Hypoxia-Induced Endothelial Proliferation Requires Both mTORC1 and mTORC2

Weimin Li,* Marco Petrimpol,* Klaus D. Molle, Michael N. Hall, Edouard J. Battegay, Rok Humar

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 (P<0.05; n≥4). Similarly, hypoxia induced Akt-dependent and rapamycin-sensitive proliferation in mouse embryonic fibroblasts. This response was partially blunted by hypoxia-inducible factor-1α knockdown and not affected by TSC2 knockout. Finally, mTORC2 inhibition by rictor silencing, especially (P<0.001; n=7), and mTORC1 inhibition by raptor silencing, partially (P<0.05; n=7), 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. (Circ Res. 2007;100:79-87.)

Key Words: hypoxia ▪ mTORC1 ▪ mTORC2 ▪ S6K1 ▪ Akt ▪ proliferation ▪ endothelial

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 proliferation2,3 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 fibroblasts20 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. Here we examine activities of mTOR under hypoxia in detail and translation of this signal into endothelial cell proliferation.

Materials and Methods

Rat aortic endothelial cells (RAECs) were prepared, cultured, and characterized as described previously. 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. Predesigned short interfering RNAs (siRNAs) against rat mTOR (frapi_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 cttgaactagcacttg 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. neo-based plasmid targeting ctgtgaactagcacttcag in rictor mRNA; and pKDM-162, a pSuper.gfp/neo-based siRNA-expressing 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 and as described elsewhere. 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. Ruegg (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.

Data (mean±SEM) were analyzed for normal distribution (1-way 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 P<0.05 was considered as significant.

Results

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. 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 β-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 pathophysiologic O2 concentration range.

An additional regulatory mechanism of mTOR signaling may occur via cytoplasmic/nuclear shuttling. 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. Serum-deprived 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 5S. mTORC2 phosphorylates Akt on the primary phosphorylation site Ser473. 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. Furthermore Akt and GSK3 are implicated in the regulation of cell cycle regulators Cyclin D1 and p21. 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
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) 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 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.
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 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% O₂ was 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. 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 proliferation

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**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<0.001; 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).
specifically 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 \( (P<0.05, n=4) \) decreased the proliferation response to hypoxia compared to transfection with control siRNA, whereas mTOR silencing had no significant \( (P>0.05, n=4) \) effects on proliferation under normoxia when compared with proliferation in endothelial cells that were transfected with control siRNA (Figure 2C).

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 \( (P<0.001, n=5) \). Also raptor silencing decreased hypoxia-induced proliferation significantly \( (P<0.05, n=3) \), 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 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.\(^{19,22-24}\) 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 \( (P>0.05, n=3) \) 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-1α-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 \( (P>0.05, n=5) \), 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 knock-out MEFs. These results suggest that HIF-1α is a partial but not crucial effector of mTOR-dependent, hypoxia-induced proliferation in MEFs.

**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, ie, 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% O2. 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, Figure 4.
and rapamycin also decreased endothelial sprout formation in endothelial spheroids under hypoxia alone. Finally, rictor knockdown, 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 mTOR1 or mTORC2 to the same extent.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.39

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

Figure 5. Scheme representing summary of most important results.

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.

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MATERIALS & METHODS

Cells & culture conditions

Rat aortic endothelial cells (RAEC) were derived from adult male Sprague-Dawley rat (Charles River Laboratories, France) aortic endothelium and characterized as described previously 1. RAECs of passage 2 to 6 were seeded in 150 mm culture dishes at an initial density of 4.0 x 10^6 in DMEM complemented with 10% fetal calf serum, 1% sodium pyruvate, 1% non-essential amino acids, 1% penicillin-streptomycin. At 70% confluence, cells were washed twice in PBS and starved in serum-free DMEM for 30 h.

Tsc2 defective MEFs were obtained from Michael Hall (Biocenter, Basel, Switzerland), HIF-1α knockouts from Max Gassmann, Clinic for Small Animal Internal Medicine, University of Zürich, Switzerland. Following inhibitors were used: 5 -500 nM rapamycin for mTOR, 0.5 – 2 µM Akt IV inhibitor for Akt (Calbiochem).

Angiogenesis assay in vitro of endothelial spheroids

Endothelial cell spheroids of defined cell number were generated as described elsewhere 2. The spheroid containing fibrin bilayer 3 was overlaid with serum-free DMEM. Rapamycin (20 nM) was added to cultures prior to a 24 h incubation under 21% or 1% O2. Sprouts emerging from spheroids were photographed digitally on an inverted light microscope (Olympus).

si- and shRNA silencing and transfection

Predesigned siRNAs against rat mTOR (frap1_3 siRNA) were purchased from Qiagen. shRNA containing vectors against raptor an rictor are pKDM-132, a pSuper.gfp/neo-based (Oligoengine) siRNA expressing plasmid targeting cttctgtagactcacttcag in rictor mRNA; pKDM-162 is a pSuper.gfp/neo-based plasmid
targeting ggacaacggccacaagtac in raptor mRNA. RAEC were transfected with si/shRNA by nucleofection using the basic endothelial transfection kit. Briefly, cells were trypsinized, counted and 10E6 cell per cuvette were mixed with 3µg of si/shRNA and nucleofected by program T27 on a Amaxa nucleoporator. Cells were seeded and used 24 h later for experiments. Ivan Beuwink (Novartis, Basel, Switzerland) provided HA-mTOR cDNA containing pBABE-Puro retroviral vectors necessary for MEF infection.

**Cell proliferation assay**

To determine cell numbers, 1000 cells/well were seeded into 96-well plates and after 24 h the normal culture medium was replaced with serum-free DMEM. Inhibitors were added 30 min before cultures were incubated for 24 h at 21% O₂ and at 1% O₂ in octuplicates. After 24 h, cell numbers were assessed using Cell Proliferation Reagent WST-1 (Roche Molecular Biochemicals) according to the manufacturer’s specifications.

**Immunoblotting**

Total cell lysis was prepared with RIPA buffer as described before. Nuclear lysates were prepared as described elsewhere. After SDS-PAGE, proteins were transferred onto Polyvinylidene fluoride (PVDF) membrane (Millipore). The membrane was blocked with 4% skim milk powder in TBS-Tween solution or 4% BSA and probed with following antibodies: primary chicken polyclonal anti-HIF-1α (provided by Max Gassmann, Clinic for Small Animal Internal Medicine, University of Zürich, Switzerland), polyclonal anti-raptor and anti-rictor (by Markus A. Rüegg, Biocenter Basel, Switzerland), polyclonal anti-S6K1, polyclonal anti-phospho-S61 Kinase
(Thr^{389}), polyclonal anti-mTOR, polyclonal anti-phospho-mTOR (Ser^{2448} and Ser^{2481}), polyclonal anti-phospho-Akt (Ser^{374}), polyclonal anti-phospho GSK3α/β (Ser^{21/9}), polyclonal anti GSK3β, all from Cell Signaling Technology, MA, USA, polyclonal anti-Cyclin D1 and monoclonal anti-p21 (Santa Cruz Biotechnology). HRP-conjugated IgGs (Transduction Laboratories) were used for visualization of relevant proteins on X-ray films by a chemiluminescence reaction (Amersham). Protein bands were analyzed by densitometry and quantified by ImageJ 1.31v software (Wayne Rasband, NIH, USA).

**Immunestaining**

Immunestaining was performed as described previously \(^5\).

**Statistical analysis**

Unless otherwise described, data (mean±SEM) were analyzed for normal distribution (one-way or repeated measures ANOVA), followed by multiple or pairwise comparison with the Bonferroni post test using the program Prism (Graphpad). The number of single experiments compiled is indicated by n. A value of P<0.05 was considered as significant.
References


Supplemental figure legend

Cytotoxicity tests.

RAEC were starved for 30 h and incubated for 24 h at 21% O$_2$ and 1% O$_2$ with indicated amount of Akt inhibitor IV. 100% of cytotoxicity was determined by cell lysis with 2% Triton X-100. Akt inhibitor IV concentrations used for proliferation assays are indicated and range in nontoxic doses. Cytotoxicity was measured with the Roche cell toxicity kit (Roche Molecular Biochemicals, Rotkreuz, Switzerland) according to the manufacturer’s specifications.
Supplemental figure

![Graph showing % cytotoxicity against Akt Inhibitor IV (μM) with two conditions: 21% O₂ and 1% O₂. The graph indicates increased cytotoxicity at higher concentrations of Akt Inhibitor IV for both conditions.](image-url)