Activation of HIF-prolyl Hydroxylases by R59949, an Inhibitor of the Diacylglycerol Kinase*

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Hypoxia-inducible factors (HIF) are heterodimeric (α/β) transcription factors that play a fundamental role in cellular adaptation to low oxygen tension. In the presence of oxygen, the HIF-α subunit becomes hydroxylated at specific prolyl residues by prolyl hydroxylases. This post-translational modification is recognized by the von Hippel-Lindau (VHL) protein, which targets HIF-α for degradation. In the absence of oxygen, HIF-α hydroxylation is compromised and this subunit is stabilized. We have previously shown that the hypoxia-induced accumulation of HIF-α protein is strongly impaired by the inhibitor of diacylglycerol kinase, R59949. Here, we have investigated the mechanisms through which this inhibitor exerts its effect. We found that R59949 inhibits the accumulation of HIF-1α protein without affecting the expression of their mRNAs. We also determined that R59949 could only block the accumulation of HIF-α in the presence of VHL protein. In agreement with this, the binding of VHL to endogenous HIF-α was significantly enhanced after R59949 treatment, even under hypoxic conditions. In addition, we found that R59949 could stimulate prolyl hydroxylase both at 21% O₂ as well as at 1% O₂. Taken together, these results reveal that R59949 is an activator of HIF prolyl hydroxylases. This is of particular interest when we consider that, to date, main inhibitors of these enzymes have been described.

The best characterized response to hypoxia is the induction of hypoxia-inducible transcription factors (HIFs) (1). These transcription factors govern the oxygen-dependent induction of different genes, including those encoding vascular endothelial growth factor, erythropoietin, and glycolytic enzymes (2, 3). The HIFs are α/β heterodimers that belong to the basic helix-loop-helix PAS (Per, Arnt, Sim) family of transcription factors (4). The α subunit (HIF-1α or aryl hydrocarbon receptor nuclear translocator, ARNT) is constitutively expressed in cells, whereas the expression of the three different HIF-α subunits that have been identified to date (HIF-1α–3α), is tightly regulated by oxygen (5). During normoxia, HIF-α subunits are very unstable proteins because of their interaction with the von Hippel-Lindau (VHL) tumor suppressor protein (6). The VHL protein is a component of a E3 ubiquitin ligase complex (7) that leads to the ubiquitination and subsequent proteosome-dependent degradation of HIF-α subunits (8). VHL interacts with HIF-α subunits through two independent sites that contain the LXXLAP motif, present in both HIF-1α and -2α (9). The hydroxylation of the specific proline residue (underlined and bold) within this conserved box is required for this interaction (10, 11). This oxygen-dependent hydroxylation is catalyzed by a novel group of mammalian proline hydroxylases, PHD1, PHD2 and PHD3, which belong to the iron (II)-2-oxoglutarate-dependent dioxygenase family (12, 13). These three mammalian PHDs each present different subcellular localization (9, 14) and tissue-specific distribution (15). Although the three enzymes have the potential to regulate HIF in cultured cells (13), PHD2 is thought to be the most important in regulating the levels of HIF-1α during normoxia (16). These enzymes use molecular oxygen and 2-oxoglutarate as co-substrates, and iron and ascorbic acid are additionally required for the hydroxylation reaction (17). Moreover, these PHDs are also thought to be effective oxygen sensors, because their Kₘ values for O₂ are slightly above the atmospheric O₂ concentration (18). Besides controlling the activity of the PHDs, the concentration of oxygen also controls the transcription of PHD2 and PHD3 mRNAs (14, 19).

In the absence of oxygen, VHL cannot recognize HIF-α subunits and as a consequence, HIF activity is concomitantly induced. After the HIF-α subunits translocate to the nucleus, they associate with the β subunit and the HIF α/β heterodimers and then bind specifically to the hypoxia response elements in different target genes (20). The transcriptional activity of HIF is also controlled by the additional hydroxylation of a conserved asparagine residue (Asn-803 in HIF-1α and Asn-851 in HIF-2α) located in the C-terminal transactivation domain of HIF-α subunits (21). This hydroxylation blocks the association of the cysteine/histidine-rich (CH-1) domain of the p300 co-activator with HIF and therefore provides a second oxygen-dependent mechanism by which HIF can be regulated.
The factor inhibiting HIF is the asparaginyl hydroxylase responsible for this post-translational modification (22, 23). Thus, PHDs and factor-inhibiting HIF are two different HIF hydroxylases that can act as oxygen sensors and control HIF activity (17, 24, 25).

We have previously shown that hypoxia produces an increase in the levels of both diacylglycerol and phosphatidic acid (PA) through the activity of DGK (26, 27). Nine DGK isoforms have so far been described, and these phosphorylate diacylglycerol to generate PA. We have found that the pharmacological inhibition of DGK activity with R59949 (28–30) prevents the accumulation of both PA and the HIF-1α subunit induced by hypoxia (27). Here we have analyzed the effect of this inhibitor at the critical steps of HIF stabilization and activation pathways. Our data indicate that the mechanism by which this pharmacological agent inhibits hypoxia-regulated HIF involves the activation of HIF prolyl hydroxylases.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Antibodies were obtained from the following sources and used at the concentrations indicated: monoclonal PHD2 (1:1000, Bethyl Laboratories); monoclonal PHD1 (1:1000, Bethyl Laboratories); polyclonal PHD3 (1:2000, Bethyl Laboratories); monoclonal α-tubulin (1:2000, Sigma); and polyclonal Sp-1 (1:2000, Santa Cruz Biotechnology). Horseradish peroxidase-coupled sheep anti-rabbit antibody was obtained from Pierce, and the anti-rabbit horseradish peroxidase antibody was from Amersham Biosciences. The DGK inhibitor II (R59949) was purchased from Calbiochem and the [32P]phosphatidic acid (carrier-free) from Amersham Biosciences. Silica gel thin layer chromatography plates (60 Å, LK6D) were obtained from Whatman. The authentic phospholipids standards 1,2-diacylglycerol and 1,2-diacylglycerophosphatidic acid (Sphingosino) were purchased from Merck. Minimum essential medium without phosphates was obtained from ICN Biomedicals.

**Cell Culture and Hypoxic Conditions**—VHL-deficient 786-O (1–115) cells or PRC3 (31), RCC10 (32), UMRC6 (33) and their corresponding clones derived from stable transfection of VHL, 786-O-WT10, VHL53, and UMRC 3–4, as well as human embryonic kidney cells 293-T, were maintained in RPMI 1640 medium with GLUTAMAX-I (Invitrogen) supplemented with 10% fetal calf serum (Linus). HeLa cells were grown in Dulbecco’s minimal essential medium (Biochrom KG) supplemented with 5% fetal calf serum (Linus). HeLa cells were routinely cultured in 95% air and 5% CO2 (nормoxic conditions) at 37 °C. To expose the cells to hypoxia, they were placed in an airtight chamber with inflow and outflow of N2, and CO2 was infused with N2 (94% N2, 5% O2, 1% CO2). Alternatively, they were placed in an in vivo 400 hypoxia work station (Ruskinn Technology). In all experiments, cells were plated at 70–90% confluence, and when completely attached, they were exposed to normoxia or hypoxia for 5–6 h. When R59949 was used, it was added 1 h before any other treatments, and for these experiments, cells were grown in fetal calf serum-free medium.

**Western Blotting**—Proteins from total cell lysates were resolved on 8–12% SDS-polyacrylamide gels. The proteins were then transferred to a nitrocellulose membrane (Bio-Rad), blocked with 5% nonfat dry milk in TBS-T (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween 20) and incubated overnight at 4 °C with the antibodies indicated. Immunoblotting was detected by enhanced chemiluminescence (ECL, Amersham). Analysis of the gel was performed, and the blots were probed with anti-HIF-2α (ab20263) with 10 μg of IgG1 isotype negative control (anti-CD45) coupled to protein G-Sepharose beads (Amersham Bioscience) for 1 h at 4 °C. The beads were collected, and 5 μg of anti-VHL antibody coupled to protein G-Sepharose beads supernatants was added to the supernatant and incubated overnight at 4 °C. The beads were collected, washed twice with IP-lysis buffer, once with PBS, and stored in PBS at 4 °C.

**RNA Analysis**—Total RNA was isolated from 5 g of the 786-O–WT10 cell lysate in a final volume of up to 45 μl with reaction buffer (20 μl Heps, pH 7.5, 5 mM KCl, 1.5 mM MgCl2, 1 mM β-mercaptoethanol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, 5 μg/ml leupeptin). The cell suspension was incubated at 4 °C for 10–15 min and then forced 10 times through a 22-gauge needle. After removal of the cell debris by centrifugation, the protein concentration was quantified. The hydroxylation reaction was performed by incubating 10 μg of GST-HIF-2α (521–542) recombinate protein (34) with 10 μg of the 786-O–WT10 cell lysate in a final volume of up to 45 μl with reaction buffer (20 μl Heps, pH 7.5, 6 mM KCl, 1.5 mM MgCl2, 1 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, 5 μg/ml leupeptin). The cell suspension was incubated at 4 °C for 10–15 min and then forced 10 times through a 22-gauge needle. After removal of the cell debris by centrifugation, the protein concentration was quantified. The hydroxylation reaction was performed by incubating 10 μg of GST-HIF-2α (521–542) recombinate protein (34) with 10 μg of the 786-O–WT10 cell lysate in a final volume of up to 45 μl with reaction buffer (20 μl Heps, pH 7.5, 150 mM NaCl, 10% glycerol, 1.5 mM MgCl2, 5 mM KCl, 0.2% Nonidet P-40, 0.5...
mg/ml bovine serum albumin) containing 15 μl of in vitro transcribed/translated [35S]VHL (TNT quick coupled transcription/translation system from Promega). Samples were then incubated at 4 °C for 1 h, the protein complexes were recovered by centrifugation, washed twice with 30 μl of 1× loading buffer. Proteins resolved by SDS-PAGE were stained with Coomassie Blue to demonstrate equal GST-HIF-2 loading, fixed (methanol 10%, acetic acid 10%, and glycerol 10%) for 30 min at room temperature, and incubated with Amplify (Amersham Biosciences) for an additional 10–15 min. The gels were exposed to an x-ray film and bound [35S]VHL was detected by autoradiography. To demonstrate the linear conditions in these assays, GST-HIF was exposed to a 2-fold concentration of control cell lysate, in which case about twice the amount of [35S]VHL was recovered. The GST-HIF-2 protein was determined by Western blot (upper panels), and as a loading control, the membranes were probed with an anti-α-tubulin protein antibody. In parallel, the abundance of each HIF mRNA was determined by quantitative RT-PCR. The amounts of HIF-1α and -2α were normalized with β-actin in each case. The absolute values obtained in three or four independent experiments performed in duplicate and their averages are shown (lower panels). The Western blots shown are the results of one representative experiment.

RESULTS

R59949 Specifically Inhibits the Accumulation of HIF Protein without Affecting Its mRNA Levels—We previously reported that R59949 inhibits the accumulation and the transcriptional activity of HIF-1α in cells maintained in atmospheres containing 1% oxygen (27). To identify the mechanisms by which R59949 affects HIF, we first analyzed whether this inhibitor affected the levels of HIF mRNA. As such, we treated 293-T, 786-O-WT10, and UMRC-3.4 cells with R59949 (30 μM) and maintained them in conditions of normoxia or hypoxia (1% O2) during 6 h. Subsequently, total protein extracts were obtained, and in parallel, total RNA was isolated and analyzed by quantitative RT-PCR. The accumulation of both HIF-1α (293-T and UMRC-3.4) and HIF-2α (786-O-WT10) protein subunits was induced in conditions of hypoxia, and the levels of these proteins was diminished in the presence of R59949 (Fig. 1, upper panels). However, neither hypoxia nor R59949 treatment significantly modified the basal normoxic mRNA levels in any of these cell types (Fig. 1, lower panels; one-way analysis of variance, p > 0.05). The minor changes observed on occasion could not account for the strong inhibition observed at the protein level. Therefore, the inhibition of HIF-α protein accumulation promoted by R59949 was not due to a decrease in its mRNA levels.

HIF Protein Inhibition by R59949 Is VHL-dependent—The degradation of HIF protein is regulated by the VHL-proteo-
in the hydroxylated forms of HIF-α subunits can only be maintained in VHL-non-expressing cells. To analyze how R59949 might affect the recognition of HIF by VHL, we performed in vitro HIF-VHL binding assays using HIF protein recovered from RCC10 and 786-O-(1–115), cells that lack VHL. These cells were treated with R59949 in conditions of hypoxia or normoxia, and then transferred to hypoxic conditions (Hx, 1% O2) or left in normoxic conditions (N, 21% O2) for a further 5 h. Total cell extracts were resolved by SDS-PAGE and transferred to nitrocellulose membranes to analyze the levels of HIF proteins using the antibodies indicated. As a loading control, α-tubulin (A) or Sp-1 (B) were also probed in the same membranes. Similar results were obtained in four experiments, and the data shown is from a representative experiment. C, 786-O VHL+/− and VHL−/− cells were metabolically labeled with [32P]orthophosphate. The cells were then pretreated with R59949 at 30 μM for 1 h or left untreated prior to maintaining them in conditions of hypoxia (Hx) or normoxia (N) for an additional 6 h. Cellular phospholipids were extracted, and the radiolabeled PA was resolved by thin layer chromatography, visualized by exposure to x-ray film, and quantified. The radiolabeled PA content was corrected to the total amount of labeled material, assigning the value of 1 to the normalized PA level on the normoxic control. Each bar represents the induction of PA (mean ± range) of two independent experiments performed in triplicate.

**FIG. 2.** R59949 does not inhibit HIF accumulation in VHL negative cells. VHL-expressing and -non-expressing 786-O (A) and RCC10 (B) cells were grown to 90% confluence in 6-well plates. The cells were pretreated for 1 h with R59949 at doses between 10 and 40 μM, as indicated, and then transferred to hypoxic conditions (Hx, 1% O2) or left in normoxic conditions (N, 21% O2) for a further 5 h. Total cell extracts were resolved by SDS-PAGE and transferred to nitrocellulose membranes to analyze the levels of HIF proteins using the antibodies indicated. As a loading control, α-tubulin (A) or Sp-1 (B) were also probed in the same membranes. Similar results were obtained in four experiments, and the data shown is from a representative experiment. C, 786-O VHL+/− and VHL−/− cells were metabolically labeled with [32P]orthophosphate. The cells were then pretreated with R59949 at 30 μM for 1 h or left untreated prior to maintaining them in conditions of hypoxia (Hx) or normoxia (N) for an additional 6 h. Cellular phospholipids were extracted, and the radiolabeled PA was resolved by thin layer chromatography, visualized by exposure to x-ray film, and quantified. The radiolabeled PA content was corrected to the total amount of labeled material, assigning the value of 1 to the normalized PA level on the normoxic control. Each bar represents the induction of PA (mean ± range) of two independent experiments performed in triplicate.
confluence in 6-well plates. The cells were pretreated for 1 h with R59949 at the doses indicated and then transferred to conditions of hypoxia (21% O2) or left in normoxic conditions (N, 21% O2) for a further 5 h. Total cell extracts were resolved by SDS-PAGE and transferred to nitrocellulose membranes to analyze the levels of VHL protein. To ensure the correct function of R59949, HIF-1α and -2α protein levels were also analyzed. As a loading control, α-tubulin was also probed in the same membranes. The experiments shown are representative of the three independent experiments performed. Arrowheads indicate VHL.

FIG. 3. R59949 does not inhibit the accumulation of VHL protein. UMRC-3.4 (A) and 786-O-WT10 (B) cells were grown to 90% confluence in 6-well plates. The cells were pretreated for 1 h with R59949 at the doses indicated and then transferred to conditions of hypoxia (Hx, 1% O2) or left in normoxic conditions (N, 21% O2) for a further 5 h. Total cell extracts were resolved by SDS-PAGE and transferred to nitrocellulose membranes to analyze the levels of VHL protein. The accumulation of the HIF-2 isoform. Additionally, we have determined whether R59949 could regulate the intracellular pools of ascorbate or iron, as well as their uptake. Thus, further studies will be necessary to fully understand the role of R59949 and these cofactors on HIF hydroxylase activity. However, under the influence of certain stimuli that promote cell proliferation, such as exposure to growth factors, oncogenic transformation, etc., these cofactors become limiting. As a result, enzyme activity is reduced and HIF accumulates (45), but the addition of iron or ascorbic supplements under these conditions will restore enzyme activity. It remains to be determined whether R59949 could regulate the intracellular pools of ascorbate or iron, as well as their uptake. Thus, further studies will be necessary to fully understand the role of R59949 and these cofactors on HIF hydroxylase activity. Recently, it has been proposed that reactive oxygen species influence HIF hydroxylase activity in junD−/− cells by promoting the oxidation of Fe(II) to Fe(III) and therefore increasing the proportion of inactive PHD in the Fe(III) oxidation state (46). Hence, according to these data, we cannot exclude that R59949 could affect the levels of ROS and that this might account for its positive effect on PHD activity. Finally, R59949 could inhibit unidentified negative regulators of PHDs. Although the regulation of PHD activity remains poorly understood, there is some evidence that novel proteins might co-operate with PHDs in the regulation of HIF. The increase of VHL-HIF binding is because of the increase in PHD activity promoted by R59949.

We have previously described that hypoxia induces an increase of the levels of phosphatidic acid in parallel with the accumulation of HIF protein (27, 37). The accumulation of both PA and HIF induced by hypoxia is abrogated by R59949 treatment. This compound has been characterized as a specific inhibitor of type I DGKs (28–30), and these enzymes have been proposed to play a role in the regulation of HIF in conditions of hypoxia (27). In the present study, we show that R59949 is a novel activator of HIF hydroxylase activity, suggesting a possible role of PA on PHD activity. Interestingly, we found that D609, a sphingomyelin synthase/phosphatidylcholine phospholipase C inhibitor that impairs hypoxia-induced PA and HIF activation (26, 38, 39), also increases the formation of VHL-HIF complexes (data not shown). This accumulation of PA might play a role in regulating the interaction between VHL and HIF. Hence, PA accumulation in conditions of hypoxia could inhibit PHD activity, contributing to HIF activation. Given that PA is a lipid second messenger critical in the response to different stimuli, such as growth factors, cytokine stimulation, etc (40–43), its potential to regulate HIF prolyl hydroxylases is an exciting possibility.

Because oxygen is a substrate for hydroxylase activity, it is possible that R59949 stimulates PHD activity by increasing the affinity of these enzymes for oxygen. At low oxygen tension (1% O2), the activity of these enzymes is reduced, R59949 could enhance the ability to bind the remaining molecular oxygen, thereby increasing the hydroxylation and degradation rate of HIF. Furthermore, given that the hydroxylation reaction requires different cofactors in addition to molecular oxygen, R59949 might also affect these cofactors. In this regard, the inhibition of HIF hydroxylases by transition metals, such as nickel or cobalt, may to some extent be due to the depletion of the intracellular ascorbate (44). It has also been shown that under normal tissue culture conditions, the levels of ascorbate and iron are sufficient to permit HIF hydroxylase activity. However, under the influence of certain stimuli that promote cell proliferation, such as exposure to growth factors, oncogenic transformation, etc., these cofactors become limiting. As a result, enzyme activity is reduced and HIF accumulates (45), but the addition of iron or ascorbic supplements under these conditions will restore enzyme activity. It remains to be determined whether R59949 could regulate the intracellular pools of ascorbate or iron, as well as their uptake. Thus, further studies will be necessary to fully understand the role of R59949 and these cofactors on HIF hydroxylase activity. Recently, it has been proposed that reactive oxygen species influence HIF hydroxylase activity in junD−/− cells by promoting the oxidation of Fe(II) to Fe(III) and therefore increasing the proportion of inactive PHD in the Fe(III) oxidation state (46). Hence, according to these data, we cannot exclude that R59949 could affect the levels of ROS and that this might account for its positive effect on PHD activity. Finally, R59949 could inhibit unidentified negative regulators of PHDs. Although the regulation of PHD activity remains poorly understood, there is some evidence that novel proteins might co-operate with PHDs in the degradation of HIF (47, 48).

In the last few years, HIF-1α and -2α have been implicated in tumor progression as well as in pathological angiogenesis (49–51). HIF expression promotes cell survival in a hypoxic microenvironment, increasing the expression of proteins that regulate metabolic adaptation (glucose transporter-1, lactate dehydrogenase), resistance to apoptosis (nitric-oxide synthase-2, transforming growth factor-α), angiogenesis (vascular endothelial growth factor, transforming growth factor-β3, vas...
cific vascular endothelial growth factor R2), and invasion and metastasis (c-MET, urokinase-type plasminogen activator receptor) (52). In addition, hypoxic cancer cells are more likely to be resistant to radiation and chemotherapy (53, 54), and therefore HIF inhibition has been proposed as a therapeutic target in these circumstances. Because PHDs are responsible for the regulation of HIF, novel anti-HIF therapies may be focused on regulating the activity of these hydroxylases (55, 56). In this regard, the effect of R59949 in stimulating these enzymes opens new possibilities for the use of this inhibitor as a therapeutic agent in cancer. One advantage of this compound is that it inhibits the stabilization of both HIF-1α/HIF-2α with the same efficiency, and it can therefore be used to treat cells regardless the HIF isoform expressed.

In addition to cancer therapy, R59949 could also be used in the treatment of diabetic retinopathies, pulmonary hypertension, or inflammation, pathologies where HIF up-regulation is also involved. Nevertheless, in vivo experiments might be needed to validate the design of R59949-related treatments and its possible use in clinical therapies.

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