Hypoxia-Induced Apelin Expression Regulates Endothelial Cell Proliferation and Regenerative Angiogenesis

Mélanie Eyries, Géraldine Siegfried, Mariana Ciumas, Kevin Montagne, Monique Agrapart, Franck Lebrin, Florent Soubrier

Abstract—Apelin has been identified as the endogenous ligand of the human orphan G protein–coupled receptor APJ. This peptide exerts a variety of cardiovascular effects and particularly acts as an activator of angiogenesis. Importantly, hypoxia has been reported to regulate apelin expression but the molecular mechanism underlying hypoxia-induced apelin expression and the relationship with the physiological response of the apelin/APJ system are still not established. Here, we demonstrate that apelin expression is induced by hypoxia in cultured endothelial and vascular smooth muscle cells as well as in lung from mice exposed to acute hypoxia. Transient transfection experiments show that hypoxia-inducible transcriptional activation of apelin requires an intact hypoxia-responsive element (+813/+826) located within the first intron of the human apelin gene. Chromatin immunoprecipitation assay reveals that hypoxia-inducible factor-1α binds to the endogenous hypoxia-responsive element site of the apelin gene. Moreover, overexpression of hypoxia-inducible factor-1α increases the transcriptional activity of a reporter construct containing this hypoxia-responsive element, whereas small interfering RNA–mediated hypoxia-inducible factor-1α knockdown abolishes hypoxia-induced apelin expression. Finally, microinterfering RNA-mediated apelin or APJ receptor knockdown inhibits both hypoxia-induced endothelial cell proliferation in vitro and hypoxia-induced vessel regeneration in the caudal fin regeneration of Fli-1 transgenic zebrafish. The hypoxia-induced apelin expression may, thus, provide a new mechanism involved in adaptive physiological and pathophysiological response of vascular cells to low oxygen level.

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Key Words: hypoxia ■ transcriptional regulation ■ endothelial cell growth-APJ ■ zebrafish model

The apelin peptide, originally isolated from bovine stomach tissue extracts, has been identified as the endogenous ligand of the human orphan G protein–coupled receptor APJ, a receptor related to the angiotensin II receptor type 1. Apelin and APJ are distributed in various tissues where it exerts a broad range of physiological actions, including heart contractility, appetite, and drinking behavior or the hypothalamic–pituitary–adrenal axis. The role of apelin in the cardiovascular system is currently the most documented. Apelin and APJ were reported to be expressed in cardiomyocytes, endothelial, and vascular smooth muscle cells. Apelin-deficient mice are viable, but they present impaired cardiac contractility with aging and pressure overload, indicating that apelin/APJ pathway is important for maintaining cardiac function. Apelin was reported to positively regulate angiogenesis both in vitro and in vivo. Apelin stimulates endothelial cell proliferation, migration, and tube formation in vitro. In the frog embryo, apelin is crucial for the normal development of the embryonic vasculature. Apelin expression is upregulated during tumor angiogenesis, and its overexpression was reported to increase the in vivo tumor growth.

The molecular mechanisms which regulate apelin expression are still largely unknown; however, recently, it has been reported that hypoxia-inducible factor (HIF)-1 regulates apelin expression in cardiomyocytes and in adipocytes. HIF-1 is an oxygen-sensitive heterodimeric transcription factor that promotes the expression of genes containing hypoxia-responsive element (HRE). Putative HREs were found in the apelin promoter by in silico analysis, but their functionality have not been confirmed. Although several studies have suggested that hypoxia regulates apelin expression, the precise regulatory mechanisms involved have not been investigated to date.

We report that hypoxia induces apelin expression both in endothelial and vascular smooth muscle cells, as well as in mouse lung. We showed that increased apelin expression by hypoxia is mediated by the binding of HIF-1 to a HRE located within the first intron of the apelin gene. We also demonstrate that modulation of apelin expression is involved in the hypoxia-induced endothelial cell proliferation in vitro. Finally, we used the caudal fin regeneration model in Fli-1 transgenic zebrafish to demonstrate that the apelin/APJ system is involved in in vivo vessel regeneration.
Materials and Methods

An expanded Materials and Methods section, available in the online data supplement at http://circres.ahajournals.org, includes resources and detailed procedures on cell culture, animals, quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR), plasmids constructs and transfection, Western blotting, chromatin immunoprecipitation (ChIP) assay, proliferation assay, colony formation assay, apoptosis assay, and statistics.

Microinterfering RNA Expression Vectors Construction and Transfection

Microinterfering RNA (miRNA) directed against APJ and apelin were designed and introduced in an expression vector using BLOCK-iT Pol II miRNA RNA interference expression vectors kit (Invitrogen, Calif) according to the protocol of the manufacturer. Mouse embryonic endothelial cells (MEECs) were plated into 6-well plates at 2 × 10⁵ cells per well and transfected the following day with miRNA expression vectors by using Lipofectamine transfection reagent (Invitrogen) according to the recommendation of the manufacturer.

Vessel Regeneration in the Zebrafish Fin Regeneration Model

The procedures used for the care and euthanasia of the animals were in accordance with the European Community Standards (Ministère de l’Agriculture, France; authorization 94276). The Fli-1 transgenic zebrafish (Danio rerio) line has been described previously. Adult zebrafish were anesthetized in 0.6 mmol/L tricaine (ethyl-m-aminobenzoate) and were injected with various morpholinos (MOs) (Gene Tools Inc, Philomath, Ore) (sequences are available in the online data supplement) into the dermal skeleton of caudal fin with microcapillaries. Immediately following the injections, 10 consecutive 50-ms electric pulses, at 15 V with a 1-second pause between pulses, were applied via a pair of electrode disks (7 mm in diameter). Twenty-four hours postinjection, caudal fins were amputated at a level proximal to the first bifurcation with a scalpel, and immediately following the surgery, the fish were returned to a 30°C tank containing or not CoCl₂ at 10⁻³ mol/L for 3 days. Vessel regeneration was observed using a binocular magnifier Lumar (Carl Zeiss, Le Pecq, France). Measurements were performed using Image J software. Vascularization surface was quantitated for each individual and expressed as a percentage of the vascularization surface measured in fins injected with control MO in normoxia.

Results

Induction of Apelin Expression by Hypoxia

Response to hypoxia of the endogenous apelin gene was studied by real-time RT-PCR analyses. Compared with cells cultured in normoxic conditions, the apelin/RPL32 ratio was significantly increased in human pulmonary artery endothelial cells (HPAECs) exposed to hypoxia for 4 and 8 hours (Figure 1A). Hypoxia time course experiments on human
Pulmonary artery smooth muscle cells (HPASMCs) revealed similar results (Figure 1B), showing an early and sustained increase of apelin expression after hypoxia exposure. Total RNA was isolated from the lung of mice exposed to normoxic and hypoxic conditions (10\% O_2). After 5 hours, the apelin mRNA was significantly increased in the lung of the mice exposed to hypoxia. By competitive enzyme immunoassay, we showed that lung apelin levels were slightly but significantly higher in lung from mice exposed to hypoxic conditions (4.497±0.086 ng/mL) compared to normoxia (3.602±0.269 ng/mL) (Figure 1D).

Identification of a Regulatory Region Located in the First Intron of the Apelin Gene Involved in Hypoxia-Induced Expression

Given that regulatory regions are often conserved among species, we performed Multi-Vista analysis to identify conserved HRE in nonprotein coding areas of the apelin gene (Figure 2A). We identified 1 potential HRE binding site conserved among human, mouse, and rat sequences located between positions +813 and +826 (relative to human sequence) within the first intron of the apelin gene. Cross-linked protein–DNA complexes were immunoprecipitated with HIF-1α antibody before polymerase chain reaction (PCR) amplification of the apelin gene. The top gel represents the 150-bp apelin first intron amplicon, whereas the bottom shows the apelin gene amplicon not containing a HRE in either direction. D, Knockdown of HIF-1α inhibits hypoxia-induced apelin expression. Cells were transfected with a nonspecific or HIF-1α–specific small interfering (Si) RNA. Twenty-four hours after transfection, cells were exposed to hypoxia for 8 hours and then total RNA was extracted. Apelin mRNA levels were determined by real-time reverse transcriptase-PCR analysis. The relative apelin expression levels were calculated as the ratio of apelin expression in experimental cells to those of nontransfected normoxic cells, both normalized to RPL32 levels. Results are mean±SE values of 3 independent experiments. *P<0.05 relative to normoxia.
Figure 3. RNA interference–mediated knockdown of APJ and of Apelin inhibits hypoxia-induced cell proliferation. A, Colony-formation assay of mouse embryonic endothelial cells (MEECs) transfected with blasticidin expression vector containing a control microinterfering (mi) RNA, a specific Apelin miRNA, or a specific APJ miRNA. Cells were cultured for 10 days in a blasticidin-selective medium in the presence or not of vascular endothelial growth factor inhibitor SU1498 (25 μmol/L) and exposed or not to hypoxia (1% O2). Results represent mean±SE of 4 independent experiments. *P<0.05 relative to miControl. D, BrdUrd incorporation in MEECs transfected with blasticidin expression vector containing a control miRNA, a specific Apelin miRNA, or a specific APJ miRNA and exposed or not to hypoxia (1% O2). Results represent mean±SE of 3 independent experiments. *P<0.05 relative to normoxia.

By site-directed mutagenesis, we introduced a mutation in the putative HRE binding site identified by computer analysis in the pApeR1-luc construct (mutpApeR1-luc). When we transfected mutpApeR1-luc, the hypoxia-induced transcriptional activity observed with pApeR1-luc was abolished (Figure 2B, top). We also introduced this element downstream a luciferase reporter gene driven by the heterologous promoter SV40 (pSV40R1-luc) and showed that the R1 (+751/+900) regulatory element is sufficient to drive hypoxia-induced apelin transcriptional activity (Figure 2B, bottom).

This finding demonstrates the requirement of the intact HRE (+813/+826) located within the first intron of the human apelin gene for hypoxia-inducible expression.

Role of HIF-1 in Hypoxia-Induced Apelin Expression
To provide evidence that HIF-1 interacts with the HRE of the endogenous apelin gene, we performed ChIP analysis on cells subjected or not to hypoxia (Figure 2C). Immunoprecipitation of cross-linked DNA–protein complexes was performed using an anti–HIF-1α antibody. The amount of cross-linked HRE-containing genomic DNA fragments derived from the apelin first intron that coprecipitated with HIF-1α was determined by PCR analysis with primers that span the first intron of the apelin gene between +751 to +900 and containing the HRE. ChIP/PCR analysis revealed that, after exposure to 1% O2 for 2 hours, increasing amounts of genomic DNA fragments containing the apelin HRE were detected only when an anti–HIF-1α antibody was used for immunoprecipitation of the DNA–protein complexes. The specificity of this signal was confirmed by the absence of PCR amplicons when primers used for PCR analysis span a region of the apelin gene that does not contain a HRE. This region is detected in the input but is not associated with an HIF-1α immunoprecipitate. These results demonstrate that HIF-1α binds the endogenous HRE located within the first intron of the apelin gene during hypoxia.

Cotransfection experiments using an expression vector encoding HIF-1α indicate that HIF-1 activates the transcriptional activity of the apelin gene through the HRE (+813/+826) (Figure I in the online data supplement). To further examine the role of HIF1-α in hypoxia-induced apelin expression, we knocked down the expression of HIF1-α subunit by using a specific small interfering (si)RNA (supplemental Figure II). Hypoxia-induced apelin expression is fully inhibited in cells transfected with HIF1-α–directed siRNA compared with those transfected with nonspecific siRNA (Figure 2D).
Figure 4. Mammalian target of the rapamycin (mTOR) signaling pathway mediates the effect of Apelin in hypoxia. A, Analysis of Apelin/APJ signaling. Cells were transfected with plasmids containing a control microinterfering (mi) RNA, a specific Apelin miRNA, or a specific APJ miRNA and exposed or not to hypoxia (1% O2) for 8 hours. Protein extracts were analyzed by Western blot using specific phospho-p70S6 kinase, p70S6 kinase, phospho-mTOR, and mTOR antibodies. B, Densitometric analysis of p70S6 kinase and mTOR activation. Kinase activation is defined as the ratio between the phospho-kinase signal and kinase signal. C, BrdUrd incorporation in mouse embryonic endothelial cells (MEECs) treated or not with [Pyr]Apelin-13 (1µM) and rapamycin (5 nmol/L). Results represent mean±SE of 3 independent experiments. *P<0.05 relative to normoxia. D, BrdUrd incorporation in MEECs treated with rapamycin (5 nmol/L) and exposed or not to hypoxia (1% O2). Results represent mean±SE of 3 independent experiments. *P<0.05 relative to normoxia.

RNA Interference–Mediated Knockdown of Apelin and APJ Inhibits Hypoxia-Induced Cell Proliferation

To determine the functional significance of hypoxia-induced apelin expression, we knocked down the expression of apelin or its receptor, APJ, by transfecting MEECs with blasticidin expression vectors containing an unrelated miRNA (miControl), an miRNA directed against apelin (miApelin) or an miRNA directed against APJ (miAPJ) and analyzed their effects on cell growth and survival by a colony-formation assay. After selection for 10 days in the presence of blasticidin, the cells that survived and formed colonies were visualized by crystal violet staining. We observed a significant increase in the number of colonies when cells were exposed to hypoxia compared to normoxic condition. This effect was partially abolished in cells expressing miRNA-mediated APJ knockdown (Figure 3A and 3B). In miRNA-mediated apelin knockdown cells, the number of colonies formed in hypoxia was reduced, but it was still statistically significantly different from normoxia. Because vascular endothelial growth factor (VEGF) is strongly induced in response to hypoxia and is a potent endothelial growth factor, we performed a similar experiment in the presence of SU1498, a VEGF receptor-2 inhibitor. In this condition, we observed a significant decrease in the number of colony in APJ knockdown cells and in apelin knockdown cells under hypoxia in both presence or absence of SU1498 (Figure 3C), suggesting that the effect of apelin on cell growth and survival under hypoxic conditions is independent of VEGF signaling. Moreover, by monitoring 5-bromodeoxyuridine (BrdUrd) incorporation, we confirmed that hypoxia-induced endothelial cell proliferation rate was reduced in APJ knockdown cells and in apelin knockdown cells (Figure 3D). Inhibition of the APJ receptor or the apelin ligand apelin affects hypoxia-induced endothelial proliferation in a similar way, suggesting that apelin could act by both autocrine and paracrine mechanisms.

Previously, it has been reported that the binding of apelin to its receptor APJ activates p70S6 kinase signaling.5 Phosphorylation of p70S6 kinase and mammalian target of the rapamycin (mTOR) were induced in MEECs transfected with control miRNA and exposed to hypoxia but was significantly reduced in MEECs expressing miAPJ or miApelin, indicating that apelin is required to activate p70S6 kinase signaling pathway in response to hypoxia in these cells (Figure 4A and 4B). To confirm the involvement of this signaling pathway, we performed BrdUrd incorporation assay in cells treated with rapamycin a specific inhibitor of mTOR. When endothelial cells were incubated with apelin for 24 hours, BrdUrd incorporation was significantly increased. This effect was fully abolished in the presence of rapamycin, demonstrating...
that apelin-induced endothelial cell proliferation requires mTOR activation (Figure 4C, left). Furthermore, rapamycin inhibited hypoxia-induced endothelial cell proliferation (Figure 4C, right). Altogether, these results suggest that apelin participates to the hypoxia-induced endothelial cell proliferation through the activation of the mTOR signaling pathway.

Because apelin was shown to have an antiapoptotic activity, apoptosis was assessed. However, no effect of hypoxia was observed on apoptosis of endothelial cells (supplemental Figure III).

**Role of Apelin in Regenerative Angiogenesis in Zebrafish Caudal Fin Model**

To examine the role of apelin in hypoxia-induced vessel regeneration in vivo, we performed caudal fin regeneration assay in the adult Fli-1 transgenic zebrafish. In fin regeneration experiments, zebrafish caudal fins are amputated at midfin level, then allowed to recover. The regenerated fin is an area of intense cell proliferation shortly after amputation where new blood vessels are formed. To determine whether apelin expression is modulated in this model in both basal and hypoxic conditions, zebrafish were treated with CoCl$_2$ (10$^{-3}$ mol/L), a hypoxia-mimicking agent, immediately after fin amputation. At 3 days postamputation (dpa), RNA was extracted from both the regenerating and the nonregenerating area of fins. By RT-PCR analysis, we observed that VEGF expression was induced in CoCl$_2$-treated fins, demonstrating that this treatment is effective to mimic hypoxic condition (Figure 5B). Apelin expression was induced in the blastema compared to the nonregenerating fin area. This induction was stronger in CoCl$_2$-treated fins. The zebrafish genome harbors 2 orthologous genes, agrt1-a and agrt1-b, of the APJ receptor. Expression of agrt1-a and agrt1-b is stable in the blastema and in the nonregenerating fin area in both basal and hypoxic conditions.

Gene expression was also examined by whole mount in situ hybridization. In the area anterior to amputation plane, in both control and CoCl$_2$-treated fins, apelin transcripts are restricted to the edges of the bony rays (Figure 5C, e and i). Cells expressing apelin appear to partially colocalize within endothelial cells expressing enhanced green fluorescent protein (eGFP) (Figure 5C, a). The same pattern of expression is detectable in the blastema (Figure 5C, f, h, j, and l) with a diffuse pattern.
To examine the role of apelin signaling in this model, we knocked down the expression of apelin or the 2 isoforms of the apelin receptor agrt1a and agrt1b by using MO technology in Fli-1 transgenic zebrafish in which endothelial cells are labeled with enhanced green fluorescent protein. Lissamine-tagged agrt1a and agrt1b (defined as MOAgrt1-1), apelin (MOApelin), or control MO (MOCt) were injected into fins, and electroporation followed. The following day, fins were amputated and zebrafish were treated with CoCl2 (10^{-3} mol/L) for 3 days (Figure 5A). Vascular architecture in the caudal fin is organized with a central artery and 2 flanking veins in each fin ray. A set of smaller blood vessels form the connecting microvasculature in the interray mesenchyme. By 3 dpa, networks of endothelial cells in the regenerated tissue formed a vascular plexus characterized by unstructured regenerating vessels that gradually remodeled from the proximal end into new arteries and veins (Figure 6A, a).15

High-magnification views of the fin tip show an arteriovenous anastomosis at the severed ends of blood vessels (Figure 6, red arrow), as well as the appearance of sprouting endothelial cells (Figure 6A, g, yellow arrow). In CoCl2-treated fins, we observed and quantified the enhanced surface of the vascular plexus compared to control conditions (Figure 6B). Furthermore, remodeling in arteries and veins occurs to a lower extent compared to control condition (Figure 6A, d). We also observed rounded ends with additional sprouts, but these protrusions are more numerous compared to control conditions (Figure 6A, j, yellow arrow and Figure 6C). The new vessel branches are thicker than untreated vessels and make numerous connections with neighboring vessels, reflecting that more anastomotic bridges are formed in the vascular plexus under hypoxic condition (Figure 6A, j, red arrow). Injection of MOAgrt1 or MOApelin did not modify significantly the vascular regeneration pattern in control condition (Figure 6A, b and c, h and i). However, injection of MOagtrl1 in CoCl2-treated fins partially restored the vascular plexus observed in control conditions (Figure 6A, e and k). Indeed, vessel sprouts returned to the level quantified in normoxia (Figure 6B). Similar results are obtained in fins injected with MOApelin (Figure 6A, f, l). These results suggest that the apelin-agtrl1 system participates in hypoxia-induced regenerative angiogenesis in the zebrafish caudal fin.

**Figure 6.** Vessel regeneration in the zebrafish fin regeneration model. A, Fin vessel regeneration observed at low (top) and high-magnification (bottom) in fin electroporated with control MO (a and g), fin electroporated with control MO and exposed to CoCl2 (10^{-3} mol/L) (d and j), fin electroporated with agrt1a and agrt1b MO (b and h), fin electroporated with agrt1-a and agrt1-b MO and exposed to CoCl2 (10^{-3} mol/L) (d and j), fin electroporated with apelin MO (c and i), or fin electroporated with apelin MO and exposed to CoCl2 (10^{-3} mol/L) (f and l). B and C, Graphic representation of the quantitative comparison of the vascularization surface (B) and number of tip cells (C) in fin electroporated with control MO (n=6), fin electroporated with control MO and exposed to CoCl2 (10^{-3} mol/L) (n=6), fin electroporated with agrt1a and agrt1b MO (b and h), fin electroporated with agrt1-a and agrt1-b MO and exposed to CoCl2 (10^{-3} mol/L) (d and j), fin electroporated with apelin MO (n=5), or fin electroporated with apelin MO and exposed to CoCl2 (10^{-3} mol/L) (n=3). Vascularization surface was quantified for each individual and expressed as a percent of the vascular surface measured in control condition in normoxia. *P<0.05 relative to normoxia. See legends to Figures 1 through 5 for definitions of abbreviations.
Discussion
The present study provides evidence that apelin expression is upregulated under hypoxic conditions in vascular cells. We observe an early upregulation of the human apelin gene expression under hypoxia (1% O2) in both primary endothelial and vascular smooth muscle cells. By analyzing in silico noncoding regions of the apelin gene, we identified three putative HRE binding sites: 2 located within the human apelin promoter (−1228/−1206 and −478/−460 relative to the transcriptional start site) and 1 located within the first intron of the apelin gene (+813/+826), but only the potential HRE within the first intron of apelin displays high interspecies similarity, suggesting that it was an important regulatory element. Using luciferase reporter construct, we identified the HRE of the first intron as the one involved in hypoxic apelin transcriptional activity induction, in contrast to the 2 HRE-like elements located within the upstream promoter of the apelin gene, because mutation of this HRE site resulted in a >90% decrease in hypoxia inducibility. Taken together, strong evidence is provided that the flanking regions surrounding the core HIF-1 binding site from position +813 to +826 function as a classic HRE. Three approaches were used to precisely define a role for HIF-1 in the induction of apelin: (1) an HIF-1 targeted siRNA is able to block apelin induction; (2) overexpression of HIF-1α mimics hypoxia-induced apelin expression; and (3) HIF-1α binds in vivo to the first intron of apelin.

Thus, we identify apelin as a HIF-1 target gene and demonstrate that on hypoxia, HIF-1 binds to the first intron of apelin leading to upregulation of apelin expression. Hypoxia is not the only condition that stabilizes HIF-1α and activates HIF-1 transcriptional activity. Several hormones and growth factors, including insulin16 or endothelin-1,17 have been shown to increase the level of HIF-1α in various cell types. In addition, inflammatory cytokines, such as interleukin-1β and tumor necrosis factor-α, also induce HIF-1 activity in normoxic cells.18 Considering that inflammatory stimulus such as tumor necrosis factor-α significantly increased apelin expression,19 it will be of interest to investigate whether this stimulus and others could regulate apelin gene expression via hypoxia-independent HIF-1 pathways. This was recently demonstrated for insulin-induced apelin expression in adipocytes.11

Hypoxia is a strong stimulator of angiogenesis both during embryonic development and in pathological conditions such as malignant growth, atherosclerosis, and diabetic retinopathy by inducing expression of several angiogenic growth factors. A few HIF-1 target genes, most notably VEGF, have been demonstrated to play integral roles in proliferation, migration and tube formation of endothelial cells that are characteristic of angiogenesis.20 Several reports suggest that apelin could be an angiogenic factor. Thus, apelin was shown to be mitogenic, to possess a chemotactic activity and an antiapoptotic activity for cultured endothelial cells.7 Furthermore, apelin was shown to have an angiogenic activity for retinal endothelial cells both in vitro and in vivo.6 Apelin could also play a role during embryonic angiogenesis because mouse APJ and xenopus ortholog of APJ are expressed in embryonic endothelium and apelin/APJ signaling is required for normal development of the vascular system in the frog embryo.7,21

Recently, apelin was shown to behave as a potent activator of tumor neoangiogenesis.9 Our results demonstrate that apelin is an HIF-1 target gene playing a key role in hypoxia-induced endothelial proliferation in cultured cells. Thus, knockdown of apelin or APJ expression results in the inhibition of hypoxia-induced endothelial proliferation in vitro. After binding to its receptor, apelin signals via both extracellular signal-regulated kinase 1/2 and phosphatidylinositol 3-kinase/Akt, leading to p70S6 kinase activation.5 P70S6 kinase is a downstream target of mTOR. mTOR pathway was shown to mediate hypoxia-induced cell proliferation and angiogenesis.22 We found that inhibition of the apelin/APJ system blocks p70S6 kinase and mTOR phosphorylation, as well as BrdUrd incorporation induced by hypoxia, suggesting that the effect of apelin on hypoxia-induced cell proliferation is mediated by this pathway. Recently, it was established that mTOR promotes the transcriptional activity of HIF-1 in hypoxic condition.23 We can hypothesize that a positive feedback loop of regulation could exist, where hypoxia induces apelin expression through HIF-1 and subsequently the mTOR pathway, which, in turn, activates HIF-1 and enhances hypoxia-induced apelin expression.

To study the role of apelin in hypoxia-induced vascular regeneration in vivo, we used a caudal fin regeneration assay in the adult Pli-1 transgenic zebrafish. Zebrafish is a small teleost fish that have the remarkable capability to regenerate their fins, optic nerve, scales, heart, and spinal cord.24 Zebrafish recovers the lost caudal fin tissue after amputation through a process of epimorphic regeneration and this occurs in a stepwise manner with the formation of an epithelial wound cap, followed by blastema formation and finally the regenerative outgrowth. Fin regeneration is associated with and requires new vessel growth. Here, we report studies on blood vessel regeneration in fins of Pli-1 transgenic zebrafish. This fish line expresses eGFP in blood vessel endothelial cells throughout normal development and fin regeneration. It was described as a model of choice for dissecting the molecular signaling that controls regenerative endothelial growth and angiogenesis.14,25 Stages of vessel morphogenesis during normal fin regeneration include vessel healing, anastomosis to reconnect arteries and veins, plexus formation, and plexus remodeling.15 We found that HIF-1 induction by cobalt chloride affects blood vessel regeneration in adult zebrafish. Indeed, in hypoxic fins, the surface of the vascular plexus and the endothelial sprouting were enhanced compared to control condition. Knockdown experiments of agtr1l, the orthologous of APJ, or of apelin, reverse partially the regenerative vascular pattern observed in normoxic condition indicating that the apelin/agtr1l system is an important regulator of hypoxia-induced neovascularization in the regenerating fin. We also observed that in hypoxia, regenerating vessels are thicker compared to control condition. It was recently demonstrated that apelin is involved in the calibre size regulation of blood vessels during angiogenesis.26 As apelin gene expression is upregulated by hypoxia, this property fits with its role in the adjustment of the vascular supply to metabolic demand. Thus, under hypoxia, blood vessels...
have an opportunity to enlarge their size and the induction of apelin under hypoxia could participate to this process.

In conclusion, we report here that apelin is a hypoxia-inducible gene both in vitro and in vivo. We showed that hypoxia-induced apelin expression is mediated by HIF through an HRE located within the first intron of the gene. Moreover we determined that hypoxic induction of apelin expression regulates endothelial proliferation in vitro. Our study demonstrates hypoxic regulation of regenerative angiogenesis in the zebrafish caudal fin and establishes the importance of apelin/APJ signaling in this process. The hypoxia-induced apelin expression, thus, may provide a new mechanism involved in adaptive physiological and pathophysiological response of vascular cells to low oxygen level where hypoxic tissues drive the formation of new blood vessels in part by the secretion of apelin which promotes angiogenesis through proliferation of endothelial cells. Taken together, these data suggest that apelin/APJ signal has the potential to modulate blood vessel growth in the mature organism. Thus, hypoxic regulation of the apelin expression could play an essential role in the context of normal and pathological angiogenesis.

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Disclosures
None.

References
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Supplemental materials and methods

**Cell culture.** Human pulmonary artery endothelial cells (HPAEC) and Human pulmonary artery smooth muscle cells (HPASMC) were obtained from Clonetics (Baltimore, MD, USA). Mouse Embryonic endothelial cells (MEEC) were obtained and cultured as previously described 1.

**Animals.** Eight weeks-old male C57Bl/6 mice were used for this study and were divided into two groups: a control group (n=8), and one group exposed to hypoxic conditions (10% O2) for 5h (n=8). After hypoxia treatment, mice were sacrificed under pentobarbital anesthesia and lungs were removed. The latters were washed in PBS and immediately frozen in liquid nitrogen and stored at -80° before RNA extraction.

**Real-time RT-PCR.** Real-time RT-PCR assay was performed as previously described 2. Data are expressed as mean fold change ±s.e.m. of three replicates.

Primers used for real time RT-PCR are indicated below:

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Apelin Assay. Apelin assay was performed as previously described 3 using the Apelin-12 EIA assay kit (Phoenix Pharmaceuticals, Belmont, CA).

Plasmid constructs and site-directed mutagenesis. To generate the apelin promoter-luciferase reporter gene construct (pApe-luc), a fragment corresponding to -1371 to -178 (relative to the transcriptional start site) of the promoter region of apelin gene was amplified by PCR and cloned into the luciferase reporter vector pGL3-basic (Promega, Madison, WI). The pApeR1-luc was obtained by cloning a fragment of the first intron of the apelin gene that corresponds to +751 to +900 relative to the transcriptional start site (genbank : AF179680) by PCR amplification. Amplified fragment was cloned into the pApe-luc. A mutation of the potential HRE binding site (+813/+826) was introduced in the pApeR1-luc construct using an oligonucleotide-directed mutagenesis system (Quick Change site-directed mutagenesis kit, Stratagene) according to the manufacturer’s recommendation. The following oligonucleotides were used to create the mutation (underlined): forward: 5’-GTGCCAACATGCAGATCTTACACTTGGTGCATAGGCAG-3’, reverse: 5’-CTGCTATTGCAAAAGTGTAGAGTGCTCAGTCATGGCAG-3’.

Transient transfection. HPASMC were transfected using the primary smooth muscle cells Nucleofector Kit (AMAXA Biosystems, Gaithersburg, MD) according to the manufacturer’s recommendation, with a machine setting of "D-33." Co-expression experiments were performed using expression vectors pcDNA3.1-HIF-1α and pcDNA3.1-HIF-1β 4. Data are expressed as mean fold change ±s.e.m. of three experiments.

siRNA transfection. HPASMC were plated into 6-well plates at 3x10^5 cells/well and transfected the following day with siRNA by using Dharmafect1 transfection reagent (Dharmacon Research, Lafayette, CO) according to the manufacturer’s recommendation. The sense and antisense strands of the siRNAs used were previously described 5. SiCONTROL Non-targeting siRNA 2 (Dharmacon D-001210-02) was used as a nonspecific control siRNA.
Western blot analysis. Western blot analysis was performed as previously described. HIF-1α (BD Biosciences, Franklin Lakes, NJ), Pp70S6 kinase, p70S6 kinase, PmTOR and mTOR (Cell Signaling Technology, Danvers, MA) and β-actin (Sigma, Saint-Louis, Missouri) antibodies were used. Densitometric analysis was performed using NIH Image software.

ChIP Assay. The chromatin immunoprecipitation (CHIP) assay was performed using a ChIP assay kit (Upstate Biotechnology, Lake Placid, N.Y.). HPASMC were exposed to hypoxia (1% O₂) for 2h. The cells were then fixed, DNA and bound proteins were cross-linked, and ChIP assays were performed by co-precipitating the DNA–protein complexes with an anti-HIF1α antibody (BD, Franklin Lakes, N.J.)

Proliferation assay and colony formation assay. Proliferation assay was performed using Cell proliferation Elisa, BrdU kit (Roche, Mannheim, Germany) according to the manufacturer protocol in the presence or absence of the VEGFR2 inhibitor SU1498 (25µM) (Calbiochem, CA, USA) or rapamycin (Cell Signaling Technology, MA, USA). Colony formation assay was performed as previously described.

Apoptosis assay. Cells were plated at a density of 1x10⁴ cells/well in 24-well plates for 24 h followed by culture in serum-free medium for 48 h in normoxia or in hypoxia (1% O₂). Cell lysates were tested for apoptosis by measurement of cytoplasmic nucleosomes using a Cell Death Detection ELISA kit (Roche Diagnostics GmbH, Roche Molecular Biochemicals, Mannheim, Germany), according to the kit protocol.

Morpholinos sequences.

Agtrl1a MO 5’-TTGGCTCCATTTGGAAAGGGCGCGT-3’
Agtrl1b MO 5’-TGGCATTCATTCCTTCAAGAAAATC-3’.
Apelin MO 5’ GAT CTT CAC ATT CAT TTC TGC TCTC 3’
Gene tools standard control MO 5’-CCTCTTACCTCAGTTACAATTATA-3’
**Computer and statistical Analysis**- The apelin gene structure was analyzed using the UCSC Genome Bioinformatics Browser (http://www.genome.ucsc.edu/) using the July 2003 assembly. Homology of human apelin to mouse and rat was identified using the Multi-VISTA Browser (http://pipeline.lbl.gov/cgi-bin/gateway2) using the July 2003 base genome.

Two-way ANOVA with time as a repeated measure was used to determine time and treatment differences between the hypoxia and control in the cultured cells. To determine the statistical difference for all other data, a non-parametric Kruskal-Wallis test was performed. Statistical significance was assumed at $P<0.05$. 
Supplemental figure 1. Effect of the HIF-1 co-expression on the transcriptional activity of the human apelin gene. Cells were co-transfected with the reporter construct and expression vectors HIF-1α and HIF-1β (HIF-1). After transfection, cells were incubated for 24h at 37°C, and the luciferase activity was measured using the Dual-Luciferase reporter assay system. The relative luciferase activity is defined as the ratio between firefly luciferase activity and the internal control Renilla luciferase activity. All data represent means ± S.E. of 3 experiments performed in duplicate. *, p<0.05 relative to empty vector.
Supplemental figure 2. HIF-1α small interfering (si)RNA specifically inhibits the expression of the targeted HIF-1α subunit in HPASMC. HPASMC were transfected with HIF-1α siRNA or nonspecific (NS) siRNA, and HIF-1α subunit and β-actin levels were determined by Western blot analysis 48h after transfection. The marked upregulation of HIF-1α level by exposure to hypoxia (1% O2 for 4h) is abolished by HIF-1α-specific siRNA but is unaffected by nonspecific siRNA.
**Supplemental Figure 3**

Supplemental figure 3. Apoptosis is not affected in hypoxia-exposed endothelial cells. Apoptosis assay in MEECs transfected with blasticidin expression vector containing a control miRNA, a specific APJ miRNA or a specific Apelin miRNA and exposed or not to hypoxia (1% O₂). Results represent means ± S.E. of 4 independent experiments performed in duplicate.
References


