Sphingosine Kinase 1: A New Modulator of Hypoxia Inducible Factor 1α during Hypoxia in Human Cancer Cells

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Abstract

Here, we provide the first evidence that sphingosine kinase 1 (SphK1), an oncogenic lipid kinase balancing the intracellular level of key signaling sphingolipids, modulates the transcription factor hypoxia inducible factor 1α (HIF-1α), master regulator of hypoxia. SphK1 activity is stimulated under low oxygen conditions and regulated by reactive oxygen species. The SphK1-dependent stabilization of HIF-1α levels is mediated by the Akt/glycogen synthase kinase-3β signaling pathway that prevents its von Hippel-Lindau protein–mediated degradation by the proteasome. The pharmacologic and RNA silencing inhibition of SphK1 activity prevents the accumulation of HIF-1α and its transcriptional activity in several human cancer cell lineages (prostate, brain, breast, kidney, and lung), suggesting a canonical pathway. Therefore, we propose that SphK1 can act as a master regulator for hypoxia, giving support to its inhibition as a valid strategy to control tumor hypoxia and its molecular consequences.

Introduction

Hypoxia, the insufficient delivery of oxygen for the demand of a tissue, is a characteristic hallmark of locally advanced solid tumors. Clinically, hypoxia contributes to the development of aggressive phenotype, resistance to radiation therapy and chemotherapy, and is predictive of a poor outcome in numerous tumor types.

The master regulator of oxygen adaptation is the transcription factor hypoxia inducible factor 1 (HIF-1), a heterodimer composed of an unstable α subunit tightly regulated by the cellular O2 concentration and a constitutively expressed nuclear β subunit (1). Under normoxic conditions, HIF-1α is hydroxylated on either one or two proline residues by a family of oxygen-dependent specific prolyl-hydroxylases (reviewed in ref. 2). The hydroxylation of HIF-1α is required for its recognition by the von Hippel-Lindau tumor suppressor gene product (pVHL). In concert with other proteins, pVHL forms an E3 ubiquitin ligase complex, targeting HIF-1α for degradation by the proteasome. Inactivation of the pVHL, hallmark of renal cell carcinomas and other tumors regrouped in the VHL syndrome, prevents HIF-1α ubiquitylation and, hence, proteosomal degradation resulting in HIF-1α accumulation (2).

Under hypoxia, HIF-1α remains unhydroxylated and thus is no longer recognized by pVHL (2). Subsequently, HIF-1α accumulates and translocates to the nucleus where it heterodimerizes with its partner, HIF-1β. The HIF-1 complex recruits a number of coactivators inducing the binding to the hypoxic response element (HRE) located in regulatory regions of target genes, enhancing their transcription, which results in cellular adaptation to a low-oxygen environment (reviewed in refs. 3, 4). Hundreds of human genes are influenced by HIF-1, and more than 70 of these have been recognized as direct targets (3, 4). HIF-1–regulated gene transcription under hypoxia contributes to angiogenesis, metabolic adaptation, resistance to apoptosis, invasion, and metastasis, leading toward a surviving phenotype with clinical aggressiveness.

Although much is known about the transcriptional activity and biological roles of HIF-1, the intrinsic mechanisms whereby cells can detect the diminution in O2 that elicits HIF-1 activation are not entirely elucidated. Over the last years, the connection between mitochondrial reactive oxygen species (ROS) production, hypoxia, and HIF-1 stabilization has emerged. The stability of HIF-1α is dependent on ROS metabolism. Earlier studies have suggested that ROS produced by mitochondria are required for proper induction of HIF-1α under hypoxic conditions (5–9), and blockade of the ROS pathway has been shown to successfully diminish HIF-1α activity (10, 11). Growth factors or cytokines via activation of the phosphatidylinositol 3-kinase (PI3K)/Akt or Ras/Raf/mitogen-activated protein kinase pathway have also been shown to modulate HIF-1α protein levels and transcriptional activity in normoxia (reviewed in refs. 3, 4). Interestingly, the PI3K/Akt signaling cascade is also clearly implicated in the regulation of HIF-1α under hypoxic conditions. Genetic and pharmacologic inhibition of PI3K indeed results in the inhibition of HIF-1α stabilization and activation in various cell models (12–19). Activated Akt, the downstream target of PI3K, has been shown to inhibit the glycogen synthase kinase 3β (GSK3β) activity via a specific phosphorylation of its Ser9 residue (20). GSK3β, initially described as a key enzyme in glycogen metabolism, is now recognized to regulate a wide array of transcription factors (Smad 1, Smad3, the c-Jun coactivator nascent polypeptide–associated complex and coactivator α, IPF1/PDX1, BCL-3, etc.) including HIF-1α (21, 22). GSK3β, in its active nonphosphorylated form, seems to exert its effect by down-regulating HIF-1α protein levels. A new paradigm has therefore been proposed where hypoxia inactivates GSK3β through the PI3K/Akt pathway (17–19, 23). Sphingosine 1-phosphate (SIP) has emerged as a critical lipid mediator that may play a major regulatory role in tumor cell growth, survival, invasion, vascular maturation, and angiogenesis (reviewed in ref. 24). SIP has been shown to either act extracellularly by binding to one of the five cell surface G-coupled SIP receptors and intracellularly by a mechanism still undefined (24). The balance between the intracellular levels of SIP and its metabolic precursors ceramide and sphingosine is regarded as a switch that could determine whether a cell proliferates or

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undergoes apoptosis (25). A decisive regulator of this sphingolipid rheostat is sphingosine kinase 1 (SphK1), the enzyme that converts sphingosine into prosurvival S1P. Whereas SphK1 activity can be stimulated by a wide array of growth factors (24), we and others have shown that anticancer treatments cause its down-regulation (reviewed in ref. 26). To further support a role for SphK1 in promoting cancer, SphK1 has been found to act as an oncogene (27), and its mRNA is overexpressed in many human solid tumors such as those of the breast, colon, lung, ovary, stomach, uterus, kidney, and rectum (28). Interestingly, the increase in SphK1 expression in tumor biopsies has been correlated with short survival rate in patients with glioblastoma (29). Furthermore, SphK1 enzymatic activity and expression are markedly increased in tumor samples from prostate cancer patients (as compared with normal counterparts) correlating with other markers such as prostate-specific antigen level, tumor grade, and the clinical outcome after prostatectomy.4

For the first time, we show in a broad range of cancer models that under hypoxia, SphK1 stabilizes HIF-1α through the Akt/GSK3β pathway and prevents its pVHL-dependent proteasomal degradation. Therefore, we propose that SphK1 can act as a master regulator for hypoxia, giving support to its inhibition as a valid strategy to control tumor hypoxia and its molecular consequences.

**Experimental Procedures**

**Cell lines.** Human prostate cancer PC-3 and lung carcinoma A549 cell lines were obtained from DSMZ. Human U87 glioblastoma cells were from American Type Culture Collection. Human breast cancer SUM-159PT cells were a gift of Dr. Piette (INSERM EMI 229, Montpellier, France). The pVHL-deficient renal cell carcinoma cell line RCC10 and its counterpart stably transfected with wild-type pVHL were kindly provided by Dr. Godinot (Centre National de la Recherche Scientifique, UMR 5534, Villeurbanne, France). Cells were cultured in RPMI 1640 containing 10% fetal bovine serum at 37°C in 5% CO2 humidified incubators. Hypoxic conditions were obtained by incubating cells at 37°C in a modular...
Heraeus incubator chamber flushed with a custom gas mixture (1% O₂, 5% CO₂, and balance N₂).

**Reagents.** Culture medium, serum, and antibiotics were obtained from Invitrogen. Sphingosine kinase inhibitor and MG132 were from Calbiochem. [γ-32P]ATP (3,000 mCi/mmol) was purchased from Perkin-Elmer, and silica gel 60 high-performance TLC plates were from VWR. N-Acetylcysteine and cycloheximide were from Sigma-Aldrich.

**RNA interference experiments.** Transient interference was achieved by double-stranded human SphK1-specific small interfering RNA (siRNA; 5'-GGGCAAGGCUUGCAGUCCdTdT-3'; ref. 30), aleatory sequence scrambled siRNA (Eurogentec Angers, France), or a pool of four siRNAs specific for GSK3β (siGENOME SMARTpool Kit, Dharmacon). SiRNA transfection was done with the siRNA using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions.

**SphK1 assay.** SphK1 activity was done as previously described (30) and determined in the presence of 50 μmol/L sphingosine, 0.25% Triton X-100, and [γ-32P]ATP (10 μCi, 1 mmol/L) containing 10 mmol/L MgCl₂. The labeled S1P was separated by TLC on silica gel 60 with 1-butanol/ethanol/acetic acid/water (80:20:10:10, v/v) and visualized by autoradiography. Activity was expressed as picomoles of S1P formed per minute per milligram of protein.

**Western blot analysis and antibodies.** Mouse HIF-1α (BD Biosciences), mouse anti-VHL (BD Biosciences), rabbit anti–GLUT-1 (NeoMarkers-Lab Vision), rabbit Ser473-phospho-Akt (CST), rabbit GSK3β (CST), and rabbit Ser9-phospho-GSK3β (CST) were used as primary antibodies. Western blot assays were done according to the manufacturer’s instructions. Proteins were visualized by enhanced chemiluminescence detection system using anti-rabbit or antimouse horseradish peroxidase–conjugated IgG (Bio-Rad). Equal loading was confirmed by probing the blots with the mouse anti–β-actin antibody (Sigma-Aldrich, clone AC-15) or mouse anti-tubulin (Sigma-Aldrich, clone DM1A).

**Statistical analysis.** The statistical significance of differences between the means of two groups was evaluated by unpaired Student’s t test, and one-way ANOVA was used to test for differences between two or more independent groups. All statistical tests were two-sided and the level of significance was set at \( P < 0.05 \). Calculations were done using Instat 3 for Macintosh (GraphPad Software). Densitometry quantitation was determined using the ImageJ software (NIH).

**Results**

**SphK1 regulates HIF-1α level and activity under hypoxia in multiple tumor cell lineages.** To investigate whether SphK1 was involved in the regulation of HIF-1α expression under hypoxia, we first used sphingosine kinase inhibitor, a specific inhibitor of SphK1 activity (28), which displays a dose-dependent inhibitory effect on SphK1 activity and S1P levels (Fig. 1A). Cells were treated with increasing concentrations of sphingosine kinase inhibitor (2.5–10 μmol/L). As shown in Fig. 1B (top), sphingosine kinase inhibitor inhibited accumulation of HIF-1α induced by hypoxia in a concentration-dependent manner in human PC-3 prostate cancer cells. The ability of sphingosine kinase inhibitor to inhibit HIF-1α accumulation was tested in three other models including the lung adenocarcinoma cell line A549, the breast adenocarcinoma cell line SUM-159PT, and the glioblastoma cell line U87. A similar dose-dependent action of sphingosine kinase inhibitor on HIF-1α content was observed in these models (Fig. 1B, bottom). We next evaluated the effect of SphK1 knockdown with siRNA targeted against SphK1. A substantial decrease in SphK1 activity was observed in SphK1 RNAi–treated cells compared with those treated with scrambled RNAi. As shown in Fig. 1C, basal SphK1 activity was decreased by 70% to 90% with SphK1 siRNA (siSphK1) as compared with scrambled siRNA in all the cell lines tested. In PC-3 cells, under hypoxic conditions, siSphK1 treatment was paralleled by a significantly lower HIF-1α content (Fig. 1D, top). A similar effect was achieved by pharmacologic inhibition of SphK1 (Fig. 1D, top). Likewise, siSphK1 treatment resulted in decreased HIF-1α level in A549, U87, and SUM-159PT tumor cells after 6 hours of hypoxia (Fig. 1D, bottom).

To establish that this down-regulation of HIF-1α protein was correlated with an inhibition of HIF-1α transcriptional activity, we examined whether SphK1 silencing or its pharmacologic inhibition could regulate the transcriptional functions of HIF-1α. Under hypoxia, the increase in HRE-mediated reporter gene activity was remarkably decreased after sphingosine kinase inhibitor treatment (data not shown). Specifically, we chose to analyze the level of GLUT-1, a marker of hypoxia. In normoxia, there was no detectable GLUT-1 protein in both PC-3 and U87 cells (Fig. 2A). Under hypoxic conditions, there was a strong induction of GLUT-1 protein that was markedly inhibited by SphK1 silencing (Fig. 2A) or sphingosine kinase inhibitor (Fig. 2B) in both cell models. Similar findings were observed in the other cell models tested (data not shown).

**SphK1 is activated under hypoxia via a ROS-dependent mechanism.** Because SphK1 inhibition could prevent HIF-1α accumulation under hypoxic conditions, it was of interest to find out whether the SphK1 activity was regulated by hypoxia. Under low oxygen concentration, an early and transient stimulation of

**Figure 2.** SphK1 inhibition by pharmacologic inhibitor or RNA silencing blocks HIF-1α activity in hypoxic PC-3 and U87 cancer cells. A, PC-3 and U87 cells were untransfected or transfected with 20 nmol/L of siSphK1 or scrambled siRNA (siScr) for 72 h, then incubated under normoxia or hypoxia for 6 h. B, human prostate PC-3 and brain U87 cancer cells were treated with 5 μmol/L sphingosine kinase inhibitor under hypoxic conditions for 6 h. Cell lysates were assayed for GLUT-1 expression by Western blot analysis. Similar results were obtained in three independent experiments.
SphK1 activity was noted, peaking at 2 hours, after which SphK1 activity returned to normal levels (Fig. 3B and D). Noteworthy, HIF-1α accumulation (Fig. 3A and C) followed the SphK1 stimulation in both PC-3 and U87 cell lineages (Fig. 3B and D). One emerging mechanism of regulation for HIF-1α stability under hypoxia involves ROS. In agreement with recent studies (10), the ROS scavenger N-acetylcysteine could markedly prevent HIF-1α accumulation under hypoxia in PC-3 cells (Fig. 3A). We thus studied SphK1 activity in the presence or absence of N-acetylcysteine. Whereas no significant effect was observed in normoxic conditions, N-acetylcysteine could abrogate the surge in SphK1 activity induced by hypoxia (Fig. 3B). A similar effect of N-acetylcysteine was detected in the U87 glioblastoma cell model (data not shown). Therefore, we hypothesize that SphK1 is a downstream target of ROS during hypoxia.

HIF-1α degradation induced by SphK1 inhibition is controlled by the proteasome pathway via pVHL. HIF-1α is known to be degraded by the ubiquitin-proteasome pathway; thus, we sought to determine whether HIF-1α degradation induced by SphK1 inhibition relied on the proteasome machinery. As anticipated, the treatment of PC-3 and U87 cells by the proteasome inhibitor MG132 under normoxic conditions was associated with increased HIF-1α levels (Fig. 4A and B). Noteworthy, no differences in HIF-1α content were detected in PC-3 (Fig. 4A) and U87 (Fig. 4B) cells treated with sphingosine kinase inhibitor or siSphK1 under hypoxia in the presence of MG132. To test whether inhibition of SphK1 had an effect on the stability of HIF-1α protein, we measured the half-life of HIF-1α protein after blocking de novo protein synthesis with cycloheximide. The half-life of HIF-1α was significantly decreased (by ~50%) when SphK1 was inhibited in both PC-3 (Fig. 4C) and U87 (Fig. 4D) cells, suggesting that SphK1 is involved in the stabilization of HIF-1α protein under hypoxic conditions.

To investigate whether pVHL was instrumental in SphK1-mediated modulation of HIF-1α levels, we took advantage of the human renal clear carcinoma cell line RCC10, which lacks pVHL, and a derivative line with reconstituted pVHL expression (RCC10 PT; Fig. 5A). In the pVHL-deficient parental RCC10 cells (RCC10 wt), HIF-1α levels were constitutively high and not influenced by hypoxia or SphK1 inhibition by sphingosine kinase inhibitor (Fig. 5). On the other hand, the pVHL-reconstituted RCC10 cells responded to hypoxia by a physiologic surge in HIF-1α levels that could be abrogated by pharmacologic inhibition of SphK1 (Fig. 5B). These observations support the idea that SphK1 inhibition decreases HIF-1α levels by stimulating HIF-1α degradation in a pVHL-dependent manner.

SphK1 modulates HIF-1α level via phosphorylation of AKT and GSK3β. One of the mechanisms regulating HIF-1α stability involves GSK3β through its inactivation by phosphorylation of its Ser9 residue by the activated protein kinase Akt. As previously reported in other cancer cell models (14, 17, 18, 23), there was an increase in phospho-Akt (Ser473) under hypoxic conditions in both PC-3 and U87 cells untransfected or transfected with scrambled RNAi (Fig. 6A). Under the same conditions, a significant increase in phospho-GSK3β (Ser9) content was observed (Fig. 6). In contrast, the hypoxia-triggered Akt phosphorylation and subsequent GSK3β
phosphorylation were abolished by SphK1 interference (Fig. 6A). We next examined the effect of GSK3β down-regulation on HIF-1α levels in SphK1-silenced U87 hypoxic cells. The treatment with siRNA directed against GSK3β (Fig. 6B) reversed the siSphK1-induced diminution of HIF-1α levels (Fig. 6C). Overall, these results establish that SphK1-mediated regulation of HIF-1α levels under hypoxia relies on the activation of the Akt/GSK3β signaling pathway.

**Discussion**

The sphingolipid S1P is a potent metabolite that has been involved in a wide array of cellular processes including cell proliferation, calcium homeostasis, apoptosis, and angiogenesis (24). Similar to other signaling molecules, the S1P content in cells is low and strictly kept under control by the balance between its synthesis and degradation. SphK1 activity, responsible for S1P production, can be rapidly and transiently stimulated by many agonists, thus reflecting its central role in controlling S1P levels. Once generated, S1P can act either intracellularly as a second messenger or be released from cells to activate cell surface S1P receptors to elicit paracrine or autocrine signaling cascades (24). Nevertheless, the agonist-induced S1P generation as well as its downstream effects can be impeded by SphK1 pharmacologic inhibition. Thus, SphK1, and its activation, plays a decisive and necessary role in the observed effects ascribed to S1P.

In cancer, overwhelming evidence has implicated SphK1 in promoting oncogenesis and response to treatments. Since the pioneering work of Xia and colleagues (27) showing the oncogenic nature of SphK1, SphK1 mRNA was found to be up-regulated in a number of solid tumors (28); high SphK1 expression in brain tumor was correlated with poor survival of patients (29). On the other hand, anticancer treatments (chemotherapies and radiation therapy) have been shown to down-regulate SphK1 activity in various cancer cell and animal models, suggesting that SphK1 could act as a “sensor” to anticancer therapies (26). Surprisingly, given the importance of hypoxia in cancer, the relationship between SphK1 and adaptation to hypoxic conditions had not been investigated to date in tumor cells, whereas preliminary data support an instrumental connection between ischemia and SphK1 in normal cells. Indeed, we and others have shown both in vitro and in vivo the implication of SphK1 in the adaptation of cardiomyocytes to ischemia (31, 32). Additionally, a connection has been hypothesized between the SphK1/S1P metabolism and hypoxia based on the SphK1-dependent vascular smooth muscle cell growth increase under acute hypoxic stress (33) and the transcriptional mediated SphK1 expression in pulmonary smooth muscle cells under both acute and chronic hypoxia (34). More recently, SphK1-null cardiomyocytes have been shown to be more susceptible to hypoxia and glucose deprivation (35). A single article has recently suggested a link between SphK1 and CoCl2 chemically induced hypoxia in cancer cells (36). Under these conditions, SphK1 expression was down-regulated, and SphK1-null cells were more susceptible to hypoxia and glucose deprivation. These findings suggest that SphK1 may play a role in the adaptation of cancer cells to hypoxia.

**Figure 4.** SphK1 regulates proteasome-dependent HIF-1α degradation in hypoxic PC-3 and U87 cancer cells. Human prostate PC-3 (A) or brain U87 (B) cancer cells were exposed to normoxia or hypoxia for 6 h and then tested for HIF-1α content by immunoblotting with an anti–HIF-1α antibody (A and B). For siRNA treatments, cells were untransfected or transfected with 20 nmol/L siSphK1 or scrambled siRNA for 3 d before the experiments. For sphingosine kinase inhibitor treatments, cells were treated with 5 μmol/L sphingosine kinase inhibitor at the beginning of the experiments. MG132 was added into the medium 2 h before the end of the incubation. Representative of at least three independent experiments. PC-3 (C) or U87 (D) cells were incubated under hypoxic conditions in the presence or absence of sphingosine kinase inhibitor for 6 h. Cells were then treated with 50 μg/mL cycloheximide, and total proteins were extracted at the indicated times and subjected to Western blot analysis to determine the relative density between HIF-1α and β-actin. Columns, mean of four independent experiments done in duplicate; bars, SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
In this report, we propose an novel function for SphK1 in cancer: adaptation to hypoxia. Indeed, we show for the first time that SphK1 regulates HIF-1α accumulation on bona fide hypoxia, and that this effect relies on pVHL-dependent proteosomal degradation. Conducted in five distinct tumor cell lineages (glioma, prostate, breast, lung, and kidney), our studies suggest a canonical role for SphK1 in cancer adaptation to hypoxic stress. With respect to hypoxia and HIF-1α, glioblastoma and prostate adenocarcinoma are of particular relevance, which prompted us to further detail the role of SphK1 in these models. Indeed, in glioblastomas, increased vascularizability is a harbinger of poor prognosis (37); increased SphK1 activity was shown to be correlated to aggressive behavior and poor survival in patients (29); and HIF-1α silencing was recently shown in a preclinical model to attenuate cell growth (38). In prostate cancer, we have shown that SphK1 activity was strikingly increased in tumor samples and correlated with prostate-specific antigen level and tumor grade. In addition, HIF-1α overexpression has been shown to be strongly correlated with prostate carcinogenesis (39), and mouse transgenic models suggested that HIF-1α accumulation preceded vascular endothelial growth factor (VEGF) and VEGF receptor overexpression (40), a hallmark of the angiogenic switch.

Accordingly, we focused on PC-3 prostate and U87 glioblastoma cell models where we showed a significant increase in SphK1 activity before HIF-1α accumulation. Since the seminal article by Wang and colleagues (41), the main regulatory mechanism of HIF-1α accumulation in hypoxia is the pVHL-mediated proteosomal degradation. Using a pVHL-deficient cancer cell model and a derivative with reconstituted pVHL expression, we found that whereas HIF-1α accumulation on hypoxia was observed in both cell models, it could be abrogated by SphK1 inhibition only in pVHL reconstituted line, suggesting that SphK1 drives HIF-1α accumulation through a pVHL-dependent mechanism. This was further reinforced by the demonstration that in U87 and PC-3 cells with functional pVHL, the proteasome inhibitor MG132 abrogated the inhibitory effect of pharmacologic and RNA silencing inhibition of SphK1 on HIF-1α accumulation.

Having shown that SphK1 was instrumental for HIF-1α accumulation and transcriptional activity, we sought to understand how low oxygen conditions could trigger SphK1 activation. Recent evidence supports a link between ROS and HIF-1α stabilization (5–9) through oxidation of the ferrous ion that is central for the catalytic hydroxylation of proline residues (10). In line with previous studies, the ROS scavenger N-acetylcysteine prevented HIF-1α accumulation. A novel finding was that it could also block SphK1 activation on hypoxia that preceded HIF-1α accumulation. We therefore hypothesize that ROS action on HIF-1α accumulation results from a dual effect: inhibition of prolyl-hydroxylases and ROS activation of SphK1 activity by a mechanism yet unknown. Interestingly, sphingomyelinase activity, which is responsible for the generation of ceramide, the precursor of the substrate of SphK1, has been shown to be regulated by N-acetylcysteine (42), suggesting that other enzymes of the sphingolipid metabolism might mediate the effect of ROS toward SphK1 activity.

Finally, another pathway for ROS regulation of HIF-1α, the PI3K/Akt/GSK3β pathway, has recently been implicated (23). The PI3K/Akt pathway is often up-regulated in aggressive cancers associated with increased angiogenesis, notably by mutated phosphatase and tensin homologue deleted on chromosome 10 (PTEN), such as in glioblastoma (43, 44) and prostate cancer (45–47). PTEN restoration, by inhibiting Akt signaling, was shown in glioma cell line to ablate hypoxia-triggered induction of HIF-1α-regulated genes (13). We here show by RNA interference that irrespective of the PTEN status, SphK1 modulation of HIF-1α under hypoxia is associated with the activating phosphorylations of Akt and GSK3β thereby positioning SphK1 upstream of the Akt/GSK3β pathway.

In human tumors, increased expression of HIF-1α is induced by physiologic stimulation as well as genetic alterations such as PTEN or p53 loss-of-function mutations. It is of importance to note that our results have shown that SphK1 inhibitory strategies were able to almost abrogate HIF-1α expression in cancer cells regardless of their PTEN or p53 status (e.g., PC-3 cells are null for p53 and PTEN; U87 cells contain wild-type p53 but are null for PTEN; A549 cells contain wild-type p53 and PTEN).

To conclude, we show for the first time the strong interaction between the SphK1/SIP pathway and the adaptation to hypoxia. Because HIF-1α mediates adaptive responses to low oxygen supply, a hallmark of aggressive solid tumors, we anticipated a strong link between this master regulator of oxygen homeostasis and the oncogenic lipid kinase SphK1, whose SIP product is one of the most potent proangiogenic agents. Indeed, we show that SphK1

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5 B. Malavaud and O. Cuvillier, in preparation.

Figure 5. The reduction of HIF-1α level by SphK1 inhibition relies on pVHL. A, VHL expression was examined by Western blotting with an anti-pVHL antibody in VHL-negative renal cell carcinoma cells (RCC10) or its VHL gene expression reconstituted counterpart (RCC10 PT). B, RCC10 and RCC10 PT were grown under normoxia or hypoxia in the presence or absence of 5 μM sphingosine kinase inhibitor. Cells were lysed and HIF-1α expression was analyzed by immunoblotting with an anti-HIF-1α antibody. Similar results were obtained in three independent experiments.
controls HIF-1α stability through a mechanism connecting ROS and the downstream Akt/GSK3β pathway.

As recently shown with PI3K inhibition (48) and direct anti-VEGF strategies (49), we propose that inhibiting the SphK1 surge in activity under hypoxia might normalize the tumor microenvironment and increase tumor sensitivity to radiation and chemotherapy in the broader concept of “normalization of tumor vessels,” as tumor oxygenation is known to enhance response to chemotherapy and radiation. This concept, with a pan-VEGF receptor tyrosine kinase inhibitor, was recently brought into the clinic and shown to be able to control tumor vessel permeability and size and inhibit in a transitory manner tumor growth in glioblastoma patients (50). Therefore, we postulate that the relevance of SphK1/S1P targeting inhibitory strategies in cancer could be extended to the realm of hypoxia.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Figure 6. SphK1 modulates HIF-1α level via phosphorylation of AKT and GSK3β. A, PC-3 and U87 cells were untransfected or transfected with 20 nmol/L of siSphK1 or scrambled siRNA for 72 h, then incubated under normoxia or hypoxia for 6 h. Cells were lysed and phospho-AKT and phospho-GSK3β expression were analyzed by immunoblotting with anti–phospho-AKT (Ser473) and anti–phospho-GSK3β (Ser9) antibodies, respectively. B, GSK3β expression in U87 cells treated in the presence or absence of 50 nmol/L of GSK3β siRNA (siGSK3β) or scrambled siRNA was examined by Western blotting with an anti-GSK3β antibody. C, U87 cells, transfected with either GSK3β siRNA (50 nmol/L) and/or siSphK1 (20 nmol/L) as indicated, were incubated under hypoxia for 6 h. Cells were lysed and HIF-1α expression was analyzed by immunoblotting with an anti–HIF-1α antibody. Similar results were obtained in three independent experiments.

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