggests a direct activation step. All together, our data suggest cooperating mechanisms between signals from both mTOR complexes in the response to hypoxia in EC.

Study 2: To study potential downstream effectors of mTOR-dependent proliferation in response to hypoxia we have focused on Hypoxia inducible factors (HIF). HIFs mainly control transcription of genes for angiogenesis, erythropoiesis and glycolysis in response to hypoxia. In normoxia HIF-α's are constantly degraded. Degradation is prevented in hypoxia, the HIF-α's form heterodimers with HIF-β's, translocate to the nucleus and become transcriptionally active. HIF-1α stabilization in hypoxia was shown to be rapamycin sensitive, and therefore to potentially require active mTOR signaling. How mTORCs stabilize HIF-α's is unclear.

In this study we have investigated the regulation and role of HIF1-α in hypoxia-induced proliferation of aortic endothelial cells. Hypoxia and growth factor stimulation induced stabilization and translocation of HIF-1α to the nucleus. By using siRNA constructs, we found that HIF-1α knock down reduces RAEC proliferation in hypoxia. The pathways potentially regulating HIF-1α have been investigated by using specific inhibitors of signaling relay enzymes. We show that mTOR is required for HIF-1α accumulation during hypoxia and growth factor stimulation, and is partially responsible for the increased proliferation of RAECs in hypoxia. Inhibition of MEK1/2 signaling only affected growth factor-induced HIF-1α stabilization under normoxia and endothelial proliferation under normoxia and hypoxia to a similar extent, thus not specifically affecting the hypoxic response. Knock down of raptor and rictor should answer the central question, which of the two mTORCs is responsible for HIF-1α stabilization in hypoxia. These experiments are ongoing.

Review: Hypertension and impaired angiogenesis are intrinsically linked. Angiogenesis is impaired in most hypertensive patients, and microvascular rarefaction contributes to hypertension-induced end organ damage. In the framework of a review we summarized

and discussed the effects of antihypertensive drugs on microvessel structure. Studies done with diuretics, α- and β- adrenergic receptor blockers and calcium antagonists are inconclusive. Most promising for an induction of angiogenesis or normalization of microvessel structure are angiotensin II type1 receptor blockers (AT_1 receptor blockers, ARBs) and ACE (angiotensin converting ezyme) inhibitors.

Study 3: ARBs and ACE inhibitors both influence the renin-angiotensin-aldosterone system (RAAS). RAAS controls blood pressure by regulating vasodilation and vasoconstriction. The vasoactive peptide Angiotensin II (Ang II) is generated by cleaving Ang I by ACE. Ang II causes vasoconstriction by activating the AT_1 receptor. The AT_2 receptor is the other potential binding domain for Ang II and can interact with the bradykinin receptor B2 (BK-B2 receptor). Bradykinin binds the BK-B1 and BK-B2 - receptors to up regulate nitric oxide, growth factors and was shown to induce angiogenesis.
Using an angiogenesis assay in vitro and tissue from left ventricular myocardium of AT_1 and AT_2 –knock out and wild type mice, we investigated the mechanism underlying the angiogenic effects of angiotensin II. AT_1 and AT_2 –receptors were expressed in normoxia and hypoxia. Ang II induced angiogenesis dose-dependently but only in hypoxia. Induction of angiogenesis by Ang II was dependent on the availability of the AT_2 and B2 receptor, as blockade or knock out of AT_2 inhibited angiogenesis in vitro. Also, Ang-II-induced angiogenesis was nitric oxide (NO) dependent. Inhibiting the formation of bradykinin with a specific kininogenase inhibitor completely abrogated Ang II-induced angiogenesis. Taken together, this study suggests an obligatory role of hypoxia in the angiogenic effect of Ang II via the AT_2 receptor through a mechanism that involves bradykinin, its B2 receptor and NO as a downstream effector.

Angiogenesis occurs in physiological but also in pathological situations and may be activated or inhibited in a therapeutic approach: Inhibiting hypoxia-driven tumor angiogenesis may reduce cancer growth whereas stimulation of angiogenesis after myocardial infarction may speed up tissue regeneration. Induction of microvessel growth may also decrease peripheral resistance and thereby reduce hypertension.
Thus, mechanisms and pathways studied in this thesis are involved in the process of angiogenesis and may contribute to the identification of potential targets to develop drugs for modulating angiogenesis in patients.

2. Introduction

2.1. Hypoxia

Oxygen is essential for all eukaryotic organisms to drive oxidative phosphorylation for generating energy. Therefore a constant oxygen supply, maintained by the vascular system in mammals, is pivotal for most organs. The supply of oxygen to the tissue is regulated by the number and function of blood vessels, whereas the number of cells in the tissue regulates the demand. Hypoxia emerges when oxygen delivery does not meet the demands of the tissue[4]. This can either occur in rapidly proliferating tissue or as a result of occlusion of blood vessels, e.g. during embryonic development, tumor growth, wound healing and ischemia (i.e. ventricular hypertrophy)[5].

The physiologic oxygen concentration in different tissues varies from 14% in arterial blood, to less than 10% in the myocardium, and to 8-2% in the liver, cartilage or bone marrow. Experimental hypoxia (in cell culture) is routinely established by placing the cultures to an incubator, containing oxygen concentrations in the gas phase of 0.5-3.0%[4,6].

When oxygen concentrations drop below their physiological levels, distinct systems respond to these environmental conditions. Energy production can be rapidly switched to anaerobic glycolysis. Therefore enzymes like phosphofructokinase or glucose transporter-1,3 are up-regulated by hypoxia to drive glycolysis[7,8]. To further correct the balance between O_2 demand and supply, the cell cycle of most cells is arrested and addition of new cells into the tissue stops to reduce the number of oxygen consuming cells[9,10]. Some specialized rescue cells, i.e. vascular endothelial cells (ECs), smooth muscle cells (SMCs) and mouse embryonic fibroblasts increase their proliferation in response to hypoxia[10,11], and participate in the formation of new microvessels to increase oxygen concentrations in particular tissues[5]. Moderate hypoxia is not typically toxic when sufficient nutrients and glucose are present. However, if energy is not sufficient or even, anoxia occurs, most cells stop proliferation and eventually undergo apoptosis[12].

The bad efficiency factor of glycolysis, the accumulation of lactate during glycolysis and the need to avoid cell death induces mechanisms to maintain aerobic energy production. To increase tissue oxygenation, hypoxia also induces regulation of a very complex series of responses necessary to increase the number of red blood cells (erythropoiesis), to relax existing- and to generate new blood vessels. Hypoxia induces vasodilatory enzymes e.g. inducible nitric oxide synthase (iNOS) and pro-angiogenic

factors e.g. vascular endothelial growth factor (VEGF) or placental growth factor (PIGF) and represses anti-angiogenic molecules[13,14]. Most hypoxia-induced genes are regulated by hypoxia inducible transcription factors (see 2.5. and 4.2.).

2.2. The vasculature – vasculogenesis, angiogenesis and arteriogenesis

Hypoxia is the main stimulus to induce angiogenesis and stimulates the expansion and remodeling of the existing vasculature to enhance blood flow in oxygen-deprived tissue. The vascular network mediates the delivery of oxygen and nutrients to all cells of the organism, removes metabolites and carbon dioxide, and maintains an adequate hydrostatic pressure[2].

Angiogenesis is a complex morphogenic process, that occurs in a stepwise fashion and is primarily induced by hypoxia that induces a variety of positively- and negatively-acting growth factors[5].

In early development, oxygenation of a cell aggregate can be maintained by diffusion of O_2. At a critical tissue size a vascular system has to be developed to keep up O_2 supply for each cell. The initial embryonal step for vascular development is called vasculogenesis, the formation of new blood vessels when there are no pre-existing ones. Angioblasts (vascular endothelial cells that have not yet formed a lumen) proliferate, migrate and differentiate to subsequently form a primitive blood vessel and the primary capillary plexus[5,15,16].

After the primary vascular plexus is formed, endothelial cells form new capillaries by sprouting or by splitting from their vessel of origin. This process is termed angiogenesis. First, blood vessels dilate, partially induced by nitric oxide (NO). NO and VEGF increase in vascular permeability, and cause pericytes, surrounding the vessels, to detach. Degradation of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs) allows ECs to migrate in response to chemotactic growth factors. VEGF and angiopoietins, together with fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF), guide the migration and proliferation of ECs to form migration columns. Behind the migration columns, endothelial cells adhere to each other and create a lumen, which is accompanied by basement-membrane formation and pericyte and SMC attachment. Finally, blood-vessel sprouts will fuse with other sprouts to build new circulatory systems. Non-sprouting angiogenesis occurs predominantly in the lung.

Endothelial cells proliferate inside a vessel, producing a wide lumen that can be split by transcapillary pillars, or fusion and splitting of capillaries[3,5,16].

Arteriogenesis describes the remodeling process of pre-existing arterio-arteriolar anastomoses, where an increase in the luminal diameter will form large functional arteries. Growth of these collateral arteries is triggered by physical forces, but does not require hypoxia as a stimulus. Fluid shear stress or other mechanical forces trigger proliferation of SMCs which surround the vessels to increase their stability[17,18].

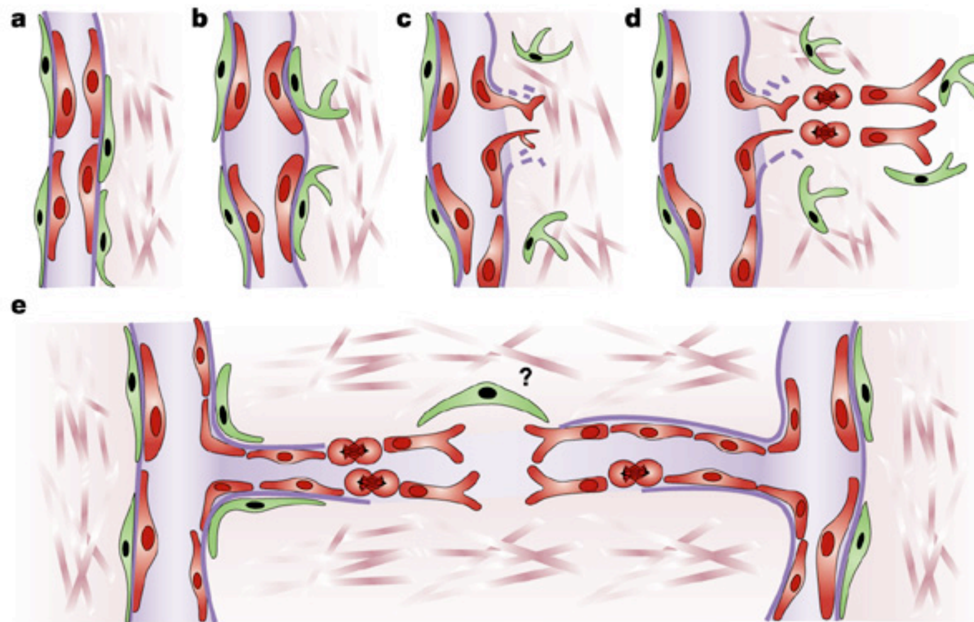


Figure 1. New blood-vessel formation. Blood vessels arise from pre-existing capillaries or post-capillary venules in tumours (a). (b) First, pericytes (green) detach and blood vessels dilate before the basement membrane and extracellular matrix is degraded. (c) This allows endothelial cells (red) to migrate into the perivascular space towards angiogenic stimuli produced by the tumour cells or host cells. (d) Endothelial cells proliferate, loosely following each other, and are presumably guided by pericytes. (e) Behind the migration columns, endothelial cells adhere to each other and create a lumen, which is accompanied by basement-membrane formation and pericyte attachment. Finally, blood-vessel sprouts will fuse with other sprouts to build new circulatory systems. Little is known about this fusion mechanism.
Figure by Bergers[3].

2.3. Hypertension – impaired angiogenesis and microvascular rarefaction

Arterial hypertension can be primary or secondary. Secondary arterial hypertension occurs only in 5-10% of hypertensive patients and can be a consequence e.g. of obesity, alcoholism and hormonal disorders. Arterial hypertension can lead to left ventricular hypertrophy, arthereosclerosis, myocardial infarction and other endorgan damages[19].

Reasons for primary hypertension are complex and not yet fully understood. Interestingly, most forms of arterial hypertension are associated with decreased numbers of microvessels. The arteriolar and microvascular circulation is important in determining blood pressure. Microvascular rarefaction can further increase peripheral resistance, raise blood pressure and therefore worsen hypertension[2].

Rarefaction can be primary or secondary. Decreased capillaryzation antedates the raise in blood pressure in primary rarefaction. Impaired angiogenesis, e.g. during development, might lead to a reduced vascular system and therefore predispose to high blood pressure[20]. Also, low birth weight can be accompanied with reduced formation of microvascular networks and increased tendency towards developing hypertension [21].

Secondary rarefaction is a consequence of prolonged elevation of blood pressure and might be caused by functional shut-off or destruction of existing capillaries[22]. Increased sensitivity to vasoconstrictors could lead to reduced perfusion of microvessels to the point of non-perfusion and therefore cause necrosis and apoptosis of these vessels[23].

However, offspring of individuals with high blood pressure have fewer capillaries on the dorsum of their fingers before the manifestation of hypertension[24]. Thus, capillary rarefaction might antedate, rather than follow, sustained hypertension and microvascular remodeling can be totally or partially blood pressure independent[24-27].

2.4. Anti hypertensive drugs and vascularization - the renin-angiotensin-aldosterone system

Treatment with anti-hypertensive drugs to avoid multiple consequences of hypertension can also normalize the microvascular system. Long-term and effective antihypertensive treatment of non-diabetic hypertensive patients increases capillary density compared with non-treated patients in a recent study[28].

There is a broad range of different antihypertensive drugs. For most substance classes, the influence on the microvasculature is unclear. Diuretics probably negatively influence the microvasculature. They attenuate expression of angiogenesis related genes and inhibit proliferation of endothelial cells in vitro[29-31]. Reports on the effects of α- and β-

adrenergic receptor blockers on microvascular rarefaction are also inconclusive. The β-blocker nebivolol, but not metoprolol, inhibits endothelial sprout formation in vitro and causes apoptosis of aortic and coronary ECs and SMCs (Petrimpol, M., unpublished)[32]. α-Blockers can either inhibit microvascular formation (doxazosin, terazosin) or increase total vascular area (prazosin)[33-35]. Nifedipine, a calcium antagonist can raise VEGF levels and induce capillary tube formation whereas an other calcium channel- and a chloride channel blocker (mibefradil and NPPB) inhibit tube formation of microvascular endothelial cells[36,37].

Influencing the renin-angiotensin-aldosterone system (RAAS) seems to be most promising for improving the microvasculature in hypertensive patients[2].

The RAAS is a main regulator for vasodilation, vasoconstriction and blood volume and thus blood pressure. In addition, the RAAS affects expression of the angiogenic molecules VEGF- and FGF levels and therefore angiogenesis[2].

Renin, a protease, cleaves the protein angiotensinogen to produce the inactive peptide angiotensin I (Ang I). Angiotensin-converting enzyme (ACE) cleaves Ang I to produce angiotensin II (Ang II), a vasoactive peptide. ACE also catalyses the breakdown of bradykinin, a vasodilator that binds to the bradykinin receptors (BK-B_1 and BK-B_2 -receptors) to induce NO and vasodilation, and the expression of VEGF and FGF. Ang II can either bind the angiotensin II receptor 1 (AT_1 receptor) inducing vasoconstriction, thus elevating blood pressure, or the angiotensin II receptor 2 (AT_2 receptor), inducing vasodilation and angiogenesis via bradykinin and by up regulating growth factors[2,38]. The AT_1 receptor is ubiquitously expressed whereas the AT_2 receptor is expressed early during development and after ischemic insult at lower levels in the adult[39,40].

In some older studies ACE inhibition blocked microvessel formation or reduced aortic and microvascular growth, suggesting anti-angiogenic properties for ACE inhibitors (or pro-angiogenic properties for Ang II, ACE inhibition prevents the formation of Ang II)[41-43]. However the majority of studies support a pro-angiogenic role for ACE inhibitors (ACE-Is). ACE-Is significantly increase myocardial capillary density and decrease ventricular hypertrophy[26,44-47]. Furthermore, several studies in ischemic hind limbs of mice and rabbits suggest a pro-angiogenic role for ACE-Is[48,49,45]. AT_1 receptor blockers (ARBs) prevent induction of vasoconstriction by Ang II but still allow activation of the AT_2 receptor. Several reports have demonstrated that ARBs increase capillary density[50-52]. The ARB losartan reversed rarefaction via induction of VEGF and increased angiogenesis in a NO and bradykinin (B2 receptor) dependent manner[50,53]. In an earlier

study in 70 hypertensive patients, losartan reduced vascular hypertrophy and rarefaction after three years of randomized, blinded treatment[54]. Thus, activation of AT_1 receptor may be antiangiogenic, or, ARBs may reveal a proangiogenic role of the AT_2 receptor[2].

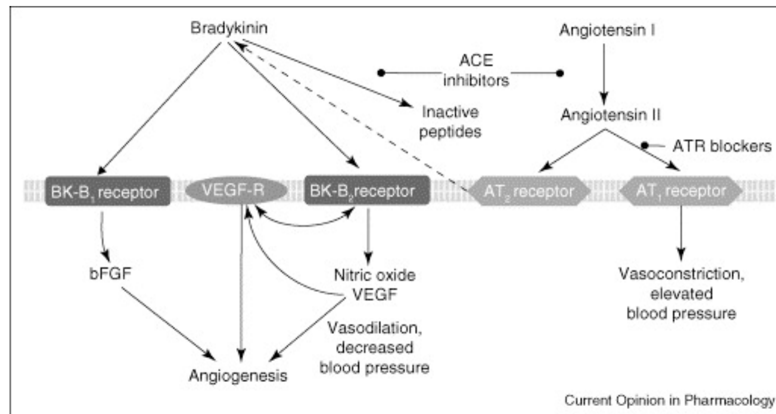


Figure 2. Angiotensin and bradykinin interact to induce angiogenesis. Bradykinin (BK), a potent vasodilator involved in regulation of blood pressure, induces angiogenesis. BK upregulates angiogenic molecules such as basic fibroblast growth factor (bFGF), via the BK B1 receptor and, or VEGF and NO, via the BK B2 receptor. The BK B2 receptor can also activate the VEGF receptor on endothelial cells. ACE inhibition results in BK accumulation and promotion of neovascularization. Moreover, angiotensin II activates the AT_2 receptor during AT_1 receptor blockade, thereby upregulating BK and contributing to an angiogenic response. ATR, angiotensin receptor. Figure by Battegay[2]

2.5. Hypoxia inducible factors (HIFs) - regulation and implication in angiogenesis

Hypoxia inducible factors (HIFs) are master regulators of O_2 homeostasis. The induction of the hypoxia inducible factor family of transcription regulators is a primary effect of the adaptive response to hypoxia in mammals. As mentioned in *2.1,* these proteins activate the expression of a broad range of genes, that mediate many of the responses to decreased oxygen concentration: enhanced glucose uptake by up-regulation of glucose transporter- and phosphofructokinase gene, increase in red blood cell production by erythropoietin and the production of new blood vessels via angiogenesis (e.g. VEGF)[5,7,8,55,56]. Beside hypoxia, growth factors, Ang II, thrombin and other hormones, can induce HIF proteins[57-59]. The hypoxic response and the HIF pathway are conserved from *C. elegans* and *Drosophila* to mice and man[5].

HIF proteins are members of a larger group of proteins known as bHLH-PAS (basic loop helix-Per ARNT Sim) proteins. Per, ARNT (aryl hydrocarbon nuclear translocator) and Sim were the three first proteins identified with such domains. Each member of this family contains an N-terminal bHLH domain that mediates binding to consensus DNA sequences, e.g. to the hypoxia response element (HRE), in the promoters of target

genes[5]. HIF proteins heterodimerize via their HLH and PAS domains in the center of each protein to form functionally active transcription factors[60].

The HIF family is comprised of three α subunits that are encoded in three different genes (HIF1A, EPAS1, HIF3A)[61]: HIF-1α, also known as MOP1/PAS1, HIF-2α also known as EPAS1 (endothelial PAS 1), MOP2 or HLF (HIF-1α-like factor), and HIF-3α also known as MOP3 or IPAS (inhibitory PAS). There are also three β subunits: HIF-1β or ARNT, ARNT2 and ARNT3. HIF-proteins form heterodimers of α and β subunits[5]. HIF-1α/ARNT and HIF-2α/ARNT complexes have been shown to be primarily responsible for the hypoxic induction of angiogenesis[62,63].

Expression of HIF-1α is ubiquitous in humans and mice whereas HIF-2α is predominantly expressed in the endothelium, lung and highly vascularized organs[61,64]. Knockout studies in mice demonstrate that HIF-1α and HIF-2α play nonredundant roles. This may result, in part, from differences in tissue-specificity and temporal patterns of induction of each isoform[1]. HIF-3α is also expressed in a variety of tissues and can also dimerize with ARNT and bind to HREs[65]. A splice variant of HIF-3α, called IPAS (inhibitory PAS), interacts with HIF-1α to prevent its DNA binding. HIF-3α in this way can act as a negative regulator to transcriptional responses to hypoxia[66,67].

HIF-1α is constitutively transcribed and constantly degraded with a half-life of only 5 min[68]. Under hypoxia or through other stimuli, HIF-1α degradation is prevented, the protein is stabilized and translocates from the cytoplasm to the nucleus, dimerizes with HIF-1β and binds to an HRE-domain; the formed HIF complex becomes transcriptionally active.

In normoxic conditions, two proline residues, located in the ODDD (oxygen dependent degradation domain) of HIF-1α are hydroxylated by a prolyl hydroxylase (PHD). The hydroxylated protein is now a target for the pVHL (van Hippel-Lindau) /E3 ubiquitin ligase complex, ubiquitinated and rapidly degraded by the proteasome. The prolyl residues targeted by the PHD are conserved in HIF-2α and HIF-3α. Oxygen and iron ions (Fe^{2+}) are absolutely required cofactors for PHD. In the absence of oxygen PHD is inactive and HIF-1α cannot be hydroxylated and thus not be bound by pVHL and its degradation is prevented[5,69]. PHDs might therefore act as direct oxygen sensors within the cells[70].

Stabilization alone is not sufficient for full transcriptional activation of HIF-1. The second major mechanism controlling HIF activity is through recruitment of transcriptional co-

activators (e.g. CBP and p300) to HIFs' C and N –terminal transactivation domains (C-TAD, N-TAD). Under normoxia, a dioxygenase (factor inhibiting HIF-1 (FIH)) hydroxylates asparagine residues within HIFs' TADs and prevents binding of the coactivators. Since FIH requires oxygen for catalytic activity, HIFs' TADs remain unmodified under hypoxia and can interact with its cofactors to activate transcription of its target genes. HIF is also modified by phosphorylation, acetylation, s-nitrosylatin and sumoylation[71]. The function of these modifications is not fully understood yet.

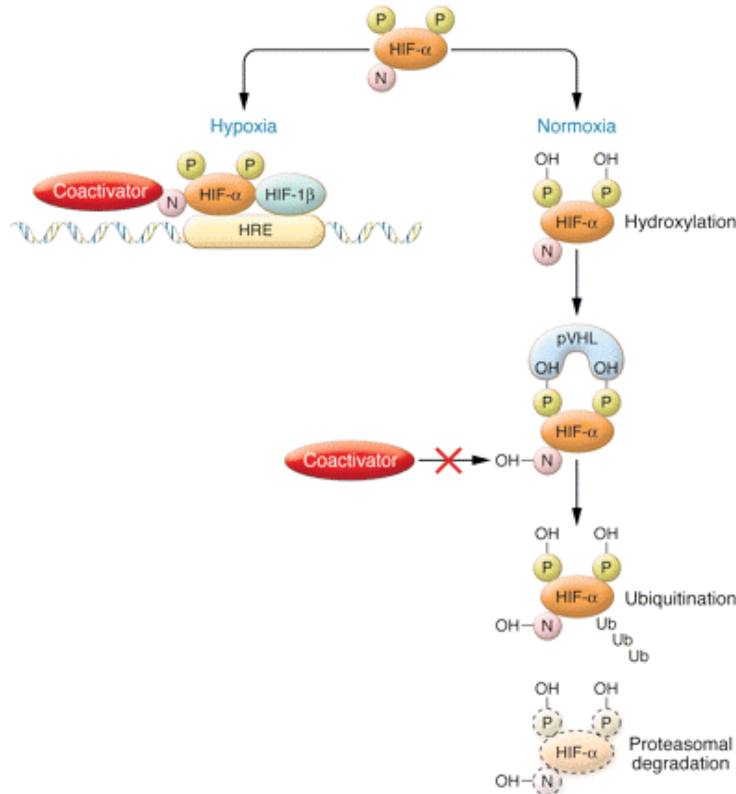


Figure 3. HIF activity under hypoxic and normoxic conditions.
In normoxia, hydroxylation at 2 proline residues promotes HIF-α association with pVHL and HIF-α destruction via the ubiquitin/proteasome pathway, while hydroxylation of an asparagine residue blocks association with coactivators.
In hypoxia, these processes are suppressed, allowing HIF-α subunits (both HIF-1α and HIF-2α) to escape proteolysis, dimerize with HIF-1β, recruit coactivators, and activate transcription via HREs. N, asparagine; P, proline; OH, hydroxyl group; Ub, ubiquitin. Figure by Ratcliffe[1]

Ang II and hormones induce HIF-1α through the production of reactive oxygen species (ROS)[57,58]. ROS can influence Fe2+ availability and thereby PHD and FIH activity[72].
Expression of HIF-1β is constitutive and not influenced by hypoxia; protein and mRNA levels are maintained at constant levels[73].
Also active mTOR (mammalian target of rapamycin) signaling has been shown to be necessary to stabilize HIF-1α under hypoxia (HIF-1α carries a potential phosphorylation site for mTOR)[74,75].

2.6. mTOR signaling – central regulator of cell growth and proliferation

Control of cell growth and proliferation in yeast, plants or humans and in any physiological context seems always to involve the same protein – the evolutionarily conserved serine/threonine kinase mammalian target of rapamycin (mTOR, also known as FRAP or RAFT) - and its signaling network (for reviews, see Harris and Lawrence, 2003[76] and Jacinto and Hall, 2003[77]). mTOR was discovered as the target for its specific inhibitor[78], rapamycin, an immnuesuppressant and antibiotic, which was isolated from the bacteria s*treptomyces hygroscopicus*, from a soil sample of Rapa Nui (Easter Island)[79].

Two mTOR complexes

RNAi-technology brought evidence that rapamycin does not affect all mTOR functions, suggesting mTOR may be present in different (iso)forms or complexes. Indeed, two TOR complexes, (m)TORC1 and (m)TORC2, have been identified in yeast and later in a variety of eukaryotes[80-82].

mTOR complex 1 (mTORC1) is rapidly and specifically inhibited by FKBP12-bound rapamycin. mTOR complex 2 (mTORC2) is not acutely rapamycin-sensitive. However, long-term treatment with rapamycin can avoid the formation of complex 2 in some cell types, e.g., in endothelial cells[83]. The newly synthesized mTOR-protein immediately forms a complex with FKBP12-rapamycin, which prevents association with rictor[83].

mTOR complex composition defines their function and identity. mTOR, Regulatory associated protein of mTOR (Raptor, 150 kDa) and PRAS40 are functional parts of TORC1. mLST8 is also present in mTORC1, but is probably not required for all of mTORC1's functions[80,84-87]. Formation of mTORC2 requires mTOR to assemble the rapamycin-insensitive companion of mTOR (Rictor) and mSIN1 (mammalian stress-activated protein kinase [SAPK]-interacting protein). In mTORC2, mLST8 is a functionally and structurally required component. The mTOR regulatory protein rictor (also known as mAVO3) is a large protein (200 kDa) and contains no obvious catalytic motifs, but repetitive domains, similar to mSIN1[80,84,88,89].

Upstream Regulators of the TOR Signaling Network

mTOR integrates various signals to regulate cell growth. Four major inputs have been implicated in TOR signaling: growth factors, nutrients, energy, and stress[90].

Growth Factors

The mTOR pathway responds to growth factors via the PI3K pathway (Figure 4). Binding of insulin or insulin-like growth factors (IGFs) to their receptors leads to recruitment and phosphorylation of the insulin receptor substrate (IRS), and subsequent recruitment of PI3K. PI3K bound to IRS converts phosphatidylinositol-4,5-phosphate (PIP2) in the cell membrane to phosphatidylinositol-3,4,5-phosphate (PIP3). PIP3 accumulation is antagonized by the lipid phosphatase PTEN. PIP3 co-recruits PDK1 and Akt to the membrane, resulting in the phosphorylation and activation of Akt by PDK1. mTOR is wired to the PI3K pathway through the tuberous sclerosis proteins TSC1 (hamartin) and TSC2 (tuberin). TSC1 and TSC2 act as a heterodimer that negatively regulates mTOR signaling. TSC2 is phosphorylated and functionally inactivated by Akt in response to insulin (reviewed in Manning, 2004[91]).

TSC1-TSC2 regulation of mTORC1

TSC2 acts as a GAP (GTPase-activating protein) for the small GTPase Rheb (reviewed in Li et al., 2004[93]). Rheb binds directly to the kinase domain in mTOR and activates mTOR in a GTP-dependent manner[94]. Long et al. suggest that GTP loading of Rheb, rather than mediating mTORC1 recruitment, enables Rheb to induce a conformational change in mTORC1 leading to mTORC1 activation and phosphorylation of downstream targets.

However, the significance of TSC2 phosphorylation by Akt may vary depending on physiological context. Recently, PRAS40 was identified as a raptor binding protein that potently inhibits mTORC1 kinase activity in vitro and mTORC1 signaling within cells. Insulin-stimulated phosphorylation of PRAS40 by Akt suppresses its mTORC1 inhibitory activity. It has been suggested that insulin activates mTORC1 through the coordinated regulation of rheb, an mTORC1 activator, and PRAS40, an mTORC1 inhibitor[87,92].

Nutrients

Nutrients, especially amino acids, regulate mTORC1 signaling. Amino acid starvation, in particular the absence of leucine, results in a rapid dephosphorylation of the mTORC1 effectors S6K1 and 4E-BP1, whereas readdition of amino acids restores S6K1 and 4E-BP1 phosphorylation in an mTORC1-dependent manner[95]. The mechanism(s) by which nutrient status is communicated to mTORC1 requires further study. Amino acids have been shown to activate mTORC1 via inhibition of TSC1-TSC2 or, alternatively, via

stimulation of Rheb. Gao et al. (2002)[96] have demonstrated that inactivation of TSC2 renders cells resistant to amino acid withdrawal, suggesting that the amino acids signal via TSC1-TSC2. Other studies have proposed a model in which amino acids signal to mTORC1 independently of TSC2. Amino acid withdrawal still downregulates mTORC1 signaling in TSC2-deficient cells[97].

Energy

Cell growth (the accumulation of cell mass) depends on a high rate of protein synthesis and consequently requires a high level of cellular energy. mTORC1 senses the energy status of a cell through AMP-activated protein kinase (AMPK). AMPK is activated in response to low cellular energy (high AMP/ATP ratio). Activated AMPK downregulates energetically demanding processes like protein synthesis and stimulates ATP-generating processes. The tumor suppressor LKB1 has been identified as an upstream kinase for AMPK, suggesting that LKB1 is linked to the TSC-mTORC1 signaling pathway[98,99]. Thus, upon energy deprivation LKB1 in conjunction with AMP activates AMPK, which in turn phosphorylates and activates TSC2, resulting in the inhibition of mTORC1.

Stress and Hypoxia

Cells respond to environmental stress, such as hypoxia, or low energy by downregulating energy-demanding processes and arresting growth. TOR has been demonstrated to play a role in the response to stress. Upon hypoxia, TOR signaling is inhibited and protein synthesis is thereby downregulated. Hypoxia is transduced to mTORC1 via the two homologous proteins REDD1 and REDD2. The expression of REDD is upregulated upon hypoxia by the transcription factor hypoxia-inducible factor 1 (HIF-1)[100].

However, stabilization of HIF-1α under hypoxia has been shown to depend on active mTOR-signaling suggesting that hypoxia activates mTOR signaling[74]. Furthermore, hypoxia has been shown to increase proliferation of lung adventitial fibroblasts, endothelial cells and of angiogenesis in vitro in a mTOR dependent way[10,101].

It is therefore unclear how hypoxia can inhibit mTOR signaling in some processes and activate it in others.

In summary, several upstream signaling cues, growth factors, energy, stress, and possibly amino acids converge on TSC1-TSC2 to regulate mTORC1 signaling. The

recent identification of mTORC2 raises the question of how mTORC2 is regulated. It has been shown that unlike mTORC1, mTORC2 does not function downstream of Rheb[102]. Therefore one might assume that TSC1/TSC2 does not regulate mTORC2, although this has not be firmly established and a role for TSC1/TSC2 in the regulation of mTORC2 cannot be completely excluded[103].

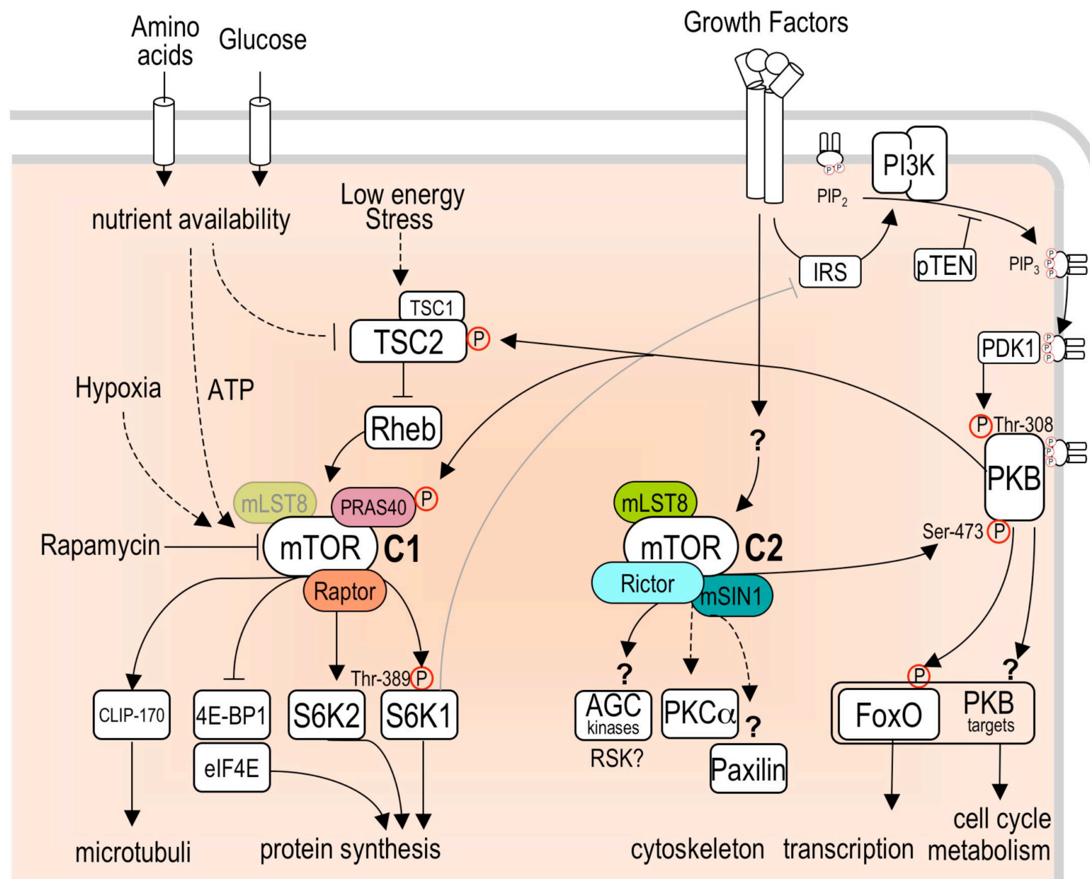


Figure 4. mTOR can be activated by growth factors, nutrients, energy and hypoxia. mTORC1 contains raptor, PRAS40 and mLST8. Downstream targets of mTORC1 are amongst others S6K and 4E-BP1. MTORC2 contains rictor, mSIN1 and mLST8. Downstream target of mTORC2 amongst others is PKB[104].

Downstream effectors and functions

mTORC1 is highly rapamycin-sensitive and is essential for regulating cell growth in response to both nutrients and growth factors. Signaling through mTORC1 promotes protein synthesis through the inactivation of the translation repressor 4E-BP1 and through the activation of S6 kinase (S6K1, phosphorylation at Thr389) (for review, see Hay and Sonenberg, 2004[95]). Raptor, a positive regulator of mTOR, appears to serve as

an adaptor protein that recruits mTOR substrates[105-108]. Raptor ko mice fail to increase inner cell mass and die on around day E7 of embryonal development[85].

mTORC2. Until recently, our knowledge of the functions of mTORC2 lagged behind that of mTORC1. Rictor knock out mice first develop normally, then exhibit growth arrest and die as embryos around E11. Rictor knock out embryos show multiple defects in fetal vascular system development. Rictor deficient MEFs have a reduced proliferation rate. Thus mTORC2 function may be generally associated with cell proliferation and cell viability[85,109].

Several studies have shown that mTORC2 can regulate the organization of the actin cytoskeleton, but the results are divergent. Rictor or mSIN1 knockdown in HeLa leads to an increase of actin fibers in the cytoplasm whereas rictor knockdown in NIH 3T3 decreases actin fibers in the cytoplasm[81,82,110]. In rictor and mLST8 knockout MEFs finally, actin distribution is not affected at all[85,109].

The demonstration that mTORC2 phosphorylates Akt/protein kinase B (PKB) on Ser473 in a growth factor-dependent manner suggests that this complex may have some vitally important functions[111,112]. siRNA mediated knock down of rictor strongly decreases Akt phosphorylation at Ser473 in adipocytes[111]. Akt Ser473 phosphorylation is also strongly reduced in rictor and mSIN1 knockout MEFs.

Akt, which is activated in a phosphatidylinositol 3-kinase (PI3K)-dependent manner, is a key intracellular mediator of diverse cellular processes, including metabolism, gene expression, cell migration, angiogenesis, proliferation, and cell survival[113,114]. Full activation of Akt requires phosphorylation at both Thr308 of the activation loop by phosphoinositide-dependent kinase 1 (PDK1) and Ser473 in the hydrophobic motif (HM) of the C-terminal tail by another kinase(s) tentatively named HM kinase or PDK2[115]. Since mTORC1 is a downstream target of Akt, the finding that mTORC2 has HM kinase activity suggests that the functional interactions between mTOR and the PI3K-Akt pathway are both more significant and complex than previously thought. Different degrees of Ser473 and Thr308 phosphorylation correlate with different degrees of enzyme activity and thereby enable a fine-tuned response. This could explain that only some downstream targets of Akt, such as FoxO but not GSK3 or TSC2 exhibit decreased phosphorylation after rictor silencing[85,88].

2.7. mTOR-related disease processes

Signaling through mTOR is essential for cell growth. It is not surprising that alteration of many upstream and downstream components of mTOR-signaling result in developmental diseases, tumor formation and cardiovascular diseases.

When tumors reach a critical volume and mass, nutrient and oxygen supply can no more be maintained by diffusion, and a vascular supply has to be generated by switching on the process of angiogenesis. Rapamycin has been shown earlier to inhibit angiogenesis and endothelial cell proliferation, whereas mTOR overexpression increased endothelial proliferation under hypoxia[10]. Clinical trials have demonstrated that mTOR inhibitors can reduce tumor vascularisation and inhibit growth of many different tumor types. Moreover, rapamycin is generally well-tolerated[117].

Unfortunately, some cell types, such as HeLa increase Akt-Ser473 phosphorylation (Akt activity) after rapamycin treatment[83]. This is due to the disruption of a feedback loop in which components downstream of mTORC1 and S6K1 (including IRS, see figure 4) block further activation of the PI3K-pathway[118,119]. In situations were Akt-Ser473 phosphorylation upregulates, rapamycin-treatment shoud be prevented or combined with other drugs, because administration of rapamycin in combination with other drugs (e.g. Akt-inhibitors, cis platin or VEGF blockers (Avastin)), leads to promising results in treating multiple types of human cancers[120].

HIF is a central regulator for angiogenesis and also depends on mTOR activity (see 2.2., 2.5.). Van Hippel-Lindau disease includes mutation of the VHL/E3 complex, thus preventing degradation of HIF-1α and leading to malignant tumor formation[121]. HIF-1 inhibitors like YC-1 or PX-478 could effectively stop tumor growth in von Hippel-Lindau syndrome patients and could potentially be interesting for a variety of angiogenesis-related disease[122].

Cardiovascular disease is a leading killer in the western world. Athereosclerosis (narrowing of arteries) is a multifactorial disease and can result from dyslipidemia, smoking or obesity. Arterial occlusions can be treated by stenting, i.e., opening and supporting narrowed vessels. Although stenting brings long-term benefits to a majority of patients, a substantial number of patients experience overgrowth of smooth muscle cells surrounding the stent, i.e., restenosis, similar to scar tissue. Inhibition of SMC and EC growth with mTOR inhibition by rapamycin-coated stents has been very effective in preventing restenosis in humans[123].

Left ventricular hypertrophy of the heart is one of the main risk factors for cardiac morbidity and mortality, and there is strong evidence that hyperactivation of the PI3K-mTOR pathway is one cause of cardiac hypertrophy. Recent studies have demonstrated that rapamycin may be a therapeutic agent for established cardiac hypertrophy[124].

3. Rationale & Aims

Hypoxia-induced endothelial proliferation

When oxygen levels are low for a longer period, erythropoiesis and angiogenesis are induced to increase tissue oxygenation[69]. In contrast to e.g. cardiomyocytes, specialized cells such as vascular endothelial cells (EC) and smooth muscle cells (SMC) are activated and increase proliferation and gene expression in response to hypoxia in order to form new vessels which supply oxygen to tissues[10]. EC proliferation in response to hypoxia was shown to be rapamycin-sensitive. Thus, we hypothesized that mammalian target of rapamycin (mTOR) is involved in the response to hypoxia in endothelial cells[10].

mTOR is central in regulating cell growth and proliferation and integrates signals from nutrients, growth factors, energy status and stress. Recent studies have identified two structurally distinct mTOR multi protein complexes (mTORC1 containing raptor and mTORC2 containing rictor) with individual downstream targets. Reports on the effect of hypoxia on mTOR are contradictive. On the one hand, hypoxia activates mTOR signalling to enhance angiogenesis[125]. On the other hand, hypoxia inhibits mTOR signalling to prevent protein synthesis[100]. Further, it is unclear which of the distinct mTOR complex-activities are affected by hypoxia

The major aim for this thesis was to elucidate the impact of hypoxia on mTOR signalling and its contribution to increased proliferation of endothelial cells.

Therefore we specifically assessed:

1. mTOR phosphorylation (at Ser 2448 and Ser2481) in dependence of oxygen concentration.
2. Time course of the levels of phosphorylation of mTOR and of mTORC1- and mTORC2 specific downstream targets, S6K and Akt, in dependence of the time of exposure to hypoxia.
3. Pathways involved in directing the hypoxia signal to mTOR.
4. The mTOR complex responsible for transducing hypoxic activation to increased EC proliferation.

Signaling in Hypoxia-Inducible Factor stabilization

Hypoxia inducible factors (HIFs) regulate the majority of hypoxia-induced genes. HIF-1α and HIF-2α are constantly degraded in normoxia. Degradation is prevented in hypoxia, thus the HIF-α's can form heterodimers with HIF-1β, translocate to the nucleus and induce transcription. Stabilization of HIF-1 in hypoxia was shown to be rapamycin

sensitive, and overexpression of mTOR enhanced HIF-1α-dependent gene transcription[74]. In the previous study we have shown that the inhibition of mTOR inhibits angiogenesis in vitro, and that mTORC1 and mTORC2 are required for hypoxia-mediated proliferation of endothelial cells. Further we have shown that mTOR-stabilized HIF-1α partially contributes to proliferation of mouse embryonic fibroblasts (MEFs) under hypoxia[11]. How mTORCs contribute to the stabilization of HIF-α's is unclear.

The major aim for this project was to assess the regulation and role of HIF-α in hypoxia-induced proliferation of aortic endothelial cells. Specifically we assessed whether:

1. HIF-α is stabilized in EC and whether this stabilization contributes to endothelial proliferation in response to hypoxia.
2. Signalling through mTORC1, mTORC2, MEK1/2, Jun kinase or p38 are necessary to stabilize HIF-1α and whether inhibition of these pathways affects proliferation of endothelial cells in response to hypoxia.

The Renin-Angiotensin Aldosteron System (RAAS) in Angiogenesis

Impaired angiogenesis can result in microvascular rarefaction that may be accompanied by arterial hypertension. The microvasculature supplies nutrients and oxygen to tissues, removes metabolites and carbon dioxide, and maintains an adequate hydrostatic pressure in tissue. Recent clinical studies with angiotensin-converting enzyme inhibitors (ACE inhibitors) and angiotensin II receptor 1 (ARBs) blockers demonstrate that long-term antihypertensive treatment increases capillary density in the skin of hypertensive patients[28]. The stimulatory effect on angiogenesis of these drugs can be mediated by activation of bradykinin pathways, resulting in the generation of vascular endothelial growth factor and nitric oxide.

The impact of antihypertensive drugs on microvessel structure were summarized and discussed in form of a review entitled *Effects of anti-hypertensive drugs on vessel rarefaction*[2], included in this thesis. We discuss the theories behind the mechanisms of primary or secondary microvascular rarefaction in hypertensive patients. Further, we discuss the potential of different antihypertensive drugs (diuretics, α- and β-adrenergic receptor blockers, ARBs, ACE inhibitors) to induce or block angiogenesis, with a main focus on ARBs and ACE inhibitors, also with respect to tumor angiogenesis.

The Role of Angiotensin II in Angiogenesis in vitro of the heart

The vasoactive peptide Angiotensin II (Ang II) is a key regulator of blood pressure. Two major subtypes of Ang II receptors are expressed in the myocardium: Ang II type 1 (AT_1) and Ang II type 2 (AT_2) receptors. Most of the effects of Ang II on the cardiovascular system, for example, vasoconstriction, are attributed to AT_1. AT_1 is ubiquitously expressed, whereas the AT_2 receptor is highly expressed early in development and at lower levels in the adult[39,40]. Interestingly, the AT_2 receptor is upregulated in response to ischemia and inflammation suggesting a potential role in myocardial angiogenesis[126,127]. Previous studies have shown that the AT_2 receptor may interact with the bradykinin receptor, the B2 kinin receptor (BK2), during signalling[128].

In this in vitro study, using an angiogenesis assay from left ventricular myocardium of AT_1 and AT_2 –knock out and wild type mice, we aimed at investigating the mechanism underlying the angiogenic effects of angiotensin II. Specifically we aimed at:

1. Studying the angiogenic potential of Ang II.
2. Investigating expression of AT_1- and AT_2 receptors.
3. Studying the angiogenic role of AT_1-, AT_2- and bradykinin receptors
4. Assessing whether nitric oxide is a downstream effectors of Ang II-induced angiogenesis.

4. Results

4.1. Hypoxia-Induced endothelial proliferation requires both mTORC1 and mTORC2

Weimin Li,* Marco Petrimpol,* Klaus D. Molle, Michael N. Hall, Edouard J. Battegay, Rok Humar; (Circ Res. 2007;100:79-87.); *both authors contributed equally to this work.

4.2. Role of mTORC1 and mTORC2 in hypoxia-induced HIF-1α stabilization and endothelial proliferation

Marco Petrimpol, Weimin Li, Lourdes Sanchez de Miguel, Edouard J. Battegay, Rok Humar; in preparation.

4.3. Effects of anti-hypertensive drugs on vessel rarefaction

Edouard J Battegay, Lourdes Sanchez de Miguel, Marco Petrimpol, Rok Humar; (Curr Opin Pharmacol. 2007;7:151-7)

4.4. Angiotensin II induces angiogenesis in the hypoxic adult mouse heart in vitro through an AT2-B2 receptor pathway

Veronica C. Munk, Lourdes Sanchez de Miguel, Marco Petrimpol, Nicole Butz, Andrea Banfi, Urs Eriksson, Lutz Hein, Rok Humar, Edouard J. Battegay; (Hypertension. 2007;49;1178-1185).

Hypoxia-Induced Endothelial Proliferation Requires Both mTORC1 and mTORC2

Weimin Li,* Marco Petrimpol,*
Klaus D. Molle, Michael N. Hall, Edouard J. Battegay, Rok Humar;
(Circ Res. 2007;100:79-87.); *Both authors contributed equally to this work.

Abstract

A central regulator of cell growth that has been implicated in responses to stress such as hypoxia is mTOR (mammalian Target Of Rapamycin). We have shown previously that mTOR is required for angiogenesis in vitro and endothelial cell proliferation in response to hypoxia. Here we have investigated mTOR-associated signaling components under hypoxia and their effects on cell proliferation in rat aortic endothelial cells (RAECs). Hypoxia (1% O2) rapidly (30 minutes) and in a concentration-dependent manner promoted rapamycin-sensitive and sustained phosphorylation of mTOR-Ser2448 followed by nuclear translocation in RAECs. Similarly, hypoxia induced phosphorylation of the mTORC2 substrate Akt-Ser473 (3 to 6 hours at 1% O2) and a brief phosphorylation peak of the mTORC1 substrate S6 kinase–Thr389 (10 to 60 minutes). Phosphorylation of Akt was inhibited by mTOR knockdown and partially with rapamycin. mTOR knockdown, rapamycin, or Akt inhibition specifically and significantly inhibited proliferation of serum-starved RAECs under hypoxia (P0.05; n4). Similarly, hypoxia induced Akt-dependent and rapamycin-sensitive proliferation in mouse embryonic fibroblasts. This response was partially blunted by hypoxia-inducible factor-1 knockdown and not affected by TSC2 knockout. Finally, mTORC2 inhibition by rictor silencing, especially (P0.001; n7), and mTORC1 inhibition by raptor silencing, partially (P0.05; n7), inhibited hypoxia-induced RAEC proliferation. Thus, mTOR mediates an early response to hypoxia via mTORC1 followed by mTORC2, promoting endothelial proliferation mainly via Akt signaling. mTORC1 and especially mTORC2 might therefore play important roles in diseases associated with hypoxia and altered angiogenesis.

Introduction

Hypoxia is associated with angina pectoris, myocardial infarction, heart failure, and peripheral artery disease. Hypoxia and tissue ischemia are caused by either arterial obstruction or functional and anatomical capillary rarefaction resulting from hypertension[1]. Hypoxia occurs during rapid tissue growth, in organ and in tumor development, and during chronic inflammation or exposure to high altitude[1]. Diminished oxygen concentration induces programmed responses, such as endothelial proliferation[2,3] and angiogenesis, that ultimately relieve tissue hypoxia and contribute to wound healing[4].

We have reported that hypoxia requires mTOR (mammalian Target Of Rapamycin) to induce angiogenesis and cell proliferation of the vascular wall in response to hypoxia[5].

The mTOR pathway is a key regulator of cell growth and proliferation, and increasing evidence suggests that its dysregulation is associated with human diseases, including cancer, diabetes, and cardiovascular disease[6]. The mTOR pathway integrates signals from nutrients, energy status, and growth factors to regulate many processes, including autophagy, ribosome biogenesis, and metabolism[6]. Recent work identified 2 structurally and functionally distinct mTOR-containing multiprotein complexes[7,8]. The first complex, mTORC1, harbors raptor, is highly rapamycin sensitive[9–13], and specifically activates protein synthesis via S6 kinase (S6K). The second complex, mTORC2[13–15], is associated with rictor and phosphorylates Akt on Ser473[16,17]. mTORC2 phosphorylates and activates Akt/protein kinase B, which promotes signaling pathways that ensure cell survival and induce cell proliferation[18].

Reports on the effects of hypoxia on mTOR are contradicting. On the one hand, hypoxia activates mTOR signaling to enhance angiogenesis[19], cellular proliferation of lung adventitial fibroblasts[20] and aortic wall cells[5], or protein levels and activity of hypoxia-inducible factor (HIF)-1α, a major transcription factor for hypoxia-inducible genes[21]. On the other hand, hypoxia has also been reported to inhibit mTOR signaling in mouse embryonic fibroblasts (MEFs), a process that dephosphorylates S6K1 and downregulates protein synthesis[22–24]. It is unclear how hypoxia can elicit both activation and inhibition of mTOR signaling and how these signals contribute to increased proliferation; Furthermore, it is currently not known whether hypoxia affects mTORC2 and mTOR-dependent Akt phosphorylation. This study further assesses the effects of hypoxia on mTOR signaling in endothelial cells[5]. Here we examine activities of mTOR under hypoxia in detail and translation of this signal into endothelial cell proliferation.

Results and Figures

Hypoxia Rapidly and Concentration-Dependently Promotes Phosphorylation of mTOR-Ser2448 and mTOR Nuclear Translocation

To investigate direct effects of hypoxia on mTOR activity, we performed time-course experiments in serum-deprived cultured aortic endothelial cells, in the absence of growth factors. We determined phosphorylation of mTOR at Ser2448 and Ser2481 in the presence and absence of rapamycin. As shown in Figure 1A, phosphorylation of Ser2448 rapidly increased after exposure to hypoxia (1% O2), peaked after approximately 3 hours of hypoxia, and remained at high levels during the period investigated (24 hours). The effect of hypoxia on Ser2448 phosphorylation was reduced

by rapamycin (Figure 1A). In contrast, phosphorylation of mTOR Ser2481 increased only slightly during hypoxic exposure and declined toward 24 hours of incubation. mTOR protein levels were not affected by hypoxia (Figure 1A).

In mammalian organs, O2 concentration ranges from 14% to 0.5%, with 14% O2 in arterial blood and 10% in the myocardium. During mild hypoxia, myocardial O2 drops to 1% to 3% or lower.28 To account for varying oxygen concentrations in the body, we investigated the effect of different oxygen saturations on mTOR Ser2448 phosphorylation. Quiescent RAECs were separately incubated under decreasing oxygen saturations (20%, 11%, 6%, 3%, and 1% O2) for 12 hours. At normoxia (21% O2), faint phosphorylation of mTOR Ser2448 was detected, which increased when O2 concentration was lowered to 11% to 6% and augmented further with a maximum at 1% to 3% O2 (Figure 1B). mTOR protein as well as -actin protein levels were not affected by oxygen saturation. HIF-1α protein levels were used as a positive control for hypoxia and increased linearly, peaking at 1% to 3% of O2 saturation (Figure 1B). Thus, mTOR phosphorylation on Ser2448 is modulated in the pathophysiological O2 concentration range.

An additional regulatory mechanism of mTOR signaling may occur via cytoplasmic/nuclear shuttling[29]. We examined whether severe hypoxia (1% O2) influences cellular localization of mTOR and mTOR–P-Ser2448. Under all tested conditions, mTOR was localized predominantly in the cytosol, as shown by immunostaining in Figure 1C. However, after quiescent RAECs were cultured in hypoxia for 6 hours, mTOR protein also appeared in the nucleus and rapamycin treatment inhibited nuclear localization (Figure 1C). Interestingly, mTOR–P-Ser2448 was only detected in distinct nuclear structures after 6 hours of exposure to hypoxia. Phosphorylation of mTOR Ser2448 was not detected under normoxia and only a very faint signal was detected under conditions of hypoxia with rapamycin treatment (Figure 1C). Similar results were obtained when assessing protein levels of mTOR and mTOR–P-Ser2448 by Western blotting. Serumdeprived RAECs were exposed to hypoxia (1% O2) for different periods of time (2 to 24 hours). At normoxia (time point, 0) basal levels of HIF-1α were detected in nuclear extracts, whereas the levels of mTOR and mTOR–P-Ser2448 were nearly undetectable (Figure 1D). mTOR and mTOR–P- Ser2448 protein levels appeared in the endothelial nuclear fraction after 2 hours of incubation under 1% O2, increased slightly with time and were maximal after 24 hours of incubation under 1% O2 (Figure 1D). Thus, hypoxia rapidly and dose-dependently promotes phosphorylation

of mTOR- Ser2448 in a rapamycin-sensitive way and causes nuclear translocation of phosphorylated mTOR.

Hypoxia Induces Rapid, but Short-Term, mTOR-Dependent Phosphorylation of S6K1-Thr389 and Sustained Phosphorylation of Akt-Ser473

mTOR is present in 2 complexes, mTORC1 and mTORC2. mTORC1 activity can be measured by analyzing the phosphorylation of the direct downstream target S6K1 on Thr389 or phosphorylation of ribosomal subunit S6[30,31]. mTORC2 phosphorylates Akt on the primary phosphorylation site Ser473[16,17]. We therefore performed time-course experiments, in which quiescent RAECs were exposed to hypoxia (1% O2) for short (10 minutes) to long (24 hours) term, and we analyzed phosphorylation of S6K1-Thr389 and Akt- Ser374. As shown representatively in Figure 2A (first 3 panels), and as averaged densitometric quantification of cumulative experiments in Figure 2B (top graph), S6K1 was highly phosphorylated at Thr389 between 10 minutes and 1 hour of hypoxic exposure but dropped to undetectable levels after more than 3 hours of culture under hypoxia. This phosphorylation step is highly rapamycin sensitive. Akt phosphorylation at Ser473 slightly increased after 10 minutes but reached maximal levels after 3 hours of hypoxic exposure before staying at a steady level for up to 24 hours. Total Akt levels remained unchanged under hypoxia (Figure 2A, middle 4 panels). Phosphorylation of Akt at Ser473 peaked after 3 hours of exposure to hypoxia as shown in Figure 2B (lower graph), representing the ratio of Akt–P-Ser473 to total Akt. Akt phosphorylation was partially inhibited by rapamycin, however, the effect of rapamycin increased with longer incubation (averaged densitometric quantification of cumulative experiments in Figure 2B, bottom graph).

Akt phosphorylates Ser21 in Glycogen synthase kinase-3 (GSK3) α and Ser9 in GSK3β and thereby inactivates GSK3 function[32,33]. Furthermore Akt and GSK3 are implicated in the regulation of cell cycle regulators Cyclin D1 and p21.[33] Similar to Akt phosphorylation, GSK3β was phosphorylated after 60 minutes of exposure to hypoxia as shown by Western blots of nuclear extracts in Figure 2A. Cyclin D1 protein gradually accumulated after 30 minutes of hypoxia in the nuclear fraction, whereas cell cycle inhibitor p21 protein levels decrease and totally disappear after 24 hours of RAEC cultivation under hypoxia (Figure 2B).

To further examine the role of mTOR on Akt–P-Ser473 under hypoxia, mTOR protein expression was silenced by mTOR-specific siRNAs that were nucleofected into RAECs

before starvation and exposure to hypoxia. Quiescent RAECs were then again exposed to hypoxia (1% O2) for short (10 minutes) to long (24 hours) term and Akt-Ser374 phosphorylation was analyzed by Western blotting. mTOR silencing, as shown by the Western blots in Figure 2C and averaged densitometric quantification of cumulative data in Figure 2D, effectively blunted hypoxia-induced Akt-Ser374 phosphorylation.

Thus, hypoxia induces sustained phosphorylation of Akt-Ser473, peaking at approximately 3 to 6 hours of hypoxia, that is blunted by rapamycin or mTOR silencing. On the other hand, rapamycin-sensitive S6K1 phosphorylation on Thr389 peaks between 10 minutes to 1 hour of exposure to hypoxia, but quickly drops to undetectable levels with further culture under hypoxia.

Hypoxia-Enhanced Endothelial Proliferation Is mTORC1 and mTORC2 Dependent

We compared our previous findings[5] in rat aortic angiogenesis with an angiogenesis assay of endothelial spheroids and endothelial proliferation assays using RAECs at severe hypoxia (1% O2). Endothelial sprout formation under 1% Owas more than twice as high when compared with the response under 21% O2 (Figure 3A). Rapamycin selectively inhibited additional sprout formation observed under 1% O2 at a low concentration (2 nmol/L) (Figure 3A). We have shown previously that hypoxia-enhanced angiogenesis in vitro is mainly attributable to enhanced proliferation[5]. A similar response was observed for RAEC proliferation under 1% O2. Hypoxia alone increased RAEC proliferation when compared with diluent normoxic control to approximately 1.5-fold (Figure 3B). Low concentrations of rapamycin (2 nmol/L) inhibited proliferationspecifically under hypoxia (Figure 3B, top graph). Akt inhibition by Akt IV inhibitor lowered overall proliferation at higher concentrations (Figure 3B, bottom graph). Akt inhibitor was used within concentrations at which cytotoxicity was absent, as shown by cytotoxicity test performed in RAECs (see the Figure in the online data supplement, available at http://circres.ahajournals.org).

To further assess the role of mTOR in transducing hypoxia into endothelial proliferation, we analyzed endothelial (RAEC) proliferation and mTOR-associated signaling after mTOR silencing. mTOR protein was consistently knocked down or reduced (up to 95%) by mTOR siRNA, whereas control siRNA had no effect on mTOR protein, as shown by Western blotting (Figure 2C). After silencing, quiescent endothelial cells were cultured for 30 hours under 1% O2 and 21% O2 and proliferation was measured. mTOR silencing significantly (P0.05, n4) decreased the proliferation response to hypoxia compared to

transfection with control siRNA, whereas mTOR silencing had no significant (P0.05, n4) effects on proliferation under normoxia when compared with proliferation in endothelial cells that were transfected with control siRNA (Figure 3C).

To assess whether a specific mTOR complex is responsible for transducing hypoxia into endothelial proliferation, we knocked down raptor, specific for mTORC1, or rictor, specific for mTORC2[14], by nucleofection of RAECs with vectors containing specific shRNAs. As shown in the top part of Figure 3D, shRNA silencing effectively inhibited expression of raptor or rictor proteins as compared with negative control transfection.

Rictor but not raptor silencing also clearly decreased phosphorylation of mTORC2 downstream substrate Akt-Ser473 after 6 hours of incubation under hypoxia (Figure 3D). At these time points, S6K1 phosphorylation at Thr389 is repressed (Figure 3D). Importantly, inhibition of mTORC2 by rictor silencing effectively blunted hypoxia-induced endothelial proliferation, with no effect on proliferation under normoxia (P0.001, n5). Also raptor silencing decreased hypoxia-induced proliferation significantly (P0.05, n3), however, not to the extent of rictor silencing. Thus, both mTORC1 and especially mTORC2 silencing significantly reduce hypoxia-induced endothelial proliferation.

Tsc2 Knockout Does Not Blunt Hypoxia-Induced Proliferation in MEFs

To extend the validity of our data to other cell types that are commonly used in molecular biology research, we have assessed MEF cells for their proliferative response under hypoxia. The broad availability of transgene MEFs allows for rapid and easy determination of the role of a specific gene.

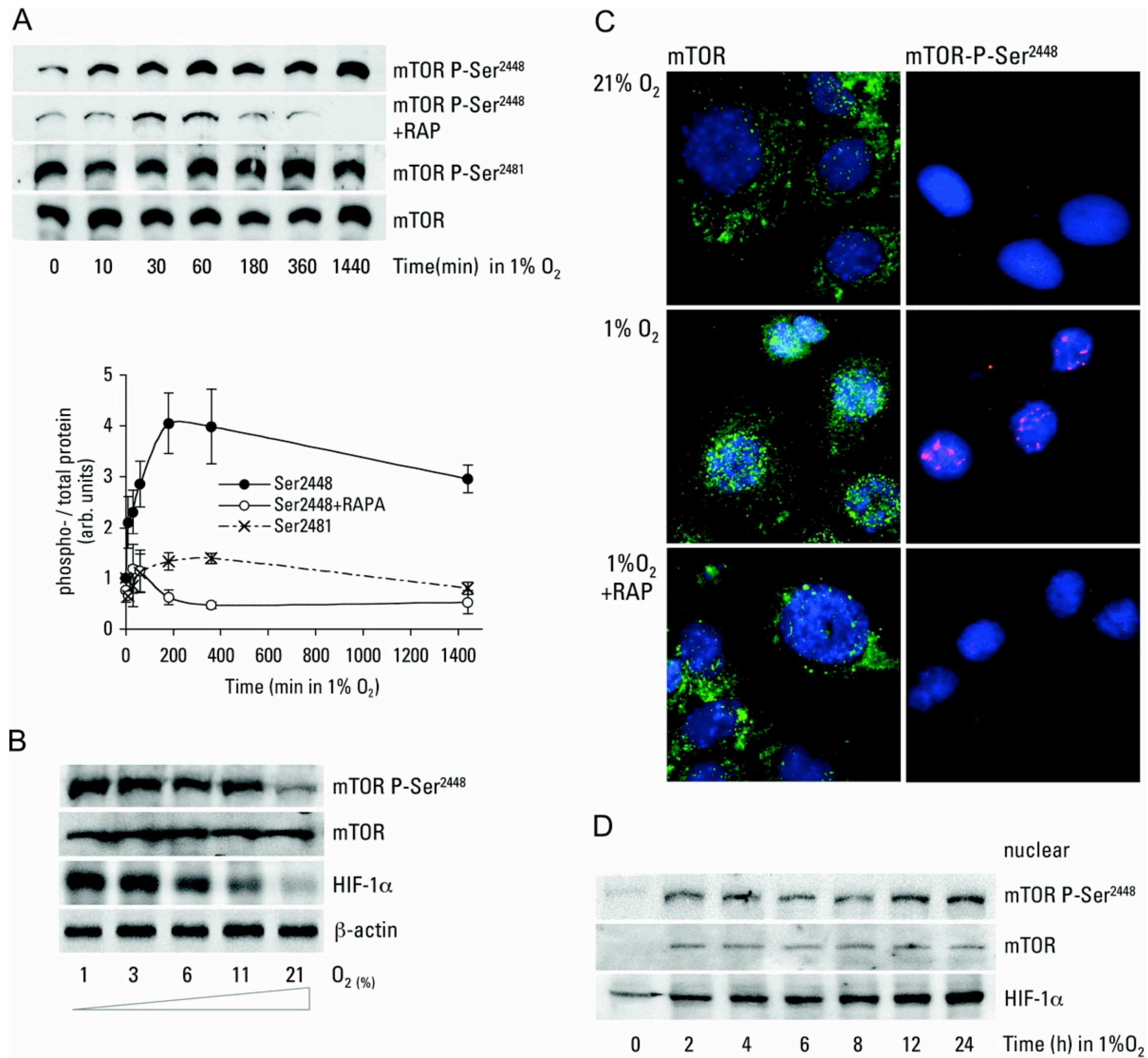
Tsc2 has been implicated to regulate proliferation under hypoxia in MEFs[24]. Tsc1 and Tsc2 proteins form a physical and functional complex in vivo and inhibit mTOR. Tsc2 is inactivated by Akt-dependent phosphorylation or nutrient availability, which destabilizes Tsc2 and disrupts its interaction with Tsc1[34,35]. We therefore investigated whether a disrupted Tsc1/ Tsc2 complex in Tsc2-defective MEFs affects hypoxia-induced cell proliferation when compared with wild-type MEFs or Tsc2-mutated MEFs with a reintroduced Tsc2 wild-type gene. As shown in Figure 4A, proliferation was clearly increased in TSC2/ MEFs (top), both under normoxia and hypoxia. Hypoxia-induced proliferation was decreased by rapamycin and the Akt inhibitor, as demonstrated for endothelial cells (Figure 4A, top). Importantly, proliferation in TSC2-defective MEFs was enhanced under hypoxia to a ratio comparable to intact MEFs. No significant decrease (P0.05, n3) was observed when comparing the ratio of proliferation indices under

hypoxia to that under normoxia (Figure 4A, bottom graphs). These results suggest that loss of Tsc2 generally increases proliferation but does not regulate the specific activating effects of hypoxia on mTOR-mediated proliferation in MEFs.

mTOR Induces HIF-1α–Dependent and –Independent Ways to Promote MEF Proliferation Under Hypoxia

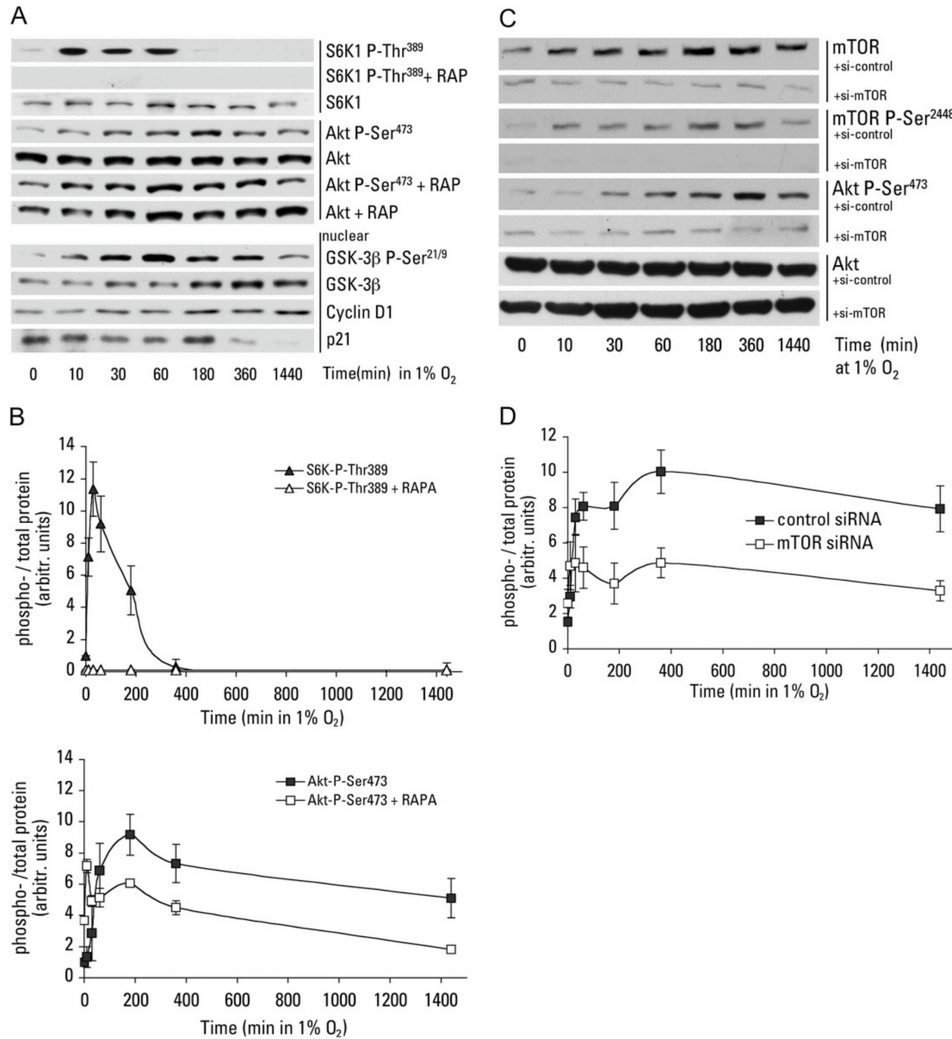
mTOR was shown to contribute to the stabilization of HIF-1α protein in cells exposed to hypoxia and is thus a positive regulator of HIF-1– dependent gene transcription[21,36,37]. We therefore asked whether HIF-1α, downstream of mTOR, is pivotal for increased cell proliferation under hypoxia. For this purpose, we measured proliferation of wild-type MEFs and those lacking the HIF-1α gene. As shown in Figure 4B, hypoxia-induced proliferation was only partially, though significantly (P0.05, n5), inhibited in HIF-1α$^{-/-}$ cells (open squares) as compared with wild-type cells under hypoxia (open circles). Both wild-type (filled circles) and HIF-1α$^{-/-}$ (filled squares) cells did not increase proliferation under normoxia. Increased proliferation under hypoxia was rapamycin sensitive both in HIF-1α$^{-/-}$ cells and wild-type MEFs. To further assess whether mTOR requires HIF-1α to induce proliferation under hypoxia, we overexpressed mTOR in wild-type and HIF-1α$^{-/-}$ cells (Figure 4C, top) and measured proliferation (Figure 4C, bottom left). mTOR overexpression increased overall proliferation in all conditions to approximately the same levels when compared with corresponding mock-transfected cells; the ratios (Figure 4C, bottom right) between proliferation under hypoxia and under normoxia were the same in wild-type and in HIF-1α knockout MEFs. These results suggest that HIF-1α is a partial but not crucial effector of mTOR-dependent, hypoxia-induced proliferation in MEFs.

Figure 1



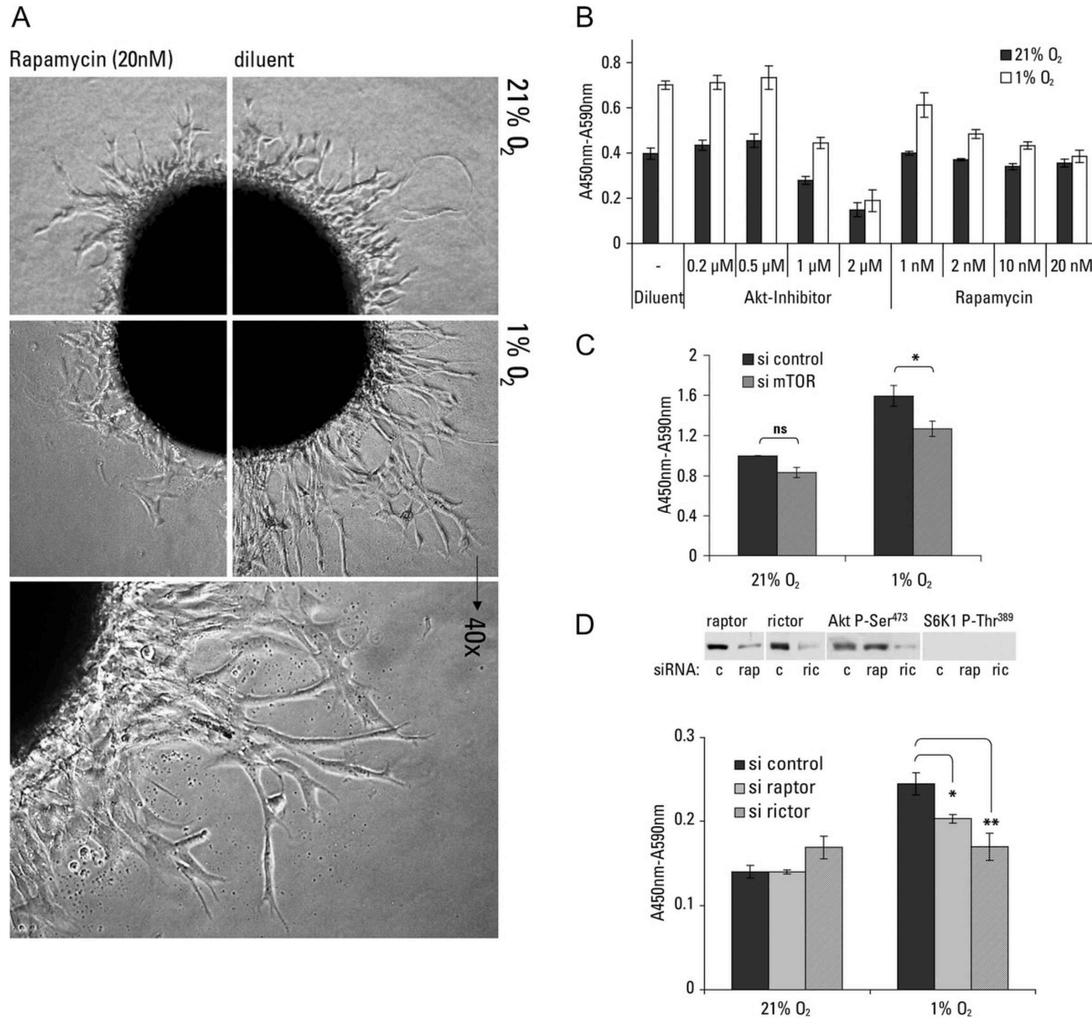
Hypoxia (1% O2) mediates rapid, dose-dependent, sustained phosphorylation of mTOR Ser 2448 and translocation to the nucleus in RAECs. A, Western blot (top) showing total cell lysates of serum-deprived RAECs exposed to increasing duration of hypoxia (10 minutes to 24 hours) and probed for phosphorylated Ser2448, phosphorylated Ser2481, and total mTOR in the presence or absence of rapamycin (20 nmol/L). Averaged densitometric quantification (bottom) shows rapid, sustained, and rapamycin-sensitive phosphorylation on Ser2448 relative to total mTOR protein that was statistically significant after 30 minutes of hypoxia ($P<0.001$, n=4). Data are given as mean±SEM. B, Quiescent primary RAECs were cultured for 12 hours under normoxia (21% O2) and decreasing oxygen saturations (11%, 6%, 3%, 1% O2). Total cell lysates were subjected to SDS-PAGE and protein levels of mTOR, mTOR Ser2448, HIF-1α hypoxia (B), and hypoxia together with 20 nmol/L rapamycin (C). Cells were fixed and immunostained with anti-mTOR (fluorescein iso-thiocyanate; green stain) and anti-mTOR phospho-Ser2448 antibodies (Cy3, red stain) and nuclear compartment (DAPI, blue stain). Rapamycin was included during serum deprivation (24 hours). D, Western blot showing nuclear cell lysates of serum-deprived RAECs exposed to increasing periods of hypoxia (2 to 24 hours) and probed for phosphorylated Ser2448, HIF-1α, and total mTOR.

Figure 2



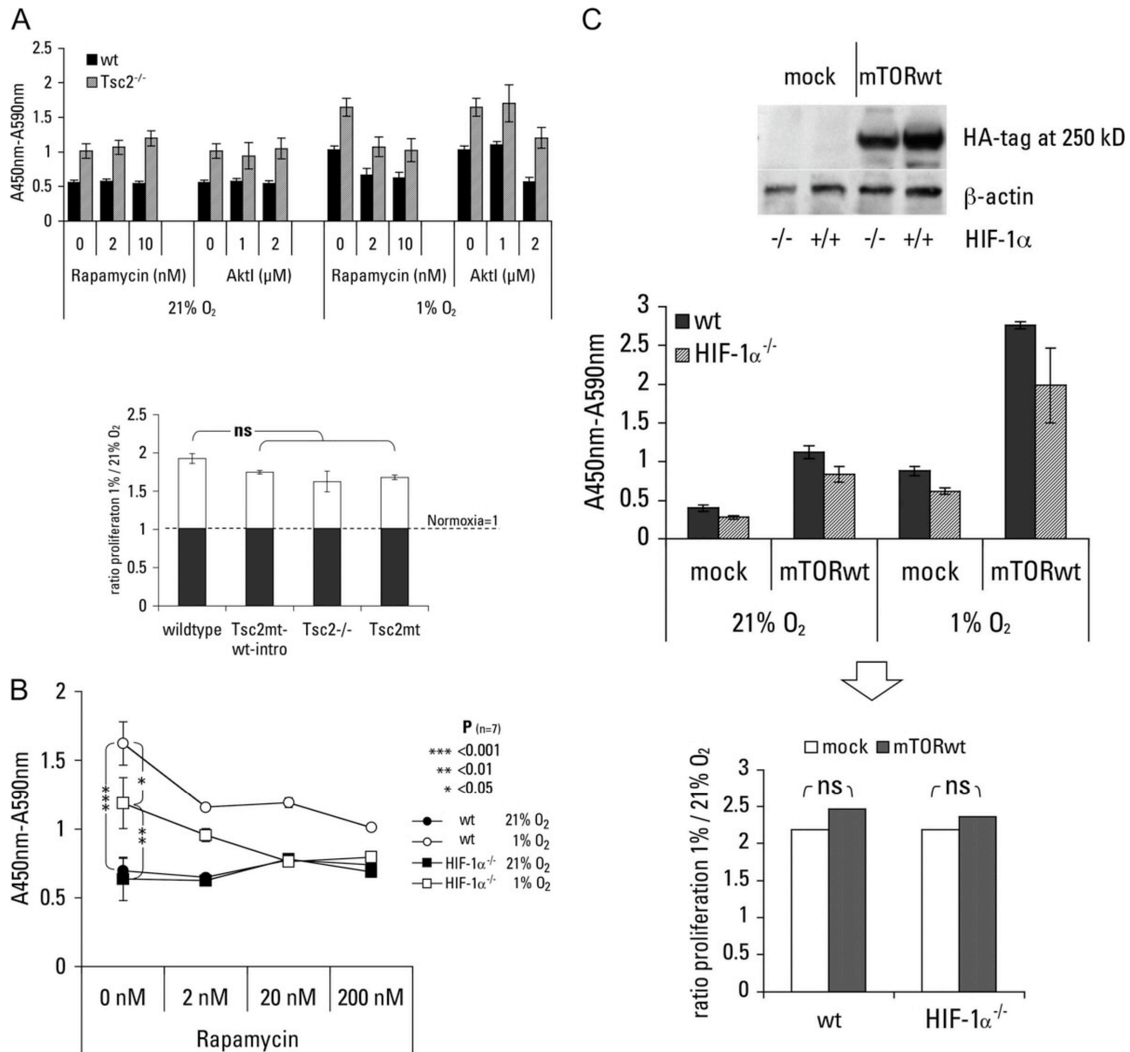
Hypoxia increases transient or prolonged activity of distinct mTOR downstream targets. A, Western blots showing total cell lysates of serum-deprived RAECs exposed to increasing duration of hypoxia (10 minutes to 24 hours) and probed for (numbering from top to bottom) (1) phosphorylated S6K-Thr389, (2) phosphorylated S6K-Thr389 in the presence of 20 nmol/L rapamycin, (3) total S6K, (4) phosphorylated Akt-Ser473, (5) total Akt, (6) phosphorylated Akt-Ser473, and (7) total Akt in the presence of 20 nmol/L rapamycin. Nuclear extracts probed for (8) phosphorylated GSK3-Ser21/9 and (9) total GSK3, (10) cyclin D1, and (11) p21. B, Top graph represents ratios of S6K–P-Thr389 to total S6K protein levels with and without rapamycin. Significant phosphorylation on S6K-Thr389 occurred within time points 10 to 180 minutes of hypoxia ($P<0.05$, n=3). Bottom graph represents ratios of Akt–P-Ser473 to total Akt protein levels with and without rapamycin as calculated from compiled densitometric quantification. Akt-Ser473 phosphorylation was significant after 30 minutes of hypoxia ($P<0.01$, n=3). Rapamycin treatment resulted in significant reduction of phosphorylation after 180 minutes of hypoxia ($P<0.05$, n=3). Data are given as mean±SEM. C, Western blots showing total cell lysates of serum-deprived RAECs transfected with negative control siRNA (+si-control) or siRNA directed against mTOR (+si-mTOR) exposed to increasing duration of hypoxia (10 minutes to 24 hours) and probed for (numbering from top to bottom) (1) total mTOR, (2) phosphorylated mTOR-Ser2448, (3) total Akt, and (4) phosphorylated Akt-Ser473. D, Graphs represent ratios of Akt–P-Ser473 to total Akt protein levels as calculated from compiled densitometric quantification. Phosphorylation on Akt Ser473 was significant after 30 minutes of hypoxia ($P<0.001$, n=4). mTOR silencing significantly inhibited Akt–P-Ser473 after 30 minutes of hypoxia ($P<0.05$, n=4). Data are given as mean±SEM.

Figure 3



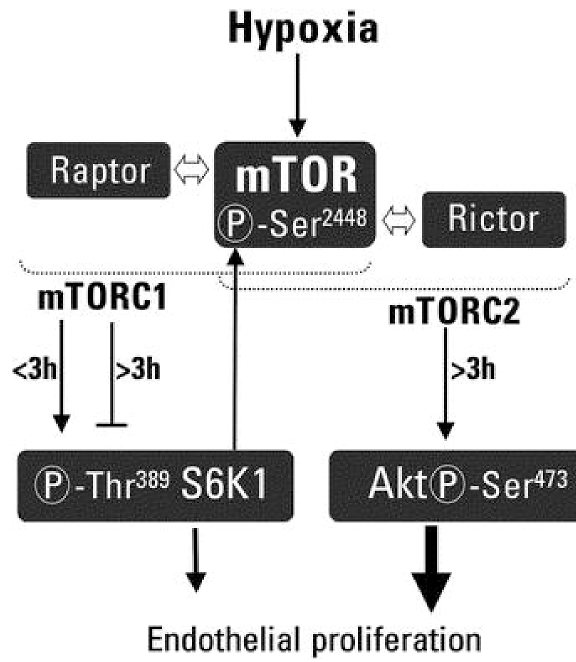
mTOR and downstream targets are required for hypoxia-induced endothelial cell proliferation. A, Typical micrographs (×10) of rat aortic endothelial spheroids (2000 cells) embedded in a fibrin gel after a 24-hour incubation under normoxia (21% O2) and hypoxia (1% O2), normoxia (21% O2) with 20 nmol/L rapamycin, and hypoxia (1% O2) with 20 nmol/L rapamycin. An additional panel shows a ×40 magnification of endothelial sprouts emerging into the fibrin gel under hypoxia. B, Cell numbers of serum-deprived (for 30 hours) RAECs were determined after 24-hour culturing under normoxia (21% O2) or hypoxia (1% O2) with inclusion of the indicated concentrations of Akt IV Inhibitor and rapamycin. Y-axis represents the mean of cell number, compiled from 3 experiments with octuplicate samples. Data are given as mean±SD (n=3). C, RAECs were nucleofected with control siRNA (si control) (filled columns) and siRNA directed against rat mTOR (si mTOR) (hatched columns). Quiescent cells were exposed to hypoxia for 30 hours, and proliferation was measured. Significance was calculated by repeated ANOVA followed by pairwise comparison with the Bonferroni post test (ns indicates not significant, P>0.05; *P<0.05; n=4). D, RAECs were nucleofected with control siRNA (c and black columns) and vectors containing shRNA directed against raptor (rap) (lightly shaded columns) or rictor (ric) (hatched columns). Western blots (top display) show efficiency of silencing and effects on Akt-Ser473 and S6K-Thr389 phosphorylation after 6-hour incubation under hypoxia. Quiescent cells were exposed to hypoxia for 30 hours, and proliferation was measured (bottom display). Significance was calculated by repeated ANOVA followed by pairwise comparison with the Bonferroni post test (*P<0.05, **P<0.001; n=5).

Figure 4



Hypoxia-induced proliferation in MEFs via mTOR does not depend on TSC2 and only partially on HIF-1α. A, Cell numbers of serum-starved TSC2 knockout (TSC2-/-) and wild-type (top) MEFs were determined after 30 hours of incubation under normoxia and hypoxia. Rapamycin or Akt inhibitor was included 30 minutes before incubation under hypoxia (top graphs). Bottom graph shows proliferation of wild-type, TSC2 mutated MEFs with a reintroduced TSC2 wild-type gene (TSC2mt-wt-intro), and TSC2-mutated (TSC2mt) MEFs as ratio of proliferation under hypoxia divided by proliferation under normoxia (ns indicates not significant, $P>0.05$; n=3). B, Cell numbers of serum-starved HIF-1α knockout (squares) and wild-type (circles) MEFs were determined after 30 hours of incubation under normoxia (filled symbols) and hypoxia (open symbols). Rapamycin (2 to 200 nmol/L) was included 30 minutes before incubation under hypoxia. Significance was calculated by 1-way ANOVA, followed by multiple comparison with the Bonferroni post test (***$P<0.001$, **$P<0.01$, *$P<0.01$; n=7). C, HIF-1α knockout and wild-type MEFs were transfected with mock and HA-tagged wild-type mTOR and expression verified by Western blot (top). Cell numbers of serum-starved, mock-transfected (filled columns) and mTOR-transfected (open columns) MEFs were determined after 30 hours of incubation under normoxia and hypoxia. The ratios between proliferation under normoxia and hypoxia were compared, and no statistical difference among all groups was observed as calculated by 1-way ANOVA followed by multiple comparison with the Bonferroni post test (ns indicates not significant, $P>0.05$; n=3).

Figure 5



Scheme representing summary of most important results.

Discussion

In this report, we have investigated the mechanisms responsible for hypoxia-induced proliferation of serum- and growth factor– deprived endothelial cells and found that mTORC1 and mTORC2, i.e., the large multidomain kinase mTOR and its regulator-associated proteins raptor and rictor, mediate an early response to hypoxia promoting endothelial proliferation via Akt signaling. Our results also clarify the apparent contradiction in the mTOR field, arising from earlier observations that hypoxia activates mTOR signaling[38], resulting in angiogenesis[19], proliferation[5,20], and HIF-1α stabilization[21] and at the same time appears to inhibit mTOR signaling, as seen by deactivation of mTORC1 substrate S6K1, 4E binding protein 1, and protein synthesis[22–24]. Our data suggest that both mTORC1 and mTORC2 participate in the response to hypoxia in a cooperative and timed program that allows an early activation and late inhibition of mTORC1 and delayed and maintained activation of mTORC2 (Figure 5).

We demonstrate that hypoxia (1% O2) induces phosphorylation of mTORC2 downstream target Akt-Ser473 (3 to 6 hours) and a short phosphorylation peak at mTORC1 substrate S6K-Thr389 (10 to 60 minutes). Thus, hypoxia activates mTOR, S6K1, and Akt in different ways. mTORC1 signaling appears to be activated only at a very early stage and is inhibited with prolonged (3 hours) exposure to hypoxia. In contrast, mTORC2 signaling is maintained; Akt-Ser473 phosphorylation increased under hypoxia at more than 3 hours and was sustained in 1% O. Importantly, phosphorylation of Akt was partially inhibited by rapamycin and strongly by mTOR silencing. It has initially been reported that mTORC2, ie, the rictor/mTOR complex, is rapamycin insensitive[14,15] However, later studies have shown that prolonged rapamycin treatment inhibits mTORC2 assembly and, as a consequence, Akt/protein kinase B in certain cell types, including endothelial cells (HUVECs) in particular[39].

In line with these phosphorylation studies, mTOR silencing, rapamycin, and Akt inhibition all specifically and significantly inhibited proliferation of serum-starved RAECs under hypoxia, and rapamycin also decreased endothelial sprout formation in endothelial spheroids under hypoxia alone. Finally, rictor knock-down, and therefore inhibition of mTORC2 signaling, clearly decreased hypoxia-induced phosphorylation on Akt-Ser473 and totally blunted hypoxia-induced endothelial proliferation. On the other hand, raptor silencing, and therefore inhibition of mTORC1, did not affect Akt phosphorylation and partially, although significantly, reduced hypoxia-induced endothelial proliferation.

The differences of hypoxic activation of mTOR1 and mTORC2 hypothetically may

involve distinct effects of hypoxia-induced phosphorylation of mTOR at Ser2448. We show that hypoxia rapidly (10 minutes) and concentration-dependently promotes rapamycin-sensitive and sustained phosphorylation of mTOR-Ser2448 and mTOR nuclear translocation in RAECs. Phosphorylation of mTOR-Ser2448 was modulated in the physiological oxygen saturation range (1% to 11% O2) also covering moderate hypoxic conditions. This is consistent with other responses to hypoxia to prevent or delay the onset of more severe hypoxia[40]. However, the functional significance of the mTOR phosphorylation site in Ser2448 is still unknown. Phosphorylation of this site has been suggested to be part of a feedback mechanism regulating mTOR activity[41]. However, it is still unclear whether this feedback loop is positive or negative and whether it affects mTORC1 or mTORC2 to the same extent[7,41]. Further investigations assessing whether hypoxia-induced nuclear mTOR-Ser2448 phosphorylation is associated with a specific mTOR complex or function will therefore be necessary. As shown by immunofluorescence, mTOR-Ser2448 phosphorylation is localized to subnuclear macromolecular structures resembling promyelocytic leukemia (PML) nuclear bodies. These PML bodies represent distinct yet dynamic intranuclear structures involved in apoptosis, proliferation, and senescence and also associate with nuclear phosphorylated Akt.[42] Indeed, very recently, PML was shown to be a novel suppressor of mTOR and neoangiogenesis during ischemia[19].

Hypoxia also induces proliferation in lung adventitial fibroblasts[20], cardiac fibroblasts[28], and MEFs[24]. To extend the validity of our data to other cell types, we have assessed how MEF cells increase proliferation under hypoxia. In MEFs, a loss of Tsc2 confers a growth advantage to hypoxic cells[24], suggesting that hypoxia inhibits mTOR via the tuberous sclerosis complex (TSC). TSC, consisting of Tsc1 and Tsc2, is the main upstream inhibitor of mTOR activity. The disruption of the complex by Tsc2 phosphorylation results in mTOR activation[38]. Indeed, we confirm that disrupting the Tsc2 gene increases proliferation under hypoxia. However, the same advantage is present in wild-type MEFs or Tsc2-mutated MEFs with a reintroduced Tsc2 wild-type gene. Increased proliferation to hypoxia, however, was specifically decreased by rapamycin and Akt inhibitor. Based on these experiments, we conclude that mTOR mediates hypoxia-induced cell proliferation independent of regulation by TSC. An autonomous role of mTOR, in sensing and transducing oxygen saturation, was suggested by recent work revealing that a redox-sensitive switch may contribute to the regulatory mechanism that controls the mTOR pathway[43,44]. Furthermore, oxidative

capacity as displayed by mitochondrial activity was shown to regulate mTORC1 assembly[45].

The proliferation studies in HIF-1α knockout MEFs suggest that HIF-1α is a partial downstream effector of mTOR- dependent proliferation under hypoxia. However, mTOR can promote hypoxia-induced proliferation also in the absence of HIF-1α, as shown by overexpression of mTOR in HIF-1α knockout MEFs. Still, further studies will have to assess the role of HIF-1α in mTOR-dependent proliferation in endothelial cells[46], as well as the contribution of mTORC1 and mTORC2 complexes to HIF-1α stability but also to the activity of cell cycle regulators such as cyclin D1 and p21.

In conclusion (see Figure 5), hypoxia-induced proliferation in endothelial cells requires signaling from both mTOR complexes, mTORC1 and mTORC2. mTOR activation by hypoxia is monitored by an early and sustained rapamycin-sensitive phosphorylation and nuclear translocation of mTOR, specifically phosphorylated at Ser2448. Activation of mTORC2 is monitored by a sustained phosphorylation of Akt-Ser473, which is decreased by mTOR and mTORC2 silencing and partially by prolonged rapamycin treatment. On the other hand, mTORC1 (rapamycin)-dependent S6K1 phosphorylation at early time points (3 hours) is likely involved in the early events that lead to hypoxia-mediated endothelial proliferation, whereas at later time points (3 hours), mTORC1 signaling is repressed as seen by complete dephosphorylation of S6K-Thr389. Blunting of hypoxia-induced endothelial proliferation by siRNA-mediated knockdown of raptor or rictor demonstrates the importance of mTORC1 and especially mTORC2, respectively. This indicates cooperating mechanisms between signals from both mTOR complexes in the response to hypoxia in endothelial cells. Thus, mTORC1 and specifically mTORC2 may be interesting novel targets to regulate hypoxia-induced endothelial cell proliferation and angiogenesis for inhibition of tumor vascularization and potential induction of reparative angiogenesis during ischemic cardiovascular disease.

Materials and Methods

Rat aortic endothelial cells (RAECs) were prepared, cultured, and characterized as described previously[5]. Tsc2-defective MEFs were obtained from Michael Hall (Biocenter, Basel, Switzerland), HIF-1α knockouts from Max Gassmann (University of Zürich, Switzerland). Endothelial cell spheroids were generated as described elsewhere[25]. Predesigned short interfering RNAs (siRNAs) against rat mTOR (frap1_3 siRNA) were purchased from Qiagen. Short hairpin RNAs (shRNAs) containing vectors against raptor

and rictor are pKDM- 132, a pSuper.gfp/neo-based siRNA-expressing plasmid targeting ctgtgaactagcacttcag in rictor mRNA; and pKDM-162, a pSuper.gfp/ neo-based plasmid targeting ggacaacggccacaagtac in raptor mRNA. RAECs were transfected with si/shRNA by AMAXA nucleofection. Cell numbers were assessed using Cell Proliferation Reagent WST-1 (Roche Molecular Biochemicals) according to the specifications of the manufacturer.

Cell lysis was prepared as described previously[5] and as described elsewhere[26]. Primary chicken polyclonal anti–HIF-1α antibodies were provided by Max Gassmann (University of Zürich, Switzerland), polyclonal anti-raptor and anti-rictor antibodies were generated by Markus A. Rüegg (Biocenter Basel, Switzerland). All other antibodies were commercially available. Protein bands were analyzed by densitometric quantification by ImageJ 1.31v software (Wayne Rasband, NIH). Immunostaining was performed as described previously[27].

Data (meanSEM) were analyzed for normal distribution (1-way or repeated-measures ANOVA), followed by multiple or pairwise comparison with the Bonferroni post test using the GraphPad software Prism. The number of single experiments compiled is indicated by n. A value of P0.05 was considered as significant.

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Disclosures

None.

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The role of mTORC1 and mTORC2 in hypoxia-induced HIF-1 α stabilization and endothelial proliferation

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(in preparation)

Abstract

Hypoxia prevents degradation of hypoxia-inducible factor (HIF) α subunits, and therefore facilitates heterodimerization with HIF-β subunits. HIF controls the adaptive response to hypoxia by driving expression of genes for angiogenesis, erythropoiesis and glycolysis. HIF-1α stabilization under hypoxia has previously been shown to be disturbed by rapamycin suggesting requirement of active mTOR signaling.

Here we have investigated the regulation and role of HIF-1α and mTOR signaling in hypoxia-induced proliferation of aortic endothelial cells (RAEC). As shown before in other cell types, hypoxia and growth factor stimulation with PDGF-BB induces stabilization and translocation of HIF-1α to the endothelial nucleus. Moreover, HIF-1α knock down reduces RAEC proliferation in hypoxia. To investigate pathways potentially regulating HIF-1α stabilization we used specific inhibitors of signaling relay enzymes mTOR, MEK1/2, JunK and p38, that are involved in stress responses. mTOR inhibition with rapamycin significantly reduced HIF-1α accumulation in response to hypoxia and PDGF-BB stimulation, whereas MEK1/2 inhibition reduced HIF-1α accumulation only during PDGF-BB stimulation under normoxia. Rapamycin dose dependently reduced proliferation of RAEC during PDGF-BB stimulation under hypoxia and hypoxia alone. MEK1/2 inhibition reduced proliferation under normoxia and hypoxia to similar extents, thus not specifically affecting the hypoxic response. Inhibition of JunK and p38 did not affect HIF-1α accumulation and RAEC proliferation in the tested conditions.

Here we demonstrate that mTOR signaling under hypoxia is partially required to stabilize HIF-1α, and that HIF-1α is a co-effector of mTOR-dependent, hypoxia-induced endothelial proliferation. HIF-1α stabilization and RAEC proliferation under hypoxia does not depend on signaling by MEK1/2, Jun kinase and p38. Recent studies have identified two structurally und functionally distinct mTOR multi protein complexes. Further ongoing studies will determine the individual or cooperative contribution of the two mTORCs in hypoxia-mediated stabilization of HIF-1α.

Introduction

Cellular hypoxia occurs when the demand for molecular oxygen necessary to generate ATP levels sufficient to sustain cell function exceed the vascular supply. Hypoxia can occur during development, is associated with various cardiovascular diseases such as peripheral artery disease, myocardial infarction, and heart failure, and also with tumor

growth or wound healing[1,2]. As part of an adaptive response, cells and tissues react to hypoxia and induce transcriptional changes that may lead to induction of genes that improve tissue perfusion (i.e. in angiogenesis and vasodilation), a switch to anaerobic energy winning in glycolysis, or to an increase in erythocyte count[3]. This is initiated in part by upregulation of transcription factors such as the well-studied hypoxia-inducible factors HIF-1 and HIF-2.

The HIF-1 nuclear transcription complex is ubiquitously expressed and consists of a stably expressed component HIF-1β (ARNT) and a hypoxia inducible protein HIF-1α. Under normoxic conditions HIF-1α subunits are hydroxylated by prolyl hydroxylase domain containing proteins (PHDs), polybiquinated in a von Hippel-Lindau protein (pVHL) dependent way and constitutively degraded by the proteasome[3-5]. Hypoxia prevents activity of the prolyl hydroxlases and thereby inhibits HIF-1α degradation.

We have shown previously that rapamycin, the inhibitor of mammalian target of rapamycin (mTOR) inhibits angiogenesis in vitro, and that mTOR complex 1 (mTORC1) and -complex 2 (mTORC2) are required for hypoxia-mediated proliferation of endothelial cells[6,7]. Active mTOR signaling has been shown to stabilize HIF-1α under hypoxia[8,9]. Moreover, mTOR-stabilized HIF-1α partially contributes to proliferation of mouse embryonic fibroblasts (MEFs) under hypoxia[7]. mTORC1 is highly rapamycin sensitive and is a key controler of cell growth and size by its capacity to control protein synthesis via S6K1/2 and 4E-BP1[10]. The function of mTORC2 is less clear, but involves regulation of cell proliferation and phenotype modulation[13,14]. mTORC2 was also proposed to play an important role in the formation of the vasculature[13,14], similarly as HIF-1α and VEGF[5].

In this study we investigated whether signaling through mTORC1, mTORC2, MEK1/2, Jun kinase and p38 are necessary to stabilize HIF-1α and whether the inhibition of these pathways affects proliferation of endothelial cells in response to hypoxia.

Results and Figures

Hypoxia and growth factor stimulation results in nuclear accumulation of HIF-1α protein.

To determine the effect of hypoxia and growth factor stimulation on HIF-1α, primary rat aortic endothelial cells (RAECs) were stimulated with or without PDGF-BB for 4.5 h under 21 % and 1 % O_2 (normoxia and hypoxia) and analyzed for HIF-1α protein expression by immunofluorescence confocal microscopy (Fig 1A) and westernblot analysis with consecutive densitometric quantification (Fig 1B). HIF-1α was almost undetectable under normoxia, whereas stimulation with PDGF-BB and hypoxia and the combination of both led to HIF-1α stabilization and nuclear accumulation (Fig 1A). Stimulation by PDGF-BB under conditions of normoxia induced HIF-1α to 4.2 fold, and hypoxic stimulation alone to 5.2 fold compared to normoxic controls. Stimulation of RAEC by PDGF-BB under hypoxia boosted HIF-1α protein levesl to 11.9 fold compared to normoxia (Fig. 1B), suggesting a slight synergistic effect of growth factor stimulation and hypoxia.

HIF-1α silencing reduces hypoxia-induced proliferation of RAECs.

Angiogenesis depends on the coordination of several independent but temporally orchestrated processes, which includes endothelial cell proliferation as a crucial component[4]. We have shown previously that hypoxia increases proliferation in RAEC[6]. In order to investigate the role of HIF-1α in RAEC proliferation in response to hypoxia, HIF-1α protein was knocked down by siRNA silencing. HIF-1α protein was silenced to more than 90 % (Fig. 2A). Hypoxia increased proliferation of RAEC significantly compared to culturing under normoxia. Increased proliferation in response to hypoxia was significantly reduced with HIF-1α knock down (Fig. 2B).

Nuclear HIF-1α accumulation and hypoxia-induced RAEC proliferation is sensitive to rapamycin-mediated mTOR inhibition.

In PC-3 prostate cancer cells, rapamycin, the specific inhibitor of mTORC1, was shown to decrease HIF-1α stabilization[8], MEK1/2, Jun kinase and p38 were also reported to contribute to the stability of HIF-1α[3,15,16]. We investigated whether mTOR, MEK1/2, Jun Kinase or p38 contribute to the stabilization of HIF-1α in response to hypoxia and PDGF-BB stimulation in RAEC. Starved RAECs were treated with rapamycin (5 – 500 nM),

PD98059 (5 – 80 µM), JNK-Inhibitor-II (5 – 200 nM) and SB203580 (0.1 – 3 µM) prior to PDGF stimulation and incubation in normoxia and hypoxia for 4.5 h. Rapamycin significantly, albeit incompletely reduced nuclear HIF-1α protein levels induced by hypoxia and PDGF-BB stimulation to a similar degree at all rapamycin concentrations tested. Inhibition of MEK1/2 by PD98059 reduced PDGF–BB-induced HIF-1α protein levels under normoxia only and did not affect HIF-1α stabilization under hypoxia. Inhibition of Jun Kinase with JNK-Inhibitor-II and inhibition of p38 with SB203580 had no effect on HIF-1α protein accumulation in any tested condition (Fig. 3A).

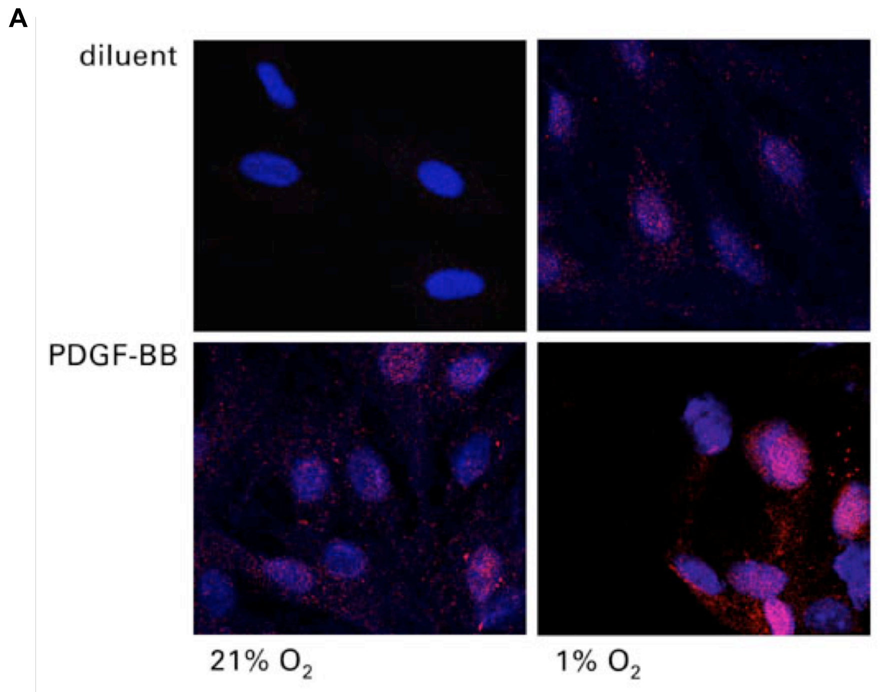
To investigate whether HIF-1α-dependent, hypoxia-induced RAEC proliferation requires mTOR, MEK1/2, JunK or p38, we pretreated the starved cells with the inhibitors rapamycin, PD98059, SB203580 or JNK-inhibitor-II respectively, and incubated RAECs under normoxia and hypoxia for 28 h. As shown previously[7] hypoxia induced a significant increase in proliferation of RAEC compared to normoxia. In the absence of growth factor stimulation, rapamycin reduced RAEC proliferation with increasing dose, and significantly at a concentration of 200 nM and higher (Fig 3B) under hypoxia only. The MEK1/2 inhibitor dose dependently and significantly lowered hypoxia-induced RAEC proliferation at a concentration of 20 µM, but also lowered proliferation under normoxia to a similar degree. JunK and p38 -inhibition did not affect RAEC proliferation (Fig 3B).

Similar results were obtained when including PDGF-BB together with hypoxia to stimulate RAEC, which significantly increased proliferation when compared to normoxia plus PDGF-BB as shown earlier[6]. Under hypoxia, rapamycin significantly blunted RAEC proliferation already at a concentration of 20 nM to levels when stimulated with PDGF-BB only. Inhibition of MEK1/2, JNK and p38 had no significant effect on proliferation of RAECs in response to PDGF-BB (Fig. 3B).

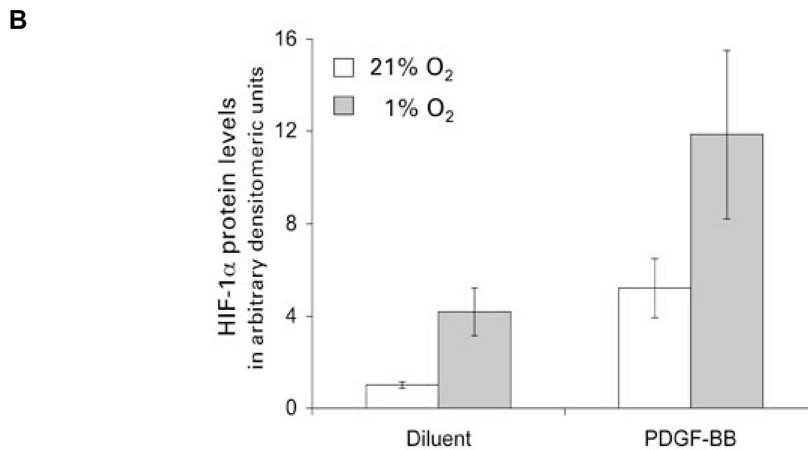
Thus, rapamycin-mediated reduction of HIF-1α levels is in parallel to rapamycin- and HIF-1α knockdown-mediated reduction of RAEC proliferation in response to hypoxia and/or growth factors. MEK1/2 mediated RAEC proliferation and contribution to HIF-1α stabilization occurs only under conditions of normoxia with PDGF-BB stimulation.

Figure 1

A

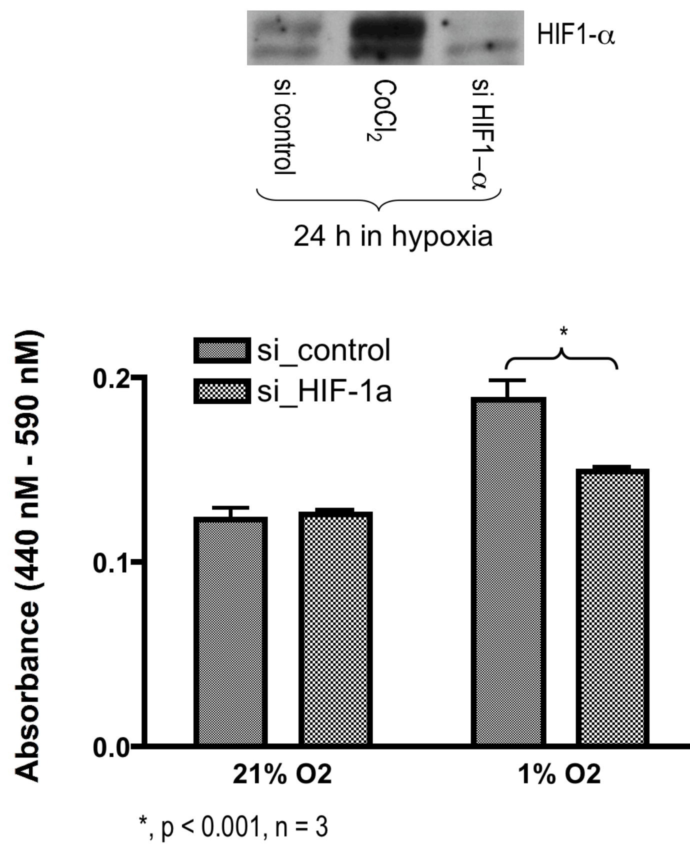


B



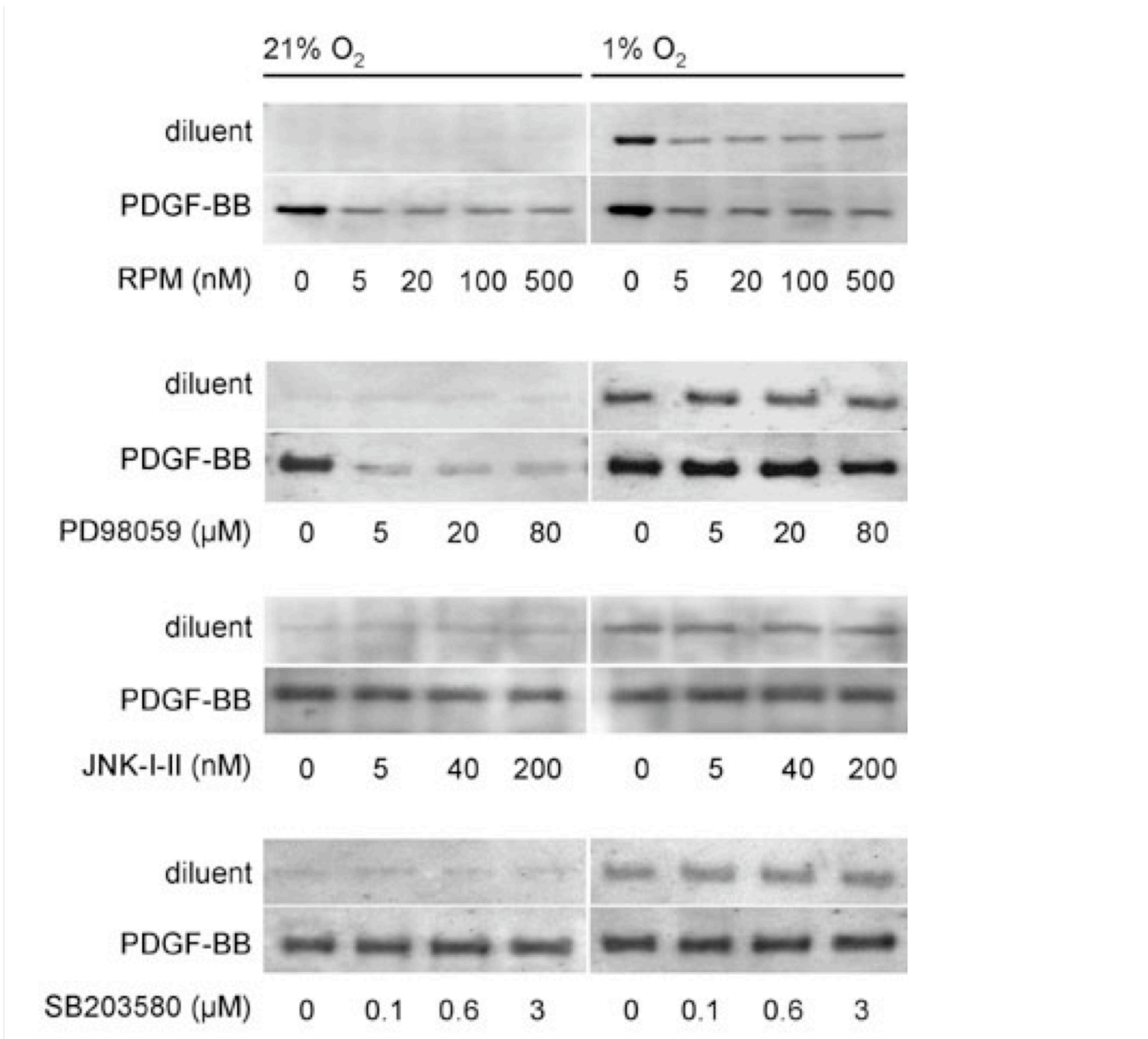
Hypoxia and growth factors induce nuclear accumulation of HIF-1α protein. A. Confocal fluorescence image of RAEC, immune stained for HIF-1α, nucleus stained with Hoechst. Cells were plated on cover slips, serum deprived, stimulated with or without PDGF-BB (10 ng/ml) and incubated for 4.5 h in 21%- or 1% O_2. B. Densitometric quantification of nuclear HIF-1α protein levels detected by Western blot analysis. RAEC were serum starved, stimulated with or without PDGF-BB (10 ng/ml) and incubated in 21%- or 1% O_2 for 4.5 h.

Figure 2



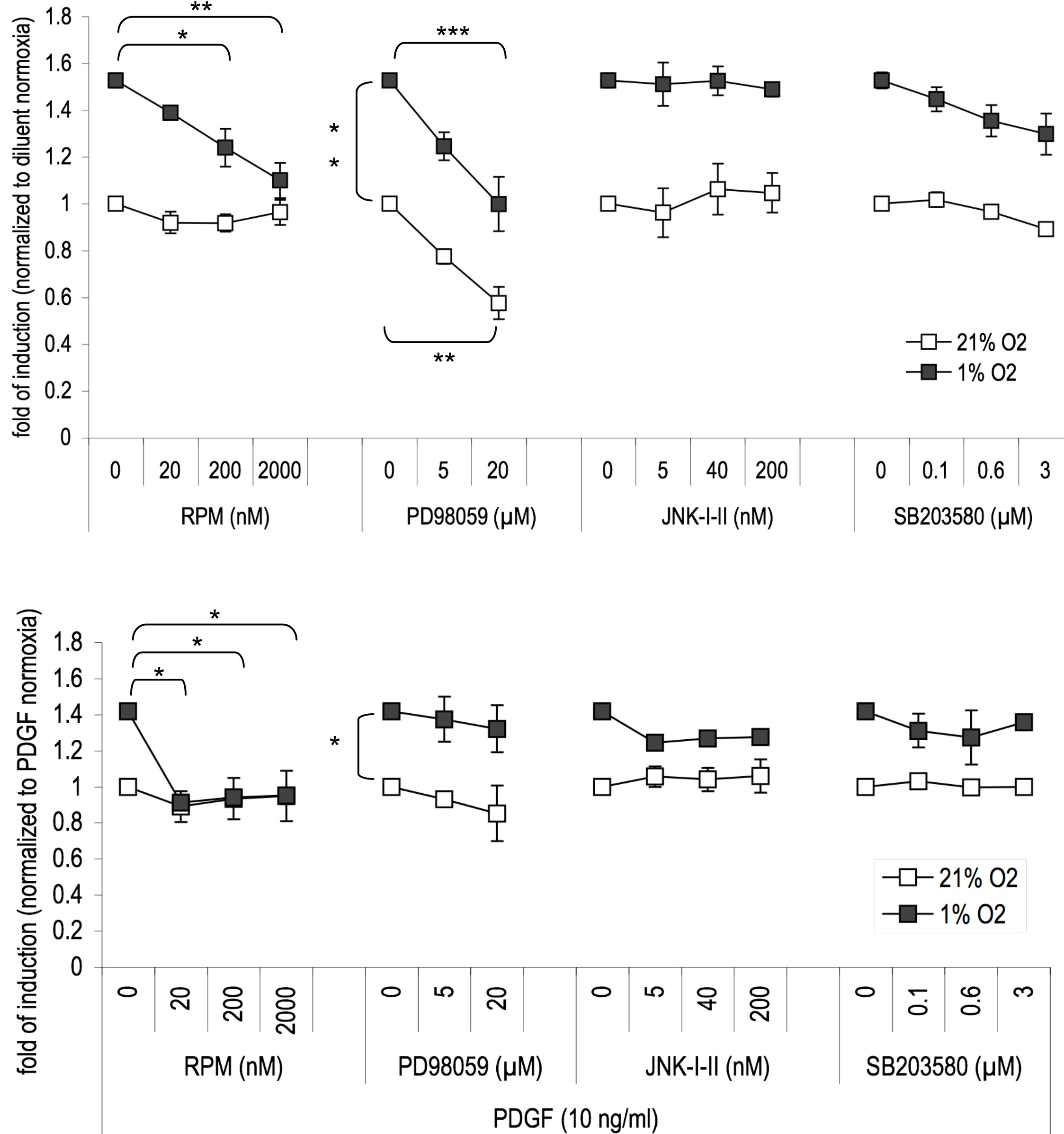
HIF-1α silencing reduces proliferation of RAEC. Serum starved RAEC were nucleofected with HIF-1α siRNA or control siRNA and incubated for 30h in 1%- or 21% O_2. Proliferation was determined using WST-1 cell proliferation reagent.

Figure 3A



mTOR inhibition prevents nuclear accumulation of HIF-1α protein under hypoxia and normoxia whereas inhibiton of MEK 1/2 only prevents an accumulation under normoxia. Western blot analysis of nuclear HIF-1α protein. Serum deprived RAEC were treated with mTOR inhibitor (Rapamycin, 5 – 500 nM), MEK 1/2 inhibitor (PD98059, 5 – 80 μM), jun kinase inhibitor (JNK-I-2, 5 – 200 nM), p38 inhibitor (SB203580, 0.1 – 3 μM) and incubated for 4.5 h in 21%- or 1% O_2 in the presence or absence of PDGF-BB (10 ng/ml).

Figure 3B



mTOR inhibition lowers proliferation of RAEC under hypoxia whereas MEK 1/2 inhibition reduces RAEC proliferation under normoxia. Serum starved RAEC were treated with inhibitors similar to the cells in Figure 2. Proliferation rates were determined using WST-1 cell proliferation reagent, after 30 h of incubation in 1%- or 21% O_2,

Discussion

In this study we have investigated the signaling pathways mTOR, MEK1/2, JNK and p38 – pathways reported to respond to stress[3,15,16] – for their contribution to HIF-1α stabilization and RAEC proliferation under hypoxia and growth factor (PDGF-BB) stimulation.

We have confirmed, that hypoxia and PDGF-BB–stimulation induce nuclear accumulation of HIF-1α (reviewed in Ke and Costa, 2006[3])[18,19]. Greater than additive amounts of nuclear HIF-1α were observed when stimulating with PDGF-BB under hypoxia, suggesting a synergistic effect of hypoxia and PDGF-BB in HIF-1α stabilization.

Inhibition of mTOR by rapamycin significantly reduced nuclear HIF-1α levels under hypoxia alone or when stimulating with PDGF-BB under hypoxia. In parallel, rapamycin specifically reduced hypoxia-induced proliferation in RAECs but did not affect proliferation under normoxia. Thus, HIF-1α stabilization under hypoxia is not only negatively regulated by PHD but also positively by mTOR. PDGF-BB induced HIF-1α stabilization under normoxia was rapamycin sensitive as well, suggesting that mTOR signalling is also essential for growth factor-induced HIF-1α stabilization under normoxia. Thus, mTOR signalling activated by growth factors or hypoxia is required for HIF-1α stabilization.

In contrast MEK1/2 inhibition reduced HIF-1α levels when RAECs were stimulated with PDGF-BB under normoxia and did not affect HIF-1α stabilization in hypoxic conditions. MEK1/2 inhibition reduced proliferation of RAECs under normoxia and hypoxia to a similar extent. Thus, we suggest that MEK1/2 is critical for HIF-1α stabilization and is involved in regulating default proliferation of RAEC only by growth factor stimulation under normoxia. The pharmacologic inhibition of JNK- and p38-signaling neither affected HIF-1α stabilization nor proliferation of RAEC in response to hypoxia or growth factor stimulation.

mTOR-signaling is required for hypoxic and PDGF-BB mediated proliferation of RAECs and HIF-1α stabilization. In agreement, knock down of HIF-1α in RAECs inhibited proliferation under hypoxia. This partial inhibition may be due to an incomplete knock down of HIF-1α. However, MEFs completely lacking the HIF-1α gene reduced proliferation under hypoxia also to a similar degree[7], thus, not completely. We therefore speculate, that HIF-1α is a co-effector of mTOR-dependent, hypoxic proliferation of endothelial cells.

mTORC1 is rapamycin-sensitive, and rapamycin destabilizes HIF-1α under hypoxia. Is mTORC1 therefore the pivotal mTOR complex for hypoxia- or growth factor mediated HIF-1α stabilization? In endothelial cells, prolonged (~24 hr) treatment of rapamycin can also prevent formation of mTORC2. We have also shown that both mTORCs are required to promote proliferation of RAECs under hypoxia[7]. It remains therefore to be elucidated which of the two mTOR complexes is responsible for HIF-α accumulation.

Multiple pathways for HIF-1α regulation under hypoxia could potentially integrate different regulatory signals for different conditions and ensure that HIF-1α is only up-regulated if all criteria for e.g. cell growth were fulfilled. mTOR as an integrator of stress, energy, nutrient and growth factor signals and major controller of cell growth therefore absolutely copes with this concept. Interestingly, HIF-1α carries a potential phosphorylation site for mTOR[8], thus further studies are required to determine whether hypoxia-activated mTOR could serve as a directly acting kinase for HIF-1α phosphorylation and stabilization.

Thus, HIF-1α is a major and directly oxygen-sensing factor in determining the cellular response to hypoxia. A significant number of secondary pathways such as mTOR-signaling may additionally modulate HIF-1α but also other cellular effectors and transcriptional responses.

MATERIALS & METHODS

Cells & culture conditions

Rat aortic endothelial cells (RAEC) were derived from adult male Sprague-Dawley rat (Charles River Laboratories, France) or WISTAR rats (RCC Ltd, Itingen) and characterized as described previously[6]. RAECs were cultured in DMEM (Oxoid AG, Basel, Switzerland) complemented with 10% fetal calf serum (FCS, Oxoid AG), 1% sodium pyruvate (Oxoid AG), 1% non-essential amino acids (Oxoid AG), 1% penicillin-streptomycin (GIBCO™, Invitrogen AG, Basel, Switzerland). At 70% confluence, cells were washed twice in PBS (Oxoid AG) and starved in serum-free DMEM for 30 h.

Reagents, Antibodies and Plasmids

Inhibitors of cell signaling used were: Rapamycin 5 mM stock, PD98059 10 mM stock, JNK-Inhibitor II 10 mM stock, SB203580 10 mM stock, all inhibitors were dissolved in DMSO (Calbiochem, Läufelfingen, Switzerland). PDGF-BB was from R&D systems Europe Ltd., Abingdon, UK.

Following primary antibodies for western blotting were used: chicken polyclonal anti-HIF-1α (kindly provided by M. Gassmann, Clinic for Small Animal Internal Medicine, University of Zürich, Switzerland). Secondary antibodies were HRP-conjugated anti chicken (Transduction Laboratories, San Diego, CA). Antibodies used for for immune fluorescence (confocal microscopy) were goat polyclonal IgG against HIF-1α (Santa Cruz, California, USA), anti-goat Cy2-conjugated IgG (LabForce AG, Nunningen, Switzerland). Nuclei were stained with TOTO-3 (Juro Supply GmbH, Lucerne Switzerland).

Angiogenesis assay in vitro of endothelial spheroids

Endothelial cell spheroids of defined cell number were generated as described elsewhere [20]. The spheroid containing fibrin bilayer was overlaid with serum-free DMEM and stimulated according to experimental protocol for 24 h. Sprouts emerging from spheroids were analyzed and quantified as described elsewhere[21,22].

Cell proliferation assay

To determine proliferation rate, 5500 cells/well (if transfected 11000 cells/well) were seeded into 96-well plates, after 24 h the normal culture medium was replaced with serum-free DMEM for 30 h. In all experiments using inhibitors and PDGF-BB, inhibitors were added 30 min, PDGF-BB 5 min before incubation for 28 h at 21% O_2 and at 1% O_2. Each condition was tested in octuplicates. Cell numbers were assessed using Cell Proliferation Reagent WST-1 (Roche Molecular Biochemicals, Rotkreuz, Switzerland) according to the manufacturer's specifications.

Immunoblotting and transfections

Cells were seeded in a confluency of ~70 % and after 24 h starved in serum free DMEM for 30 h. Inhibitors were added 30 min, growthfactors 5 min prior to incubation at 1% O_2. Cells were lysed with RIPA lysis buffer. To obtain nuclear extracts for HIF-1α protein alnalysis cells were lysed with high and low -salt buffer as described elsewhere[23]. 15 µg, for HIF-1α blots 20 µg –protein respectively were subjected to SDS-gel electrophoresis and blotted on nitrocellulose membrane (Schleicher & Schuell GmbH, Dassel, Germany). 5 % skim milk /TBST was used for blocking. Antibodies were diluted in 5 % skim milk/TBST or according to manufacturers protocol. Protein bands appearing on X-ray films were quantified by Image J software (Wayne Rasband, NIH, USA).

Immunofluorescence microscopy

RAECs were seeded on cover slips at a density of 4.0 x 10^4/well in 24 well tissue culture plates. At 70% confluence, cells were starved in serum-free DMEM for 30 h. After 4.5 h of culturing in different conditions (± 10 ng/ml PDGF-BB under 21% O_2 or 1% O_2) cells were rinsed and fixed with 4% Paraformaldehyde for 15 min at RT. Samples were blocked with goat serum (Fluka Chemie GmbH, Buchs, Switzerland) in PBS with 0.25% BSA. Fixed cells were probed with primary goat polyclonal IgG against HIF-1α and secondary anti-goat Cy2-conjugated. TOTO-3 was used for nuclear DNA staining. Cover slips with cells were embedded on microscope slides with FluorSave reagent (Calbiochem, Läufelfingen, Switzerland) and analyzed by confocal laser scanning microscopy (LSM 510, Carl Zeiss AG, Feldbach, Switzerland).

Statistical analysis

Data (mean ± SD) were analyzed for normal distribution (one-way ANOVA), followed by Bonferroni posttest with multiple or pair wise comparison using GraphPad Prism 4.0a software (GraphPad Software Inc., San Diego, USA).

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Effects of anti-hypertensive drugs on vessel rarefaction

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(Curr Opin Pharmacol. 2007 Apr;7(2):151-7)

Summary

The microcirculation largely determines peripheral vascular resistance and substantially contributes to arterial hypertension. In both human arterial hypertension and animal models of hypertension, genetic, fetal and other mechanisms associated with hypertension can reduce the formation and number of microvessels (i.e. parallel-connected arterioles and capillaries). Impaired formation of microvessels (impaired angiogenesis) and microvascular rarefaction can, on the other hand, contribute to increased peripheral resistance and raise blood pressure. Interestingly, drugs targeting the renin-angiotensin-aldosterone system (i.e. angiotensin-converting enzyme inhibitors and AT_1 receptor blockers) induce angiogenesis *in vivo* in the majority of animal studies. Furthermore, recent clinical studies demonstrate that long-term antihypertensive treatment increases capillary density in the skin of hypertensive patients without diabetes. These effects of angiotensin-converting enzyme inhibitors and AT_1 receptor blockers can be mediated by activation of bradykinin pathways, resulting in the generation of vascular endothelial growth factor, nitric oxide and, consequently, angiogenesis. In conclusion, specific antihypertensive drugs can induce angiogenesis and reduce or even reverse microvascular rarefaction. This might improve target organ damage in, and slow the development of, hypertension.

Introduction

The microvasculature supplies nutrients and oxygen to tissues, removes metabolites and carbon dioxide, and maintains an adequate hydrostatic pressure in tissues. The microcirculation is extremely important in determining blood pressure via peripheral resistance, vasoconstriction and vasodilatation. Hydrostatic pressure drops largely in this microcirculation, sinking gradually from small arteries to capillaries without a specific, single site of resistance control. Capillaries further contribute to resistance because of their narrow caliber and their contractility. The number of capillaries, and therefore their combined luminal area, also affects peripheral resistance. Stressors such as hypoxia and inflammation can induce microvessels to dynamically form and, after reversal of the stressors, to functionally or anatomically disappear. Similarly, factors associated with vasodilatation and vasoconstriction can affect the formation of microvessels (i.e. angiogenesis). Most forms of human and experimental arterial hypertension are associated with decreased numbers or densities of microvessels. This microvascular rarefaction can further increase peripheral resistance, raise blood pressure and therefore

aggravate hypertension and hypertension-induced target organ damage (Figure 1).

This review aims to explore the role of angiogenesis and its mechanisms in relation to arterial hypertension. It also describes the effects of antihypertensive drugs on angiogenesis and microvascular rarefaction in hypertension.

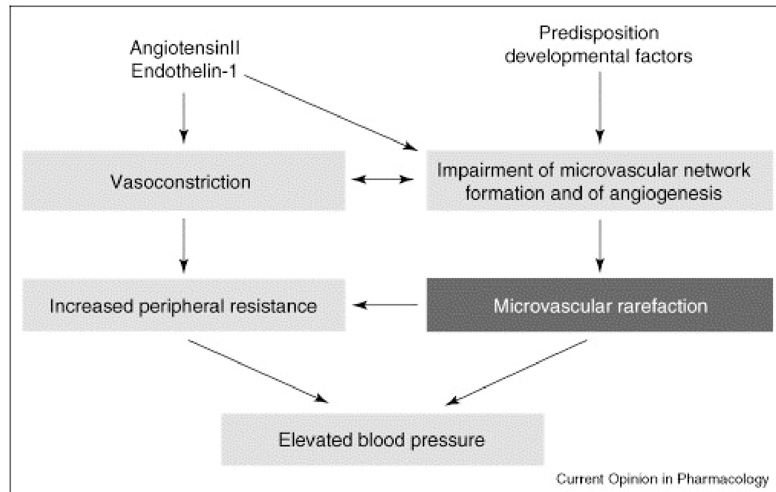

Figure 1. Microvascular rarefaction contributes to increased peripheral resistance and high blood pressure. In most studies of human hypertensives and in animal studies of hypertension, there are less microvessels in hypertensives versus normotensives. Microvascular rarefaction can be caused by either predisposition or an impaired angiogenic reponse. The reduction in total luminal microvascular diameter results in increased peripheral resistance and could contribute to elevated blood pressure in essential hypertension.

Microvascular rarefaction in hypertension

Several theories explain microvascular rarefaction in hypertension. Rarefaction can be either primary (i.e. it antedates the onset of hypertension) or secondary (i.e. it occurs as a consequence of prolonged elevation of blood pressure). Secondary rarefaction might be caused by functional shut-off or destruction of existing capillaries Primary rarefaction, by contrast, might result from impaired angiogenesis and microvascular network formation[1]. In patients with essential hypertension, anatomic rather than functional rarefaction reduces the density of capillaries in the skin of the dorsum of the fingers[1,2]. Increased blood pressure can also lead to microvascular rarefaction (for review, see[4]); however, resistance vessel and microvascular remodeling can be totally[5,6] or partially[7] blood pressure independent. Patients with borderline essential hypertension have skin capillary densities that are as equally low as, or even lower than, patients with established hypertension, and capillary density does not correlate with blood pressure[8]. Moreover, impaired microvascular vasodilatation and capillary rarefaction are associated

with a familial predisposition to essential hypertension[9]. Offspring of individuals with high blood pressure have fewer capillaries on the dorsum of their fingers before the onset of definitive hypertension[10]. Thus, capillary rarefaction might antedate, rather than follow, sustained hypertension[10]. Therefore, impaired angiogenesis during development or early in life might predispose to high blood pressure[9]. In addition, deficient embryonic vascular development, low birth weight and impaired postembryonic vascular growth could impede the formation of microvascular networks[11].

Mechanisms of formation and upkeep of new microvessels

During fetal growth, the vascular bed expands by sprouting and matures into a network of stable vessels. When tissues grow beyond the limit of oxygen diffusion, hypoxia can lead to the formation of new vessels in every mature tissue, triggering vessel growth by signaling through hypoxia-inducible transcription factors[12]. These factors upregulate several angiogenic genes, such as the gene encoding vascular endothelial growth factor (VEGF); VEGF stimulates physiological and pathological angiogenesis[12]. A hypoxic microenvironment also controls many other factors and modulates signaling pathways to promote angiogenesis[12-14]. Endogenous VEGF upholds vascular homeostasis, and insufficient VEGF levels initiate apoptotic pathways in mouse skeletal muscle[15].

Effects of antihypertensive drugs in human clinical studies

Clinical studies in hypertensive patients have focused on lowering blood pressure, vasodilation and vasoconstriction, vascular rheology, vascular stiffness and reversal of target organ damage in the heart, kidney, brain and arteries. Only very few studies have explored the effects of antihypertensive drugs on the microvasculature. Interestingly, long-term and effective antihypertensive treatment of non-diabetic hypertensive patients increased capillary density compared with non-treated patients in a recent study[16]. In an earlier study in 70 hypertensives, losartan reduced vascular hypertrophy and rarefaction after three years of randomized, blinded treatment when compared with atenolol[17]. However, further clinical prospective studies are needed to explore the anatomical effects of anti-hypertensive treatment on the microvasculature.

Effects of antihypertensive drugs in animals or *in vitro*

Angiotensin-converting enzyme inhibitors

Angiotensin-converting enzyme inhibitors (ACE-Is) lower blood pressure and reduce the media-to-lumen ratio of big arteries by blocking the conversion of angiotensin I to the vasoconstrictive and trophic decapeptide angiotensin II, and by blocking the breakdown of the vasodilator peptide bradykinin. In addition, ACE-Is substantially change microvessel structure and density[18-20]. In some studies, ACE-Is blocked microvessel formation: captopril reduced aortic and microvascular growth in hypertensive and normotensive rats[21] and the capillary-fiber ratio in ischemic hind limbs of rats[22]. The ACE-I benazepril reduced both arteriolar density and small venule density in dorsal microcirculatory chambers of spontaneously hypertensive rats (SHRs)[23]. In these studies, suppression of the renin-angiotensin system promoted microvascular rarefaction, suggesting that angiotensin II might powerfully induce angiogenesis[21,24]. Conversely, ACE-Is significantly have been shown to increase myocardial capillary density in several studies. This effect correlates with the decrease in ventricular hypertrophy seen after treatment of SHRs and Wistar-Kyoto rats with captopril[25]. Moreover, ACE-Is increased myocardial capillary density in SHRs[6], in *in vivo* models of hind limb ischemia of the rabbit[26], in stroke-prone SHRs[27], and in different rat models of obesity[28]. Furthermore, several studies designed to investigate the induction of angiogenesis in ischemic hind limbs of mice[29,30] and rabbits[26] suggest a pro-angiogenic role for ACE-Is.

However, although the majority of studies supports a pro-angiogenic role for ACE-Is, it is not entirely clear why the results from some studies, mentioned earlier, contradict this viewpoint. It is possible that captopril, with its reactive sulfhydryl group, exerts additional anti-angiogenic effects in studies that depend upon inhibition of metalloproteinases rather than ACE-Is[31]. Most other ACE-Is, which differ from captopril chemically, might not possess these specific anti-angiogenic properties. Furthermore, ACE-Is might exert distinct effects depending upon the specific tissue and model investigated (see below).

Inhibition of the breakdown of bradykinin, rather than angiotensin-II-mediated effects, most likely accounts for the pro-angiogenic effects of ACE-Is[32]. Knockout of the bradykinin B_2 receptor blunted ACE-I-induced vascularization of mouse ischemic legs[29]. In a model of myocardial infarction-induced heart failure, icatibant, an antagonist of the B_2 receptor, prevented the increase in heart vessel density that had been induced by both high and low doses of an ACE-I[6,33]. Interestingly, icatibant itself did not show any

effect on angiogenesis[6,33]. It is possible that kinin levels are too low to affect vessel density without ACE-Is, or that blockade of the renin-angiotensin system is necessary for kinins to affect vessel density[33]. Bradykinin *per se* also required the B_2 receptor pathway, as shown both by our own studies in an *in vitro* model of angiogenesis in the heart and by the use of corresponding knockout animals (Sanchez de Miguel L *et al.*, abstract in *Microcirculation* 2006, 13:145,). Thus, ACE inhibition leads to angiogenesis via the bradykinin B_2 receptor pathway (Figure 2).

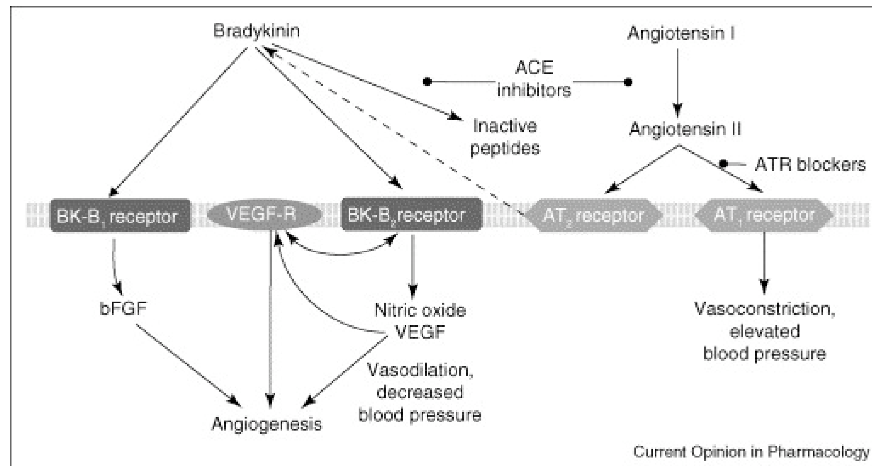

Figure 2. Angiotensin and bradykinin interact to induce angiogenesis. Bradykinin (BK), a potent vasodilator involved in regulation of blood pressure, induces angiogenesis. BK upregulates angiogenic molecules such as basic fibroblast growth factor (bFGF), via the BK B_1 receptor[70,71], or VEGF and NO, via the BK B_2 receptor[72]. The BK B_2 receptor can also activate the VEGF receptor on endothelial cells[73]. ACE inhibition results in BK accumulation and promotion of neovascularization[26,29,30,35]. Moreover, angiotensin II activates the AT_2 receptor during AT_1 receptor blockade, thereby upregulating BK and contributing to an angiogenic response (Munk *et al.*, unpublished)[74,75]. ATR, angiotensin receptor.

The pro-angiogenic effect of bradykinin on vessel density might also require nitric oxide (NO) and VEGF and/or prostaglandins[34]. Myocardial capillary rarefaction in obese rats was accompanied by decreased expression of VEGF and endothelial NO synthase[35]. Similarly, increased microvascular density in ischemic rat hind limbs after treatment with ACE-Is was associated with upregulation of endothelial NO synthase[29]. Decreased NO bioavailability also contributed to microvessel rarefaction in skeletal muscle of obese Zucker rats, a model of the metabolic syndrome[36]. In a rat model of secondary hypertension with inhibition of NO biosynthesis, the development of vascular connective tissue was substantially reduced; however, this effect was not seen in a different model with a NO-independent mechanism of secondary hypertension[37].

Angiotensin II receptor blockers

Angiotensin II type 1 receptor blockers (ARBs) block the effects of angiotensin II at the AT_1 receptor and thus decrease blood pressure and many of the other damaging trophic effects of angiotensin II on target organs. Several reports have demonstrated that ARBs increase capillary density[38-40]. When ARBs block AT_1 receptors, angiotensin II can still exert effects via AT_2 receptors. Plasma renin and angiotensin II levels also increase when the AT_1 receptor is blocked[41]; therefore, the effects of angiotensin II on the AT_2 receptor might include the release of kinins and NO[42] — molecules that mediate angiogenesis.

Both the AT_1 and the AT_2 receptor subtypes have been implicated in angiogenesis; in a model of renal peritubular capillary rarefaction in Sprague-Dawley rats, the ARB losartan reversed rarefaction via induction of VEGF expression, and the AT_2 blocker PD123319 increased capillary density via upregulation of the VEGF receptor, as well as angiopoietin and its receptor Tie-1[38]. The ARB losartan completely attenuated the decrease in rat coronary capillary density seen during angiotensin-II-induced hypertrophy[39]. By contrast, the ARB valsartan had no effect on the microcirculation of the cutaneous maximus muscle of young SHRs[23].

Diuretics

The few studies performed with diuretics do not allow us to draw a conclusion on their effects on the microcirculation. A randomized clinical trial in 40 hypertensive patients showed that hydrochlorothiazide did not affect indices of endothelial damage/dysfunction and angiogenesis[43]. *In vitro*, furosemide, a loop diuretic, attenuated expression of angiogenesis-related genes in the ischemic rat kidney[44]. Spironolactone, a potassium-sparing diuretic that antagonizes the mineralocorticoid aldosterone, inhibited proliferation of endothelial cells[45] and basic fibroblast growth factor-induced angiogenesis in the rabbit corneal assay[46].

α- and β-adrenergic receptor blockers

Reports on the effects of α- and β-adrenergic receptor blockers on microvascular rarefaction are inconclusive. Nebivolol, a β-blocker that also induces release of NO, reduced proliferation and caused apoptosis of aortic and coronary endothelial cells[47]. Similarly, nebivolol (but not metoprolol) inhibited endothelial sprout formation of the heart *in vitro* (Petrimpol, unpublished).

The α_1-adrenergic receptor doxazosin inhibited human vascular endothelial cell adhesion, migration and invasion[48], whereas terazosin inhibited the proliferation and tube formation of endothelial cells[49]. However, the α_1-adrenergic receptor blocker prazosin increased total vascular area and capillary density both in the rabbit ear chamber model[50] and in a rat hindlimb model of ischemia[51].

Calcium antagonists

Reports on the effects of calcium antagonists on microvascular rarefaction are again inconclusive. Nifedipine and amlodipine raised VEGF levels and thereby induced capillary tube formation of human coronary artery endothelial cells *in vitro*[52] and increased total capillary density in the dilated cardiomyopathic hamster heart[53]. Mibefradil and the chloride channel blocker NPPB [5-nitro 2-(3-phenylpropylamino) benzoate] inhibited tube formation of rat microvascular endothelial cells[54].

Angiogenesis and hypertension in human clinical studies

Increased VEGF levels during hypertension

In early hypertension, myocardial microvascular remodeling is accompanied by increased oxidative stress, inflammation and VEGF expression[55,56]. Plasma levels of VEGF are increased in hypertensive patients when compared with normotensives (for review, see[57]). Raised VEGF levels would imply that angiogenesis is stimulated in these hypertensive patients. Additionally, these findings might question whether capillary rarefaction is associated with hypertension[58]. Alternatively, functional rarefaction (i.e. vasoconstriction) and anatomical microvascular rarefaction might lead to an increase in shear stress of microvessels and the subsequent activation of angiogenic pathways (for review, see[59]). Furthermore, hypertensive subjects might not respond adequately to angiogenic growth factors, displaying defects in VEGF-associated signaling cascades[60,61] and endothelial dysfunction of newly formed microvessels[55,56]. Moreover, in a mouse ear model of angiogenesis, a defined dose of VEGF in the presence of platelet-derived growth factor-B dimer (PDGF-BB) induced stable, non-leaky, pericyte-covered normal capillaries. Conversely, the same does of VEGF in the absence of PDGF-BB induced aberrant vascular structures that developed into hemangiomas[62]. Thus, increased plasma VEGF levels, induced by endothelial dysfunction, shear stress or local hypoxia, might not necessarily lead to a denser or functional parallel-connected capillary network.

ACE inhibition and tumor angiogenesis

ACE-Is and their associated potential pro-angiogenic effects have raised concerns about a possible increased risk of cancer. Theoretically, induction of angiogenesis by ACE-Is to reverse microvascular vascular rarefaction in hypertension might stimulate growth of dormant non-vascularized tumors[63,64]. Reassuringly, neither data in animal tumor models *in vivo*[31,65,66] nor a retrospective cohort study based on the records of 5207 patients[67], nor post-marketing surveillance, suggests tumorigenicity for ACE-Is or other antihypertensive drugs. Potentially, the local microenvironment could influence the final biological response; however, distinct effects of ACE-Is on reparative angiogenesis in the heart versus tumor angiogenesis have yet to be fully elucidated.

Anti-VEGF anti-angiogenic tumor therapy and hypertension

Fascinatingly, new anti-angiogenic therapies to treat cancer using an anti-VEGF antibody (bevacizumab; Avastin®) result in hypertension in a significant proportion of patients[68,69], possibly resulting from the blockage of vasodilation induced by VEGF. Hypothetically, use of anti-VEGF antibodies might also reduce microvascular networks in hypertension. However, this still needs to be investigated.

Conclusions

Recent clinical studies have shown that long-term antihypertensive treatment can prevent microvascular rarefaction in hypertensive patients. ACE inhibition increases microvascular density in many animal models of hypertension; this effect is mostly mediated by activation of bradykinin pathways, resulting in stimulation of VEGF formation and increased NO bioavailability. In animal models of hypertension and tissue ischemia, these molecules act locally to enable the formation of stabilized and perfused microvessels.

It will be important to better understand the mechanisms of microvascular rarefaction and, generally, to prospectively investigate the impact of antihypertensive therapy on microvascular rarefaction in clinical studies. Drugs with a potent anti-remodeling effect or the potential to induce new microvessels might be more desirable if structural rarefaction precedes the onset of hypertension or is found in hypertension-induced target organ damage. By preventing microvascular rarefaction, hypertension and/or hypertension-induced target organ damage might be reversed. Further studies on the genetics of

impaired angiogenesis during development should add to our understanding of the predisposition to hypertension.

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Angiotensin II induces angiogenesis in the hypoxic adult mouse heart in vitro through an AT_2–B2 receptor pathway

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Abstract

Angiotensin II is a vasoactive peptide that may affect vascularization of the ischemic heart via angiogenesis. In this study we aimed at studying the mechanisms underlying the angiogenic effects of AngII under hypoxia in the mouse heart *in vitro*.

Endothelial sprout formation from pieces of mouse hearts was assessed under normoxia (21% O_2) and hypoxia (1% O_2) during a 7-day period of *in vitro* culture. Only under hypoxia, angiotensin II dose-dependently induced endothelial sprout formation, peaking at 10^{-7} mol/L of angiotensin II. AT_1 receptor blockade by losartan did not affect angiotensin II-induced sprouting in wild-type mice. Conversely, the AT_2 receptor antagonist PD 123319 blocked this response. In hearts from $AT_1^{-/-}$ mice, angiotensin II-elicited sprouting was preserved but blocked again by AT_2 receptor antagonism. In contrast, no angiotensin II-induced sprouting was found in preparations from hearts of $AT_2^{-/-}$ mice. Angiotensin II-mediated angiogenesis was also abolished by a specific inhibitor of the B2 kinin receptor in both wild type and $AT_1^{-/-}$ mice. Furthermore, angiotensin II failed to induce endothelial sprout formation in hearts from $B2^{-/-}$ mice. Finally, nitric oxide inhibition completely blunted sprouting in hearts from wild type mice, while nitric oxide-donors could restore sprouting in $AT_2^{-/-}$ and $B2^{-/-}$ hearts. This *in vitro* study suggests the obligatory role of hypoxia in the angiogenic effect of angiotensin II in the mouse heart via the AT_2 receptor through a mechanism that involves bradykinin, its B2 receptor and nitric oxide as a downstream effector.

Introduction

Ischemic heart disease and left ventricular hypertrophy are characterized by impaired cardiac function caused, amongst others, by inadequate blood supply to the myocardium. In order to relieve this condition, blood flow to the myocardium needs to be restored by remodeling of pre-existing unused collateral blood vessels (arteriogenesis) and by the growth of new microvessels (angiogenesis). This process may also prevent the death and promote regeneration of damaged myocardial tissue.

Angiogenic stimuli are generated by hypoxia through activation of endothelial cell signalling[1] and gene transcription of key angiogenic molecules such as Vascular Endothelial Growth Factor (VEGF)[2]. In mice, activation of pre-existing collateral vascularization that restores blood flow to the acutely ischemic heart was shown to be induced by Angiotensin II (Ang II)[3], a key regulator of blood pressure and the main effector of the renin angiotensin aldosterone system (RAAS)[4]. During ischemia or

cancer, Ang II was shown to induce angiogenesis[5]. Two major subtypes of Ang II receptors are expressed in the myocardium[6], AT_1 and AT_2 receptors[7,8]. Most of the Ang II cardiovascular effects, e.g. vasoconstriction, are attributed to AT_1[9]. AT_1 is an ubiquitous receptor which presents two subtypes in rodents of a high homology (AT_1a and AT_1b)[10]. On the other hand, AT_2 receptor is highly expressed early in development and at lower levels in the adult[9]. Interestingly, AT_2 receptor is upregulated in response to ischemia and inflammation suggesting a potential role in myocardial angiogenesis[11]. Previous studies have shown that the AT_2 receptor may interact with the bradykinin receptor, the B2 kinin receptor (B2) during signalling[12].

In the present study we have investigated the mechanism of angiogenesis in response to Ang II in an *in vitro* model of sprout formation in the mouse heart under conditions of normoxia (21% O_2) and severe hypoxia (1% O_2) by dissecting the role of AT receptor subtypes and identifying the downstream effectors.

Results and Figures

Ang II induces vascular sprouting of the adult mouse heart under hypoxia.

We analyzed the effect of Ang II in an *in vitro* model of angiogenesis of the heart[14] both under normoxia (21% O_2) and hypoxia (1% O_2). Under normoxia neither Ang II, nor the angiogenic growth factor VEGF[16] that was used as positive control elicited an angiogenic response (Figure 1A and 1B) in the mouse heart. However, under hypoxia Ang II, bradykinin and VEGF elicited a significant angiogenic response (Figure 1A and 1B) of a similar magnitude (2.2-, 1.9- and 2.4-fold increase respectively, compared to negative control $p<0.05$). Staining with fluorescently-labelled antibodies (Figure 1C) revealed that Ang II induced sprouts typically consist of endothelial cells aligned with smooth muscle cells/pericytes.

Since hypoxia was confirmed to be a prerequisite for *in vitro* angiogenesis of the adult mouse heart (see also[14]) all following experiments were performed in hypoxia.

Ang II induces dose-dependent sprouting through the AT_2 receptor.

Stimulation of heart explants with a wide concentrations range of Ang II (10^{-10}mol/L to 10^{-6}mol/L) showed that endothelial sprouting induced by Ang II was dose dependent over at least a 1000-fold range of concentrations, and was maximal at 10^{-7}mol/L (2.2 ± 0.3, n=5, $p<0.05$) (Figure 2).

Next, we evaluated the contribution of AT_1 and AT_2 receptors in Ang II-mediated sprout formation. The selective AT_2 agonist CGP-42112 induced an angiogenic response similar to that observed in Ang II-stimulated hearts (2.0 fold increase with 10^{-7}mol/L CGP-42112, $p<0.05$ versus control) (Figure 2). AT_1 and AT_2 receptor inhibitors corroborated these results (Figure 3): Losartan, a specific AT_1 inhibitor, did not affect Ang II-induced sprout formation; PD 123319, a selective AT_2 antagonist, significantly reduced Ang II-induced sprout formation to control levels ($p<0.05$). The combination of both antagonists elicited a response very similar to that seen with PD 123319 alone ($p<0.05$). CGP-42112-induced sprout formation was inhibited by PD123319 but not by losartan (data not shown). Taken together, these results suggest that the AT_2 receptor subtype mediates the angiogenic effect induced by Ang II in the mouse heart under hypoxia.

Ang II does not induce sprouting in $AT_2^{-/-}$ animals.

To confirm these latter findings we examined hearts from $AT_1a^{-/-}$ and $AT_2^{-/-}$ mice. Ang II could not induce sprouting above control levels in heart explants from adult $AT_2^{-/-}$ mice under hypoxia (Figure 4), either alone or after blocking the AT_1 receptor with losartan. However, VEGF induced a significant level of sprout formation compared to controls (2.6 fold increase, $p<0.05$) suggesting that VEGF-induced angiogenesis *in vitro* is independent of AT_2 signalling. On the other hand, Ang II induced sprout formation in heart explants from $AT_1a^{-/-}$ mice as efficiently as in wild type hearts (1.9 fold increase, $p<0.05$) (Figure 4). In these mice, Ang II also elicited sprouting in the presence of losartan, which inhibits both AT_1a and AT_1b receptors, excluding the possibility that the observed angiogenic effect could be mediated by the AT_1b receptor still present in the $AT_1a^{-/-}$ mice. On the other hand, PD 123319 completely inhibited sprout formation ($p<0.05$) in the $AT_1^{-/-}$ heart explants. These results clearly demonstrate the exclusive role of the AT_2 receptor in Ang II-mediated angiogenesis in adult hypoxic mouse heart explants.

AT_1 and AT_2 receptor expression under hypoxia.

To exclude the possibility that AT_2-dependent Ang II-induced sprout formation could be due to the down-regulation of AT_1 receptor in hypoxia, we determined AT_1 and AT_2 receptor protein and mRNA expression in wild type mouse heart explants. As shown in

figure 5, both AT_1 and AT_2 were expressed confirming that both pathways are available for signalling.

Ang II induces sprouting via an AT_2 –B2 receptor pathway.

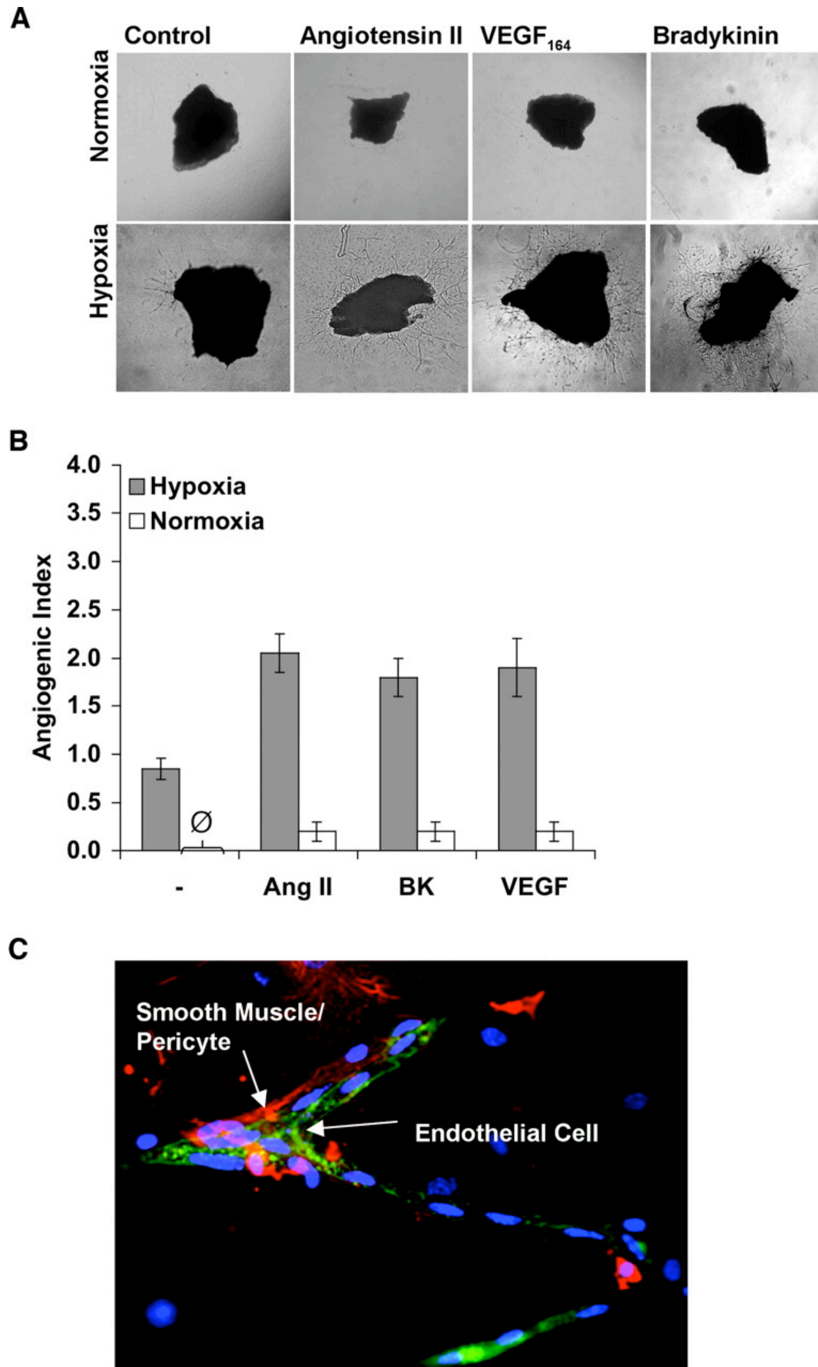
To analyze the role of the B2 receptor, we stimulated hypoxic mouse heart explants with Ang II in both wild type and $AT_1^{-/-}$ animals in the presence of HOE 140, a selective B2 antagonist (Figure 6A). We found an Ang II-induced angiogenic response (wt: 3.5 fold increase, $AT_1^{-/-}$: 3.3 fold increase versus control, p<0.05) that was completely abolished by HOE 140 (p<0.05). Bradykinin *per se* (10^{-7} mol/L) induced sprout formation both in wild type and $AT_2^{-/-}$ mouse heart (wt: 1.44 fold increase, $AT_2^{-/-}$: 1.5 fold increase versus control, p<0.001). To confirm that Ang II-induced angiogenesis requires the B2 receptor, heart explants from $B2^{-/-}$ mice were assessed. Neither Ang II nor VEGF induced significant sprouting in $B2^{-/-}$ mice (Figure 6B). To clarify whether accumulation of bradykinin was the intermediate step in Ang II-induced sprouting, we treated the heart explants with a specific kininogenase inhibitor, PKSI-527 (10^{-5} mol/L) that blocks the conversion of kinins into bradykinin. PKSI-527 completely inhibited Ang II-induced angiogenesis in wild type mouse heart (Figure 6C). Therefore, we conclude that Ang II is angiogenic in the mouse heart under hypoxia via a pathway involving both the AT_2 and the B2 receptors linked by activation of bradykinin production.

Ang II-induced sprouting requires NO release.

Since stimulation of the AT_2 receptor is associated with increased generation of bradykinin[17], NO and cyclic GMP (cGMP)[18] , we tested whether the angiogenic effects of Ang II may also require NO. As expected, Ang II (10^{-7}mol/L) and bradykinin (10^{-7}mol/L) significantly increased NO production as measured by nitrite accumulation in the medium after 7 days of incubation (in 10^{-9}xmol/L, control: 90±5; bradykinin: 121±5; Ang II: 114±15, n=3, p<0.05, ANOVA). We then inhibited NO generation using NO synthase inhibitors, i.e., SMT, L-NIO, L-NAME and 1400W. Ang II- and CGP-42112-induced angiogenesis were completely blunted by NO inhibition (Figure 7A). Heart explants derived from wild type, $AT_2^{-/-}$ and $BK2^{-/-}$ mice were then incubated with two different NO donors, GSNO (S-nitrosoglutathione,10^{-5} mol/L) and PAPA NONOate (NONO-ate 10^{-5}mol/L) (figure 7B). Both NO donors induced angiogenesis (GSNO; wt: 1.7 fold increase, $AT_2^{-/-}$ 1.7 fold increase, $BK2^{-/-}$ 1.6 fold increase versus control, p<0.05). These results demonstrate that NO is a key mediator of angiogenesis in the hypoxic mouse heart and

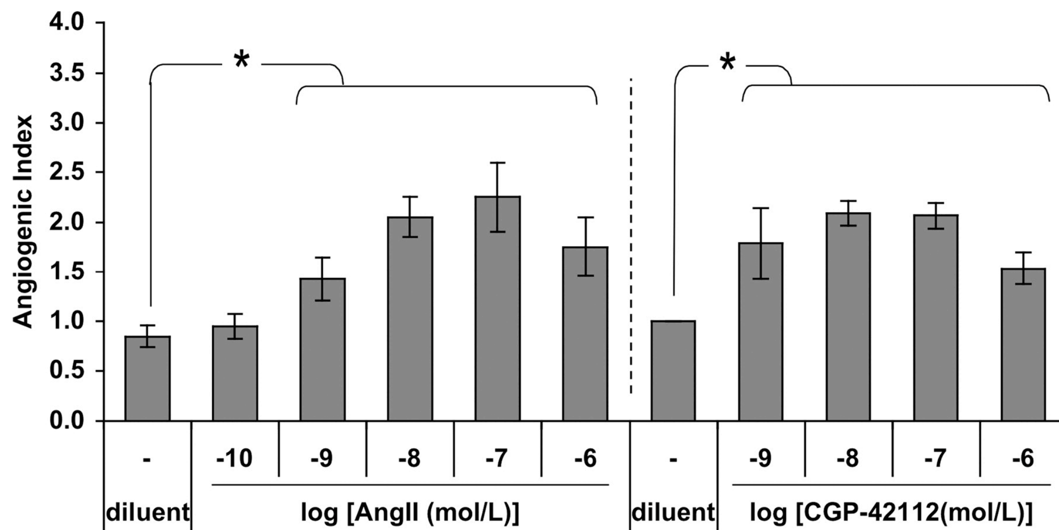
is a required downstream effector of Ang II-induced sprout formation.

Figure 1



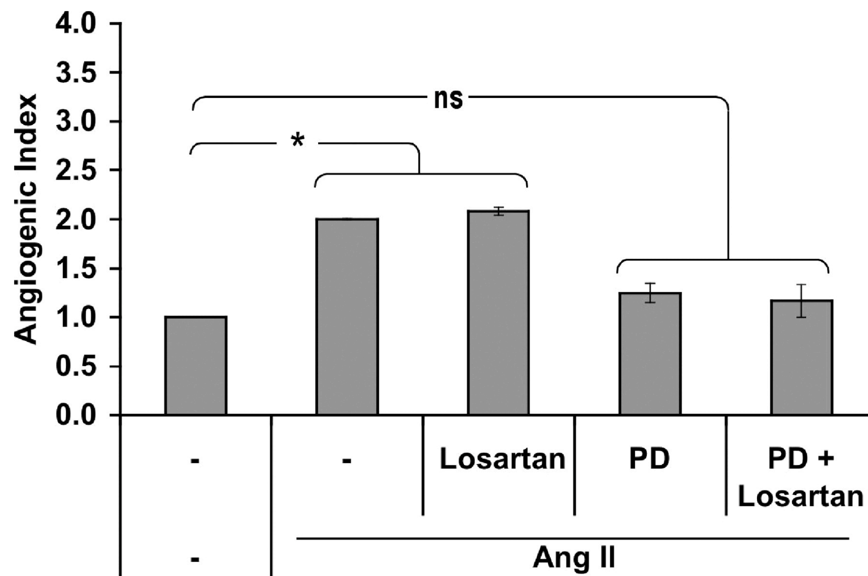
Ang II induces endothelial sprouts in a heart angiogenesis assay in vitro.
A. Mouse hearts stimulated with 10^{-7} mol/L Ang II, 10^{-7} mol/L bradykinin, 10 ng/mL $VEGF^{164}$ or diluent control after 7 days in culture under 21% O_2 (normoxia) or 1% O_2 (hypoxia). B. Ang II (10^{-7} mol/L), bradykinin (10^{-7} mol/L) or $VEGF^{164}$ (10 ng/mL) increased sprouting only under hypoxia. A standardized scale indicates the degree of cellular outgrowth (angiogenic index). Data points represent the mean of 5 independent experiments ± SEM. C. Fixed Ang II-induced sprout stained for endothelial cells by fluorescein conjugated GSL-IB_4 (green), for pericytes or smooth muscle cells by Cy3 conjugated αSMA (red), and for nuclei by Hoechst-dye (blue).

Figure 2



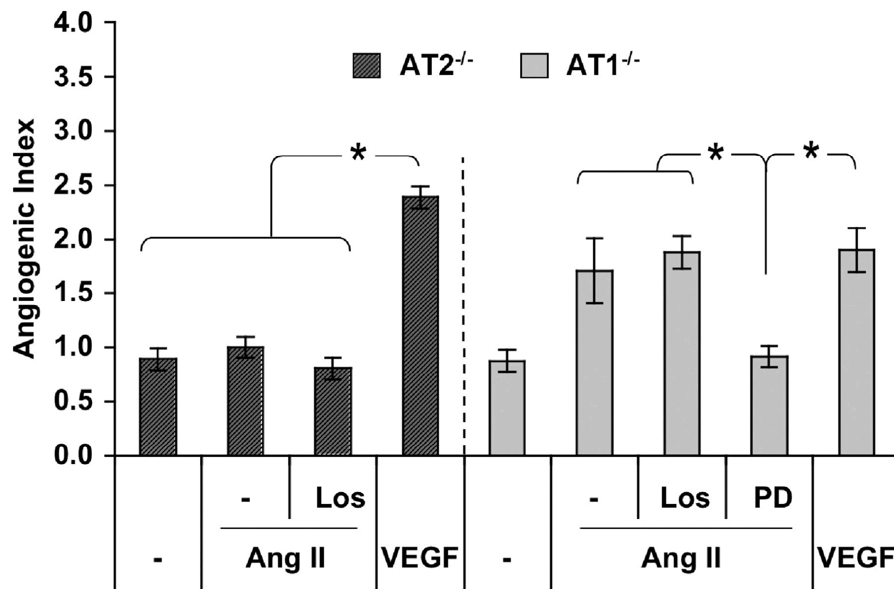
Ang II induces dose-dependent sprouting in vitro in adult mouse hearts in hypoxia. Pieces of mouse heart were stimulated with Ang II from 10^{-10} to 10^{-6} mol/L (left) or AT_2 agonist CGP-42112 from 10^{-9} to 10^{-6} mol/L (right) and incubated under hypoxia for 7 days. Data points represent the mean of 5 independent experiments ± SEM.*$p<0.05$ vs negative control.

Figure 3



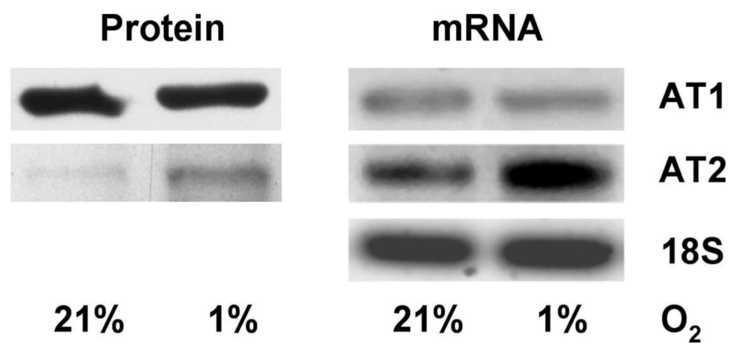
AT_1 is not required for Ang II induced sprouting. Pieces of mouse heart were incubated with Ang II (10^{-7} mol/L) alone or in combination with Losartan (10^{-6} mol/L) and/or PD 123319 (10^{-6}mol/L) under hypoxia for 7 days. The AT_2 inhibitor (PD123319) but not the AT_1 blocker Losartan significantly decreases Ang II-mediated sprout formation. Data points represent the mean of 5 independent experiments ± SEM. *$p<0.05$ vs negative control.

Figure 4



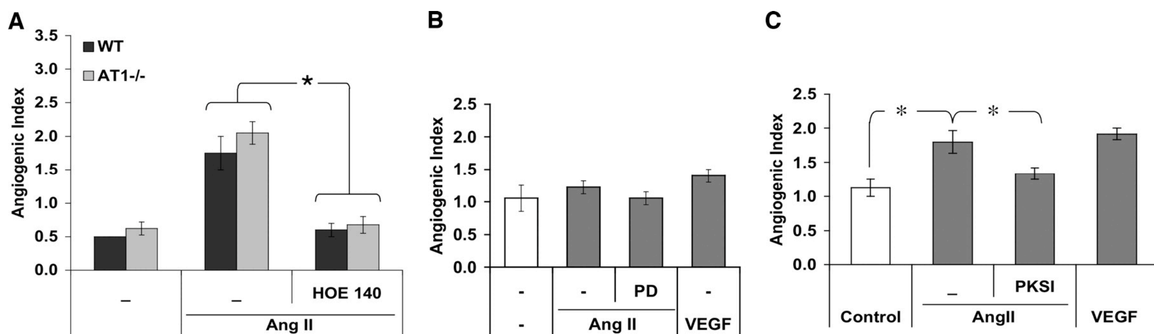
Ang II-induced angiogenesis is impaired in hearts from $AT_2^{-/-}$ but not from $AT_1^{-/-}$ mouse. Pieces of mouse heart from $AT_2^{-/-}$ (left side of the figure) and $AT_1^{-/-}$ mice (right side of the figure) were stimulated with Ang II (10^{-7} mol/L) alone or in combination with Losartan (10^{-6} mol/L) or PD 123319 (10^{-6} mol/L) and incubated under hypoxia for 7 days. $VEGF^{164}$ (10 ng/mL) was used as a positive control. Data points represent the mean of 5 independent experiments ± SEM. *$p<0.05$ vs VEGF (left side) and *$p<0.05$ vs negative control (right side).

Figure 5



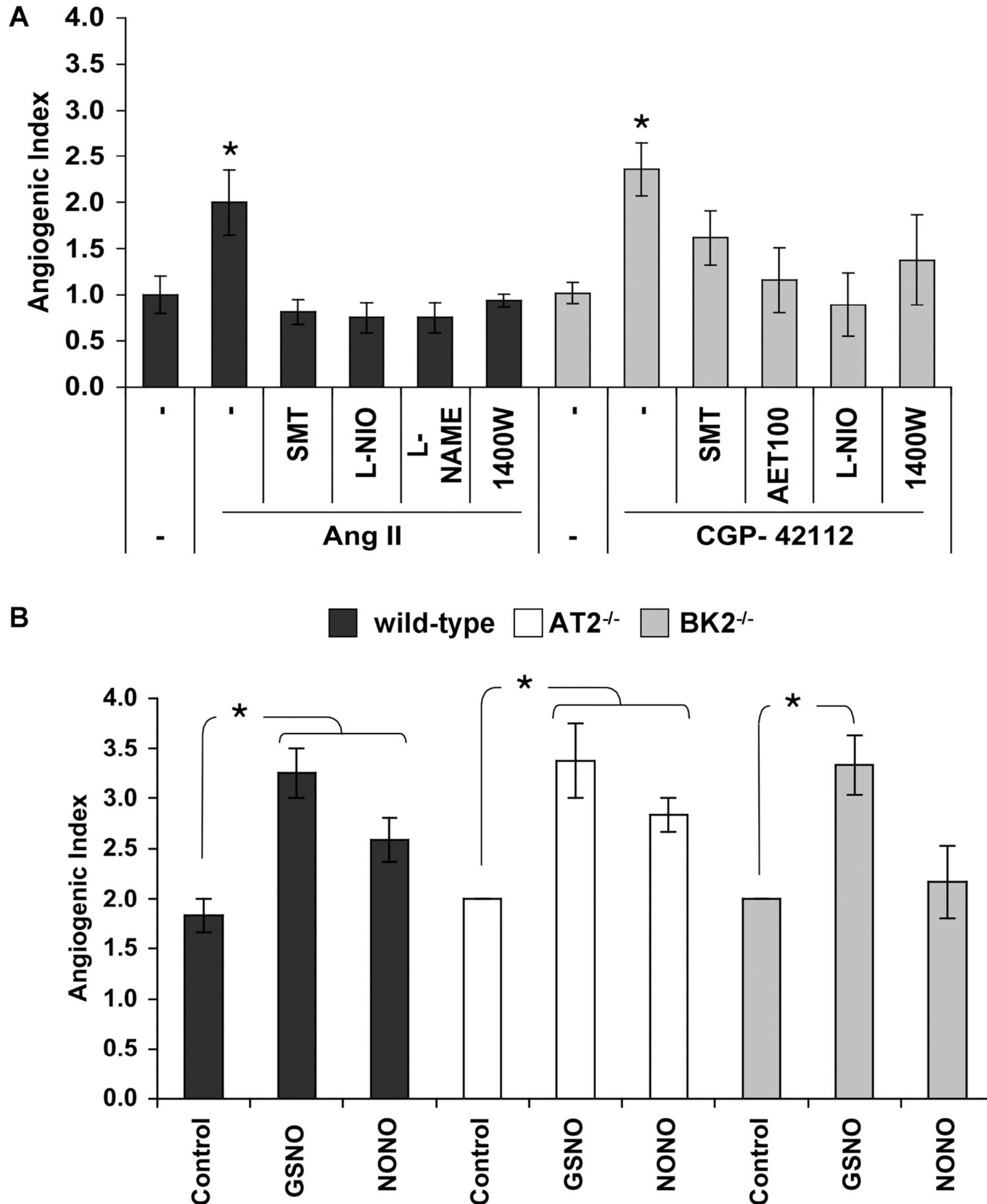
AT_1 and AT_2 mRNA and protein expression of the mouse heart in vitro under hypoxia. Pieces of heart from wild type mice were incubated under 21%O_2 or 1%O_2 during 24 hours, lysed and protein and mRNA extracted. Westernblotting and RT-PCR analysis show that both the AT_1 and the AT_2 receptor are expressed under normoxia and hypoxia.

Figure 6



Ang II-induced sprouting in wild type and AT_1 KO mice requires the B2 receptor. A. Pieces of heart from wild type or $AT_1^{-/-}$ mice stimulated with Ang II (10^{-7} mol/L) alone or in combination with the specific B2 receptor antagonist, HOE 140 (10^{-7} mol/L) and incubated under hypoxia during 7 days. HOE140 significantly (*$p<0.05$ vs negative control) decreased Ang II-induced sprout formation in wild type- and $AT_1^{-/-}$ mice compared to Ang II-treated heart pieces without the inhibitor. B. Pieces of hearts from $B2^{-/-}$ mice stimulated with Ang II (10^{-7} mol/L) alone or in combination with PD123319 (10^{-6} mol/L) were incubated in culture medium containing 5% FCS under hypoxia for 7 days. $VEGF^{164}$ (10 ng/mL) was used as a positive control. *p=ns. Data points represent the mean of 5 independent experiments ± SEM. C. Pieces of heart from wild type mice were stimulated with Ang II (10^{-7} mol/L) alone or in combination with kininogenase specific inhibitor PKSI-527 (10^{-5} mol/L) were incubated in culture medium containing 5% FCS under hypoxia for 7 days. $VEGF^{164}$ (10 ng/mL) was used as a positive control. Data points represent the mean of 3 independent experiments ± SEM. *$p<0.05$ vs control.

Figure 7



NO mediates Ang II-induced sprouting under hypoxia. A. Pieces of heart from wild type mice stimulated with Ang II (10^{-7} mol/L) (left side) or with the AT_2 agonist (CGP-42112, 10^{-7} mol/L) (right side) alone or in the presence of different NO synthase inhibitors (10^{-7} mol/L) and incubated in culture medium containing 5% FCS under hypoxia for 7 days. *p<0.05 vs control. Data points represent the mean of 5 independent experiments ± SEM. B. Pieces of heart from wild type, $AT_2^{-/-}$ and $B2^{-/-}$ mice were stimulated with two different NO donors: GSNO (10^{-5} mol/L) and PAPA NONOate (10^{-5} mol/L) and incubated in culture medium containing 5% FCS under hypoxia for 7 days. *p<0.05 vs control. Data points represent the mean of 5 independent experiments ± SEM.

Discussion

Here we show that Ang II induces angiogenesis in the adult mouse heart specifically under hypoxia, signalling through the AT_2 but not the AT_1 receptor. The mechanism requires generation of bradykinin with activation of the B2 receptor, and leads to NO biosynthesis as the downstream effector.

The RAAS is an important system in regulating vascular homeostasis. However, the precise role of the RAAS and the AT_1/AT_2 receptor pathway in angiogenesis is unclear. Clinical data has shown that blocking the AT_1 receptor preserves cardiac function after myocardial infarction[19]. Our results showing that Ang II-induced angiogenesis in the mouse heart under hypoxia is mediated exclusively by the AT_2 receptor may explain some beneficial effects of AT_1 blockade treatment in the heart. In fact, AT_1 blockade may unmask beneficial properties due to preferential AT_2 stimulation.

The role of the AT_1 and AT_2 receptor in angiogenesis is controversial. Ang II-induced angiogenesis was shown to be mediated via both the AT_1 and the AT_2 receptor in the mesenteric vasculature of Ang II-infused rats[20] or specifically via the AT_2 receptor in tumor angiogenesis in mice[21]. High AT_1 expression was associated with reduced myocardial vessel density in rats[22]. In contrast, others have shown AT_1-dependent angiogenesis in the ischemic hindlimb of mice[23] whereas AT_2 appeared to be antiangiogenic in the same animal model[24]. Tumor angiogenesis was impaired in $AT_1^{-/-}$ receptor mice[25]. Thus, the role of AT_1 and AT_2 receptors in angiogenesis is not clear and may vary upon model, tissue and conditions investigated. In particular, the vasculature of the heart has not been investigated in models of controlled hypoxia. Our model of angiogenesis in vitro of the mouse heart provides this possibility and demonstrates the key role of hypoxia in Ang II-induced cardiac angiogenesis.

Hypoxia can lead to the formation of new vessels in mature tissue, triggering vessel growth by signalling through hypoxia-inducible transcription factor (HIF-1)[26]. Interestingly, Ang II induces HIF-1α[27-29]. Hypoxia may also modulate the expression of AT_1 and/or AT_2 receptors[30]. In our experiments both AT_1 and AT_2 receptors were present under normoxia and hypoxia. Still, further studies investigating other tissues, receptor expression and intracellular signalling pathways may reveal whether AT_2-dependent angiogenesis is specific for the hypoxic heart.

The AT_2 receptor might exert downstream effects via the B2 receptor[12]. We clearly show that Ang II-induced angiogenesis is abrogated when the B2 receptor is pharmacologically inhibited or knocked out. B2 activation by bradykinin induces

vasodilation[31] which is also a prerequisite for initiation of angiogenesis[32]. AT_2 overexpressing mice blocked Ang II-induced vasopressor effects through B2 receptor[33]. Importantly, bradykinin was shown to induce angiogenesis via B2 receptor[34] or as shown by using a model of hindlimb-induced ischemia in $B2^{-/-}$ mice[35]. Collectively, these data suggest a mechanism by which a vasopressor molecule, such as Ang II, can also mediate vasodilator and angiogenic effects specifically by AT_2 receptor-dependent signalling leading to B2 kinin receptor activation[36]. Ang II-dependent activation of B2 could be achieved in different ways. A direct interaction between AT_2 and B2 leading to nitric oxide production has recently described[37] although the precise nature of this interaction has not been fully clarified. Others have pointed out an Ang II-mediated pH increase that may release kininogens to produce bradykinin[33,38]. In our study, the angiogenic effect of B2 receptor was dependent on bradykinin synthesis since kininogenase inhibition blocked Ang II-induced angiogenesis. Bradykinin induced angiogenesis in hypoxic heart explants only from both wild type and $AT2^{-/-}$ mice. Ang II, however, as mentioned before, failed to induce angiogenesis in hearts from $BK2^{-/-}$ mice. We conclude that angiogenesis induced by Ang II requires signalling through the AT_2 receptor and is mediated by an increase in bradykinin production.

Endothelium derived NO synthase is crucial for angiogenesis in vitro and in vivo[39]. In fact, NO inhibition blocked Ang II-induced endothelial sprout formation in our model of angiogenesis of the heart in vitro. Increased nitrite accumulation in the medium of Ang II-stimulated heart explants was also observed. Accordingly, NO-donors directly induced angiogenesis in pieces of heart either from wild type, $B2^{-/-}$ and $AT_2^{-/-}$ mice. Our results are in agreement with previous reports, showing that Ang II can induce renal production of bradykinin, NO and cGMP via the AT_2 receptor[40]. These data suggest that an increase in NO bioavailability downstream of AT_2 and B2 receptors is the final effector of Ang II-induced angiogenesis in the hypoxic heart.

Perspectives

The present study provides evidence for the significant role of the AT_2/B2 pathway in the Ang II-induced angiogenesis in vitro in the adult mouse heart under hypoxia. In clinical studies, AT_1 blocker treatment of hypertension has revealed additional cardioprotective effects beyond the lowering blood pressure[41,42]. A potential advantage of AT_1 blockers over ACE inhibition is the preservation of the AT_2-mediated pathway. Here we describe that Ang II-induced angiogenic effects through AT_2/B2 may provide some explanation for

these beneficial effects. Studies on neovascularization of the heart in hypertensive animals and patients after AT_1 treatment are needed to test the clinical relevance of our mechanisitic results. This may help to understand and to uncover novel therapeutic effects of AT_1 receptor blockers for patients with left ventricular hypertrophy, ischemic heart disease or myocardial infarction.

Methods

Animals

Experiments were performed with hearts of C57Bl/6: wild type-, $AT_1a^{-/-}$ - (The Jackson Laboratory, Maine USA) and $B2^{-/-}$ mice (Jackson Laboratories). FVB/J $AT_2^{-/-}$ mice were a gift from Prof. Hein and have been previously described[13]. The animals were euthanized and the hearts immediately transferred to PBS. Within half an hour post mortem, small pieces ($1mm^3$) of the mouse myocardium (left ventricle) were cut and embedded in fibrin gel. All experiments were conducted in accordance with the Swiss Federal Act on Animal Protection (1998) and were approved by the Veterinary Department of the Kanton of Basel (Switzerland). We used between 5 and 9 mice for every experiment. The age of mice ranged from 12 to 14 weeks.

Angiogenesis *in vitro* assay

A three-dimensional *in vitro* assay of heart angiogenesis was established in our laboratory as described in detail previously[14]. Briefly, 0.5 - $1mm^3$ cubes from the left ventricular myocardium of the mouse heart were placed onto fibrin-gels (Sigma-Aldrich) with 500 µl DMEM plus 5%FCS (Biochrom). Heart explants were incubated under normoxia (21% O_2) or hypoxia (1% O_2) for 7 days. Stimulants/inhibitors were added every other day: hr $VEGF^{164}$ (R&D systems); HOE140 (Sigma-Aldrich AG); Ang II Acetate (Sigma-Aldrich); Losartan (MSD), CGP-42112 (Bachem), PD123319 (Fluka) and PKSI-527 (Wako Chemicals); Nitric oxide (NO) inhibitors and donors (Sigma-Aldrich).

Inhibitors were added freshly 20 minutes before stimulants. After 7 days endothelial sprouts were photographed digitally (ColorView II-Soft Imaging System) on an inverted light microscope (Olympus IX50). The extent of sprout formation was determined as previously detailed[13]. Briefly, we used octuplicates for each condition and sprout formation was calculated and averaged by two independent investigators by comparison with a standardized scale (angiogenic index). The angiogenic index was defined with the help of an image analysis software (AnalySIS Pro, Soft Imaging System) as [sprouting

area/total area]X10 where total area corresponds to the sprouting area plus tissue area. Sprouting was computed from the area that was actually occupied by endothelial sprouts and not the space between the cells. Sprouting and tissue area were computed by AnalySIS Pro and the angiogenic index was rounded to the nearest integer and handled as a scored value.

Characterization of cells & tissue

Characterization of outgrowing cells and sprouts was performed by using specific cell markers GSL I – IB_4 (20 μg/ml; Rectolab) for endothelium and Cy3-conjugated anti-α-smooth muscle actin (1:100; SMA; Fluka Chemie) for smooth muscle cells/pericytes and Hoechst dye (Polysciences Europe) for visualization of cell nuclei as described previously[14].

NO production assay

NO concentrations were measured by the fluorometric nitrite assay[15] with the NO Assay Kit (Calbiochem). Briefly, pieces of mouse heart were incubated in phenol-free DMEM. The supernatants were collected, nitrite was detected by fluorescence and concentration (nanomoles/L) calculated according to a calibration curve in each experiment.

Western blotting

Heart tissue was lysed in RIPA buffer as described before[1]. After SDS-PAGE, proteins were transferred onto Polyvinylidene fluoride membrane (Millipore). The membrane was blocked with 4% skim milk powder in TBS-Tween solution and probed with polyclonal anti-AT_1(N-10) and anti-AT_2 (C-18) from Santa Cruz Biotechnology. HRP-conjugated IgGs from Cell Signaling Technology were used to visualize the proteins by a chemiluminescence reaction (Amersham).

Reverse transcription polymerase chain reaction (PCR)

Total RNA was isolated with TRIzol Reagent (Invitrogen), quantified and reverse-transcribed with M-MLV reverse transcriptase system (Promega).

The cDNA (1μl) was amplified in 35 cycles polymerase chain reactions. The following primer sequences were used; for mouse AT_1 receptor sense: 5′-TGAGAACACCAATATCAC TG-3′, and antisense: 5′-TTCGTAGACAGGCTTGAG-3′; mouse AT_2 receptor sense: 5′-CCTTGGCTGACTTACTCCTT-3′, and antisense 5′-GAACTACATAAGATGCTTGCC-3′; mouse 18S ribosomal RNA sense: 5′-

CCTGGATACCGCAGCTAGGA-3′, and antisense 5′-GCGGCGCAATACGAATGCCCC-3′. Specific PCR annealing temperatures were: $49°C_{AT1}$, $52°C_{AT2}$ and $57°C_{18S}$.

Statistical analysis

All results depicted represent experiments repeated using at least five different heart explants. Each single condition was performed in octuplicate wells. Data points represent the mean ± SEM. Statistical analysis was performed with SPSS for Mac OS X (SPSS Inc.). Statistical significance ($p < 0.05$) was computed using non-parametric analysis; Kruskal-Wallis and Mann-Whitney tests were performed accordingly.

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Disclosures

None

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5. General discussion

Hypoxia is a direct cause of a lack of adequate microvessel supply, but appears to be at the same time an essential stimulus to induce the formation of new microvessels by induction of angiogenesis. How is hypoxia sensed by endothelial cells, what are the signalling pathways that transduce the hypoxia signal, and what are the effector molecules that may finally lead to increased endothelial proliferation and angiogenesis? In this thesis, I have investigated these questions in three related experimental projects and discuss the interrelated and common findings below.
Detailed discussions of the individual projects are located in chapter 4.

EC proliferation is an important step in the process of angiogenesis since it provides the building blocks for nascent microvessels. We have shown previously that ECs increase proliferation in response to hypoxia. The increased proliferation was rapamycin sensitive and or could be further increased by mTOR overexpression[10]. In the study ***"Hypoxia-Induced Endothelial Proliferation Requires Both mTORC1 and mTORC2"*** we scrutinized the mechanism of EC activation under hypoxia. By analyzing mTOR and the distinct downstream targets of mTORC1 (S6 kinase 1) and mTORC2 (PKB/AKT), we found that hypoxia activates mTOR signalling O_2-concentration dependently and in a timed program, leading to an early activation and a late inhibition of mTORC1 and a delayed but sustained activation of mTORC2. Raptor and rictor knock down by siRANA demonstrated that rictor (mTORC2) is essential for hypoxia-induced endothelial proliferation, whereas raptor knock down only partially inhibited hypoxia-increased proliferation. By studying the pathways potentially directing the hypoxic stimulus to mTOR, we found that hypoxia-induced cell proliferation is independent of regulation by TSC. TSC is an upstream negative regulator of mTOR and directs signals triggered by growth factors, energy and nutrients. Thus, mTOR is a central and maybe directly activated regulation point to integrate signals from hypoxia to induce EC proliferation under hypoxia.

HIF-1α is stabilized under hypoxia and activates many genes involved in the process of angiogenesis. HIF-1α degradation is initiated by prolyl hydroxlases (PHD). PHD activity is oxygen-dependet and tags HIF-1α for proteasomal degradation only under normoxia[5,69]. In this study with the working title ***"Role of mTORC1 and mTORC2 in***

hypoxia-induced HIF-1α stabilization and endothelial proliferation" we have studied signalling pathways that are known to be induced by stress responses for their role in HIF-1α stabilization. We could exclude MEK1/2, Jun kinase and p38 for being responsible to modulate HIF-1α stability under hypoxia. We identified mTOR as an essential factor for HIF-1α stabilization under hypoxia. Interestingly, HIF-1α has been shown to carry a potential phosphorylation site for mTOR-kinase and HIF-1α stabilization under hypoxia was shown to be rapamycin sensitive[74]. Thus, HIF-1α stabilization under hypoxia is not only negatively regulated by PHD but also positively by mTOR. PDGF-BB induced HIF-1α stabilization under normoxia was rapamycin sensitive as well, suggesting that mTOR signalling is also essential for growth factor-induced HIF-1α stabilization under normoxia. Thus, mTOR signalling activated by growth factors or hypoxia is required for HIF-1α stabilization. We hypothesize that multiple hypoxia-activated pathways for HIF-1α regulation could integrate different regulatory signals for specific conditions. This might ensure, that HIF-1α is only activated if all criteria for cell growth are fulfilled such as available energy or amino acids.

Knock down of HIF-1α in RAECs inhibited proliferation under hypoxia only partially. This may be due to an incomplete knock down of HIF-1α. However, MEFs completely lacking the HIF-1α gene reduced proliferation under hypoxia to a similar degree, thus, not completely. We speculate, that HIF-1α is not the only mTOR effector responsible for increased proliferation of RAECs and MEFs under hypoxia.

mTORC1 is rapamycin-sensitive, and rapamycin destabilizes HIF-1α under hypoxia. Thus, is mTORC1 the pivotal mTOR complex for hypoxia- or growth factor mediated HIF-1α stabilization? In endothelial cells, prolonged (~24 hr) treatment of rapamycin can also prevent formation of mTORC2. We have also shown that both mTORCs are required to promote proliferation of RAECs under hypoxia. Therefore the answer, which of the two mTOR complexes is responsible for HIF-α accumulation is not clear yet. RNAi-mediated knockdown of raptor and rictor in endothelial cells and Cre-inducible knockout of raptor and rictor in MEFs are currently carried out in our lab, and HIF-1α detection in those cells will potentially reveal the responsible mTOR complex.

In our study ***"Angiotensin II induces angiogenesis in the hypoxic adult mouse heart in vitro through an AT2-B2 receptor pathway"*** we studied the angiogenic role of Angiotensin II in an in vitro model of angiogenesis of the heart. Ischemia, thus a hypoxic

environment with a lack of nutrients and excess of metabolites, often occurs in cardiovascular complications such as in angina pectoris, left ventricular hypertrophy or after myocardial infarct.

We realized that Angiotensin II, Bradykinin or even the classic angiogenic molecule VEGF could not induce angiogenesis from hearts cultured under normoxia. Incubation in hypoxia was necessary to induce angiogenesis that could be increased with the addition of VEGF, bradykinin or Ang II. This result highlights the role of hypoxia as a pro-angiogenic condition in the heart. Further, by using pharmacological agonists and antagonists of AT_1, AT_2 and BK2 receptor signalling and heart tissue from corresponding knockout mice, we determined that Ang II induces angiogenesis in vitro via the AT_2 receptor. Moreover AT_2 induces angiogenesis via Bradykinin release and activation of the BK2 receptor. Ang II induced angiogenesis was also nitric oxide dependent. Bradykinin induces NO and VEGF, both essential factors to promote angiogenesis[2]. Ang II also was shown to induce HIF-1α[57,59] and HIF-1α controls the expression of iNOS (inducible nitric oxide synthase; produces NO) and VEGF[55,129]. Thus Ang II induced angiogenesis might also be partially mediated via HIF-1.

In conclusion, these studies help us to understand the concepts, mechanisms and interactions in hypoxia-mediated or dependent-responses that ultimately lead to endothelial proliferation and formation of new microvessels.

6. Outlook

Follow up on project "Hypoxia-Induced Endothelial Proliferation Requires Both mTORC1 and mTORC2"

- To assess whether VEGF, Bradykinin, and Angiotensin II require mTORC1 or mTORC2 to induce angiogenesis in vitro.
- To assess whether FoxO transcription factors are downstream of mTORC2 and Akt and are required for angiogesis in vitro in response to hypoxia or angigenic molecules.
- To characterize the role of mTORC1 and mTORC2 in angiogenesis in vivo by induction of Cre-mediated excision of the loxed raptor and rictor gene in adult mouse endothelium. Angiogenesis and vascular morphology will be assessed after injection of myoblasts expressing distinct concentrations of VEGF in the mice ear.

Follow up on project "Role of mTORC1 and mTORC2 in hypoxia-induced HIF-1α stabilization and endothelial proliferation"

- To identify the mTOR complex responsible for HIF-1α stabilization by using raptor- and rictor knockout MEFs and raptor and rictor silenced RAECs.
- To identify the mTOR complex responsible for HIF-1α-mediated transcription by using Hypoxia-Response Element (HRE) containing Luciferase reporter plasmids in raptor- and rictor knockout MEFs and RNAi-mediated silencing of raptor and rictor in RAECs.
- To investigate role of HIF-2/3α in endothelial cells.

Follow up on project "Angiotensin II induces angiogenesis in the hypoxic adult mouse heart in vitro through an AT2-B2 receptor pathway"

- To investigate mechanisms of AT_2 and B2 receptor interaction.
- To investigate the dominant role of B2 receptor in AT_2, B1- and VEGF-induced angiogenesis.
- To analyse if mTOR (and HIF) is involved in Ang II and Bradykinin-induced angiogenesis (see also 6.1).

7. Abbreviations

ACE	–	Angiotensin Converting Enzyme
ACE-I	–	ACE Inhibitor
AMPK	–	AMP -Activated Protein Kinase
Ang I	–	Angiotensin I
Ang II	–	Angiotensin II
ARBs	–	AT_1 Receptor Blockers
ARNT	–	Aryl Hydrocarbon Nuclear Translocator
AT_1 Receptor	–	Angiotensin II Receptor 1
AT_2 Receptor	–	Angiotensin II Receptor 2
bHLH-PAS	–	Basic Loop Helix-Per ARNT Sim Proteins
B1	–	Kinin Receptor B1
B2	–	Kinin Receptor B2
BK Receptor	–	Bradykinin Receptor
C-TAD, N-TAD C	–	C and N –terminal Transactivation Domains
ECM	–	Extracellular Matrix
ECs	–	Endothelial Cells
EPAS1	–	Endothelial PAS 1
FGF	–	Fibroblast Growth factor
FIH	–	Factor Inhibiting HIF-1
GF	–	Growth Factors
HRE	–	Hypoxia Response Element
iNOS	–	Inducible Nitric Oxide Synthase
IPAS	–	Inhibitory PAS
MMPs	–	Matrix Metalloproteinases
mSIN1	–	Mammalian Stress-Activated Protein Kinase [SAPK]-Interacting Protein
mTOR	–	Mammalian Target of Rapamycin
ODDD	–	Oxygen Dependent Degradation Domain
PDGF	–	Platelet-Derived Growth Factor
PHD	–	Prolyl Hydroxylase
RAAS	–	Rennin-Angiotensin-Aldosterone-System
RAEC	–	Rat Aortic Endothelial Cells
Raptor	–	Regulatory Associated Protein of mTOR

Rictor	–	The Rapamycin-Insensitive Companion of mTOR
ROS	–	Reactive Oxygen Species
SMC	–	Smooth Muscle Cell
TORC	–	TOR Complex
TSC	–	Tuberous Sclerosis Complex
VEGF	–	Vascular Endothelial Growth Factor

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QUALIFICATIONS

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Languages:	German: Mother tongue English: Fluent in speaking and writing French: Good knowledge in speaking and writing
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EDUCATION

since 01.11.2003	Phd student, D*epartment of Research, University Hospital Basel*
2003	Graduation as dipl. Bio II, *Biozentrum, University of Basel*
1998 – 2003	Studies of BIO II (molecular biology) for nine semesters at the *Biozentrum of the University of Basel*
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WORK EXPERIENCE

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PUBLICATIONS

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PRESENTATIONS

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PERSONAL INTERESTS

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