Evidence for the Involvement of Diacylglycerol Kinase in the Activation of Hypoxia-inducible Transcription Factor 1 by Low Oxygen Tension*

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Hypoxia-inducible factor 1 (HIF-1) induces a gene expression program essential for the cellular adaptation to lowered oxygen environments. The intracellular mechanisms by which hypoxia induces HIF-1 remain poorly understood. Here we show that exposure of various cell types to hypoxia raises the intracellular level of phosphatidic acid primarily through the action of diacylglycerol kinase (DGK). Pharmacological inhibition of DGK activity through use of the specific DGK inhibitors R59949 and R59022 abrogated specifically HIF-1-dependent transcription analyzed with a HIF-1-responsive reporter plasmid. A more detailed analysis revealed that pharmacological inhibition of DGK activity prevented the hypoxia-dependent accumulation of the HIF-1α subunit and the subsequent HIF-1-DNA complex formation as well as hypoxia-induced activity of the HIF-1 transactivation domains localized to amino acids 530–582 and 775–826 of the HIF-1α subunit. Our results demonstrate for the first time that accumulation of phosphatidic acid through DGK underlines oxygen sensing and provide evidence for the involvement of this lipid kinase in the intracellular signaling that leads to HIF-1 activation.

Cells respond to insufficient oxygen delivery to tissues by inducing a gene expression program governed by the transcription factor hypoxia-inducible factor 1 (HIF-1)† (1–5). Some of the genes induced via HIF-1 are those encoding the vascular endothelial growth factor (6), which triggers the neovascularization of hypoxic regions; the glucose transporters and enzymes involved in glycolysis that facilitate the oxygen-independent provision of ATP enhancing the glycolytic metabolic pathway (7); and erythropoietin (8), which elevates the blood oxygen-transport capability by increasing the number of red blood cells. More recently, the generation of knockout mice for HIF-1 and the analysis of HIF-1-deficient cell lines has underlined the essential role of this transcription factor in embryonic development as well as in tumor progression (9–13).

HIF-1 is an heterodimer composed of α and β subunits that belong to the basic helix-loop-helix Per-Arnt-Sim family of transcription factors (14). In normoxia, HIF-1α is a very unstable protein that is rapidly degraded by the ubiquitin-proteasome pathway (15, 16). After the onset of hypoxia, HIF-1α is stabilized, resulting in the formation of the HIF-1α/β heterodimer that binds to DNA hypoxia-responsive elements to drive transcription (17). The complete activation of HIF-1 by hypoxia also requires the induction of its transactivatory function, which has been localized to amino acids 530–582 and 775–826 within the HIF-1α subunit (18, 19).

The intracellular mechanisms that connect oxygen sensing and the activation of HIF-1 remain poorly understood. The involvement of protein phosphorylation has been proposed as inhibitors of tyrosine and serine/threonine kinases block HIF-1-dependent transcription (20, 21). Recently, it has been reported that hypoxia generates mitochondrial reactive oxygen intermediates (ROIs) that are critical for the induction of HIF-1-dependent transcription (22, 23). More recently, the Ser-Thr kinase AKT and the mitogen-activated protein kinase have been also implicated in the regulation of HIF-1-dependent transcription (24–26).

The activation of signal transduction pathways in response to different extracellular stimuli often results in the accumulation of lipid second messengers, such as diacylglycerol (DAG) and phosphatidic acid (PA), due to the direct action of phospholipase C and phospholipase D (PLD), respectively (27, 28). DAG serves as an allosteric activator of classical and novel protein kinase C (PKC) isoforms that mediate many cellular responses, including cell growth and differentiation (29–31). PA has been suggested to participate in different cellular processes, such as cell proliferation and actin polymerization (32–34). The intracellular level of these two lipids can also be modulated by the action of DGK, which generates PA by phosphorylation of DAG (35–37). Although the role of DGK in signal transduction is poorly understood, it has been proposed that DGK activity might be involved in the attenuation of positive DAG signaling.
(38), as well as in the generation of PA for downstream intracellular events (39–42).

In the present work we have investigated the role of lipid second messengers in the cellular response to hypoxia and their involvement in the regulation of HIF-1-dependent transcription. Previous reports have shown that cellular exposure to hypoxia induces the intracellular accumulation of DAG (43, 44). Herein we show that exposure to hypoxia also results in a marked accumulation of the intracellular level of PA through the action of DGK. In addition, pharmacological inhibition of DGK activity with the specific DGK inhibitors R59949 and R59022 prevents HIF-1-dependent transcription due to a reduction of hypoxia-induced HIF-1-DNA complex formation, as well as HIF-1α subunit transactivation activity. These results demonstrate for the first time that PA generation through DGK is part of the cellular response to hypoxia and provide evidence for an essential role of this lipid kinase in the signal transduction pathway that culminates in the activation of HIF-1.

Experimental Procedures

Reagents—[32P]Orthophosphate (carrier free) and [γ-32P]ATP (specific activity 3000 Ci/mmol) were purchased from Amer sham Biotech. Solvents for thin layer chromatography plates (60 Å, LKGD) were from Whatman (Clifton, NJ). DGK inhibitor I (R59922) and DGK inhibitor II (R59949) were purchased from Calbiochem (La Jolla, CA). The authentic phospholipid standards 1,2-dioleoylphosphoglycerol and 1,2-dioleoylphosphatidic acid were from Sigma, and phosphatidylbutanol (PBut) was from Avanti Polar Lipids, Inc. (Alabaster, AL). Phorbol 12-myristate 13-acetate (PMA) was from Sigma. Analytic grade organic solvents for TLC were from Merck (Darmstadt, Germany).

Cell Culture, Cell Treatments, and Hypoxic Conditions—HeLa cells were grown in RPMI 1640 medium with GLUTAMAX-I (Life Technologies Ltd.), whereas 293-T and Hep3B cells were grown in Dulbecco’s minimal essential medium (Biocrom KG, Berlin, Germany) in the presence of 10% (v/v) fetal calf serum (Lutbeck International Ltd., Woodside, United Kingdom). Cells were routinely cultured in 95% air/5% CO2 (normoxic conditions) at 37 °C. To expose cells to hypoxia, they were placed into an airtight chamber with inflow and outflow valves that was infused with a mixture of 1% O2, 5% CO2, 94% N2 (S.E. Carburos Metalicos S.A., Madrid, Spain) for 30 min. In all experiments, cells were plated at 70–90% confluency, and when cells were completely attached, after 3–4 h in the case of HeLa cells or after 12–14 h in the case of 293-T and Hep3B cells, they were exposed to normoxia or hypoxia in the presence or in the absence of R59949 or R59022. The cellular incubation with both DGK inhibitors was performed in serum-free medium because this compound is inactive in the presence of serum (40). All other experiments were performed using 10% fetal calf serum-supplemented medium.

Measurement of [32P]-Radiola beled Phospholipids—Cells were cultured in phosphate-free medium supplemented with 10% (v/v) fetal calf serum (extensively dialyzed against 0.9% (w/v) NaCl) for 90 min before the addition of [32P]orthophosphate (100 µCi/ml) for an additional 90 min. Thereafter, the cells were exposed to normoxia or hypoxia in the presence or in the absence of 0.5% (v/v) butan-1-ol. Phospholipids were then extracted by the method of Bligh and Dyer (45) and separated on TLC plates developed with a solvent system consisting of ethyl acetate/isooctane/acetic acid (9:5:2 (v/v/v)) for the separation of [32P]PA and [32P]PBut or chloroform/pyridine/88% formic acid (60:30:7 (v/v/v)) for the detection of endogenous levels of [32P]PA in the absence of 1-butanol. The dried TLC plates were subjected to autoradiography, and the bands corresponding to radiola beled PA and PBut were identified by comigration with authentic nonlabeled PA and PBut standards. Quantification of the band corresponding to [32P]PA was performed using the Bio-Rad Molecular Analyst Software.

Determination of DAG Levels—Total lipids were extracted from cells exposed to normoxia or hypoxia for 6 h. The amount of DAG was determined by its conversion into [32P]PA by Escherichia coli DGK in the presence of [γ-32P]ATP as previously described (46). DAG levels were corrected to total phospholipid phosphate content. The method of Bartlett (47) was used for the assay of total phosphate.

Measurement of DGK Activity—DGK activity was determined in cell lysates as previously described (39). Briefly, cell lysates were incubated with 1,2-dioleoylphosphoglycerol in the presence of [γ-32P]ATP. Thereafter, the generation of [32P]PA by endogenous DGK was determined by its separation using TLC employing a solvent system consisting of chloroform/ methanol/4 M ammonium hydroxide (9:7:2 (v/v/v)). The band corresponding to radila beled PA was quantified as above.

Recombinant Plasmas—To generate the pHIF1-Luc reporter plasmid, we cloned nine copies in tandem of an oligonucleotide containing the HIF-1 binding sequence located between positions –985 and –951 of the human vascular endothelial growth factor gene promoter (6, 48), upstream of a minimal promoter fused to the firefly luciferase cDNA. For the generation of pBEGR Luc, we cloned a single copy of the following nucleotide sequence (5′ to 3′) containing three binding sites for the transcription factor denominated early growth response factor 1 (EGR-1) (underlined): GCGCCCCCGAAGCTTGGCCCGGACGGCCCGCA.

To generate constructs encoding the GAL4 DNA binding domain (amino acids 1–147) (GAL4 DBD) in-frame with the amino acid sequences 530–582 or 775–826 of HIF-1α subunit, we amplified these regions using the polymerase chain reaction with primers containing BamHI overhangs and using as template the HIF-1α expression vector (kindly provided by Dr. E. Huang: Brigham & Women’s Hospital, Harvard Medical School, Boston, MA). The polymerase chain reactions were performed under the previously described conditions (18). Thereafter, polymerase chain reaction products were cloned into the BamHI site of the pGAL4 DBD expression vector to generate the constructs designated pGAL4 DBD (530–582) HIF-1α and pGAL4 DBD (775–826) HIF-1α. The pGAL4 DBD vector and the pGAL4 Luc reporter plasmid were kindly provided by Dr. J. M. Redondo (Centro de Biologia Molecular, consejo superior de investigaciones cientificas, Universidad Autónoma de Madrid, Madrid, Spain).

Transfections and Analysis of Luciferase Activity—Confluent cell cultures growing in 100 mm culture dishes were transfected in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal calf serum with 20 µg of p9HIF1-Luc or pBEGR Luc plasmids by using a standard calcium phosphate method (49). For GAL4 experiments, 15 µg of the pGAL4 DBD vector or pGAL4 DBD (530–582) HIF-1α constructs were co-transfected with 7 µg of pGAL4 Luc or 7 µg of pGAL4 DBD (775–826) HIF-1α plasmid with 13 µg of pGAL4 Luc. After 10–16 h in the presence of DNA precipitates, cells were treated as indicated above prior to luciferase analysis.

Nuclear Extracts and Electrophoretic Mobility Shift Assays (EMSA)—Nuclear cell extracts were prepared as previously described (50). Nuclear extracts containing 3–5 µg were incubated with 0.5 µg of poly(dI–dC), 100 ng of calf thymus DNA, and 2.5 µl of 5× DNA binding buffer (50 mM Tris buffer, pH 7.5, 130 mM KCl, 2 mM MgCl2, 0.5 mM ZnCl2, 25 mM dithiothreitol, 5 mM EDTA, 25% (v/v) glycerol) in a final volume of 11 µl for 10 min at room temperature. For the supershift assay, 1.5 µl of a polyclonal antibody raised against residues 1–13 of the human HIF-1α protein or the corresponding preimmune serum was added to the binding reaction and incubated for an additional 10 min. Thereafter, 0.5–1.5 ng (1.5 µl) of 32P-labeled double-stranded oligonucleotide (10–20 × 106 cpm/µg) was added. After 20 min of incubation at room temperature, 2 µl of Ficoll 400 20% (w/v) were added, and DNA-protein complexes were resolved by electrophoresis. The complementary oligonucleotides annealed and used as probe in EMSA were (5′ to 3′) TGAGGCACACTGCACTGTTGGGTCACAGGTCTCCT and (5′ to 3′) TGCCAAGAGGACCTGTGAGCCCACTATGCACGT-TGG (nucleotides spanning positions –985 to –951 of the human vascular endothelial growth factor 5′ promoter sequence are underlined).

Immunoblotting—Whole cellular lysates from 2 × 106 cells were resolved after heating on an 8% polyacrylamide–SDS gel and transferred to a nitrocellulose membrane. Thereafter, immunoblotting was performed as previously described (23) using the HIF-1α antibody (dilution 1:1000) (Transduction Laboratories, Becton Dickinson) or the Sp-1 antibody (dilution 1:200) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Results

Hypoxia Induces DAG Accumulation—Because DAG is a well recognized lipid second messenger, we decided to investigate whether the level of DAG was affected upon exposure of HeLa cells to hypoxia. As shown in Fig. 1, the exposure of HeLa cells to hypoxia induces a significant increase in the intracellular level of DAG. This result is in agreement with previous reports that have shown a sustained increase of DAG level in neonatal rat ventricular myocytes, as well as in the Hep3B cell.
levels were determined by the conversion of DAG into $[^{32}P]$PA by the DAG content (mean ± S.D.) of a representative experiment performed in duplicate.

As in the case of HeLa cells, we also found that hypoxia induced a marked PA accumulation in $[^{32}P]$orthophosphate metabolically labeled Hep3B and 293-T cells that was inhibited by the DGK kinase inhibitor (Fig. 4A). Time course experiments in 293-T cells revealed a sustained hypoxia-induced PA accumulation that was detected as soon as after 3 h and remained elevated even after 15 h (Fig. 4B). The delay of 3 h in the detection of PA accumulation was probably due to the fact that 2.5–3 h are required to reach fully established hypoxic conditions in the culture medium after initial influx of hypoxic atmosphere (data not shown). In this regard, a similar delay has been observed in other hypoxia-induced intracellular events, as in the case of activation of HIF-1 that is only completely induced after 2–4 h of hypoxia (53). In addition, we performed experiments to answer the question whether the accumulation of PA was also detected with other cellular stresses or was it specific to hypoxia. We found that the exposure of HeLa cells to ultraviolet light C or heat shock during 15 min resulted in an induction of 1.2 ± 0.3-fold and 1.3 ± 0.4-fold ($n = 4$) in the level of PA, respectively, whereas an induction of 2–3-fold was observed after hypoxia exposure.

Taken together, these data indicate that a sustained PA accumulation through a R59949-sensitive DGK mechanism is a general cellular response to low oxygen tension.

**Effect of Pharmacological Inhibition of DGK Activity on HIF-1-dependent Transcription**—Next we asked whether hypoxia-dependent PA accumulation was involved in the activation of HIF-1-dependent transcription. As a first approach, HeLa cells were transfected with the HIF-1-responsive reporter plasmid p9HIF-1 Luc. Pretreatment of transfected HeLa cells with R59949, at doses identical to those that impaired hypoxia-inducible PA accumulation (1–10 μM), gradually inhibited the hypoxia-inducible transcription promoted by p9HIF-1 Luc (Fig. 5, top panel). Similar results were obtained with R59022 (10 μM), a different specific DGK activity inhibitor (Fig. 5, top panel). As a control of specificity, we analyzed in parallel the previously described PMA-dependent transcription driven by the EGR 1 (54). The response to PMA driven by the EGR-responsive reporter plasmid, pEGR Luc, was not markedly affected by pretreatment with the same doses of R59949 or R59022 (Fig. 5, bottom panel).

The binding of HIF-1 to DNA specific sequences is required to promote HIF-1-driven transcription. Therefore, we analyzed by EMSA whether R59949 pretreatment interfered with the formation of HIF-1-DNA complex. The DNA probe used in this EMSA was the HIF-1 binding sequence multimerized in the p9HIF-1 Luc. Exposure of HeLa cells to hypoxia induced the...
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The DGK inhibitor R59949 prevents hypoxia-induced PA accumulation. A, HeLa cells were incubated with vehicle dimethyl sulfoxide (0.1% Me₂SO (v/v)) or R59949 (10 μM). Thereafter, cells were lysed, and DGK activity was determined as indicated under "Experimental Procedures." Thereafter, the [32P]radiolabeled PA generated was separated from all other phospholipids by TLC (top panel) and quantified (bottom panel). Each bar represents the relative PA content (mean ± S.D.) of a representative experiment performed in duplicate. B, [32P]orthophosphate metabolically labeled HeLa cells were preincubated with vehicle (−) (0.1% Me₂SO (v/v)) or the indicated doses of R59949 for 15 min before exposure to hypoxia or normoxia for an additional 6 h. Cellular phospholipids were then extracted and the [32P]-radiolabeled PA was visualized by TLC (top panel) and quantified (bottom panel). A representative experiment performed in duplicate is shown.

appearance of two major hypoxia-inducible DNA-protein complexes indicated as H1 and H2 (Fig. 6A). A specific polyclonal antibody against the α subunit of HIF-1, but not the corresponding preimmune serum, was able to supershift both hypoxia-inducible complexes without affecting constitutive DNA-protein complexes, evidencing the presence of HIF-1α specifically in H1 and H2 complexes (Fig. 6A). The hypoxia-dependent formation of H1 and H2 complexes was markedly reduced in HeLa cells pretreated with R59949 (10 μM), whereas we did not observe any significant effect of the DGK inhibitor on the constitutive DNA-protein complexes (Fig. 6A). Furthermore, the addition of R59949 (10 μM) to the in vitro binding reaction did not affect the formation of both HIF-1-DNA complexes (data not shown), indicating that R59949 affected the cellular mechanisms that lead to the appearance of HIF-1-DNA complexes upon hypoxic stimulation. The binding of HIF-1 heterodimer to DNA requires a previous hypoxia-induced stabilization and subsequent accumulation of the HIF-1α subunit (17). Therefore, we analyzed whether the pharmacological inhibition of DGK activity was primarily preventing the hypoxia-induced accumulation of HIF-1α. We found that R59949 markedly reduces in HeLa, Hep3B, and 293-T cells the accumulation of the HIF-1α subunit upon hypoxic exposure, suggesting that the accumulation of PA in response to hypoxia is a common mechanism required to induce HIF-1 (Fig. 6B). Pretreatment with R59949 prevented HIF-1α accumulation in Hep3B and 293-T cells at doses ranging from 10 to 30 μM, which were slightly higher than those required in HeLa cells to detect the same level of inhibition (Fig. 6B and data not shown). As previously described, HIF-1 is detected by Western blotting as double band that reflects its phosphorylation state (25). As a control for the specific effect of DGK inhibitor, we also analyzed the protein level of the Sp-1 transcription factor in the same lysates. The constitutive protein level of Sp-1 detected in the three cell cultures analyzed was not affected when the same dose of R59949 was employed in either normoxia or hypoxia (Fig. 6B). Pretreatment with the other DGK inhibitor, R59022, was also able to prevent the accumulation of HIF-1α without affecting Sp-1 levels in HeLa cells (Fig. 6B).

The complete activation of HIF-1-dependent transcription also requires the hypoxia-inducible transactivation activity of its HIF-1α subunit (55). The sequences of HIF-1α subunit that mediate its hypoxia-inducible transactivation activity have been located between amino acids 530–582 and 775–826 (18, 19). Therefore, we asked whether the hypoxia-dependent PA accumulation via DGK activity was also regulating the hypoxia-induced transactivation activity driven by these two minimal sequences. HeLa cells were cotransfected with expression vectors encoding the 530–582 or 775–826 amino acid sequences fused in-frame with the GAL4 DBD (GAL4 DBD (530–582) HIF-1α and GAL4 DBD (775–826) HIF-1α) in combination with the pGAL4 Luc reporter plasmid. Pretreatment of transfected HeLa cells with R59949 at doses ranging from 1 to 10 μM gradually inhibited the hypoxia-inducible activity driven by both sequences without reducing their basal activity found in normoxia (Fig. 7). These experiments also indicated that the two transactivation domains were differentially affected by cell exposure to R59949. The hypoxia-dependent transcription driven by the HIF-1α 775–826 sequence returned almost to normoxic levels in the presence of R59949 (10 μM), whereas the same dosage of R59949 produced only a partial reduction in hypoxia-dependent activity driven by the HIF-1α 530–582 sequence (Fig. 7). We detected a basal transcriptional activity in HeLa cells transfected with pGAL4 DBD alone and GAL4 Luc that was not affected with the same doses of R59949 in either normoxia or hypoxia (Fig. 7).

DISCUSSION

The pivotal role recognized for HIF-1 in the cellular response to low oxygen environments has generated a great interest in the signal transduction mechanisms involved in its regulation. It has previously been reported that cell exposure to conditions ranging from moderate hypoxia to anoxia induce a series of intracellular events associated with signal transduction, such as the activation of c-Src kinase (56), PKC (43), p38 kinase, and c-Jun N-terminal kinase (JNK) (57–60), as well as an increase in the level of intracellular calcium (61). Despite these observations, none of these signaling mechanisms have been clearly associated with the activation of HIF-1. Chandel and coworkers showed the first evidence that connected an hypoxia-inducible intracellular event with HIF-1 activation (22, 23). They demonstrated that the generation of mitochondrial ROSs upon hypoxic stimulation mediates the induction of HIF-1-dependent transcription (22, 23). Most recently, it has been suggested that the Ser-Thr protein kinase AKT may lead to the activation of
HIF-1 by hypoxia in PTEN mutant glioblastoma cell lines (26). In addition, recent reports have provided evidence for a role of the mitogen-activated protein kinase in the regulation of HIF-1-dependent transcription (24, 25).

In the present work, we show for the first time that the generation of PA through DGK is a general response to hypoxia and provide evidence for a role of this kinase in the regulation of HIF-1. Due to the ability of this enzyme to regulate the level of the lipid second messengers DAG and PA, it has been proposed that DGK activity can participate in signal transduction by attenuating the second messenger functions of DAG as well as by inducing the generation of biologically active species of...

**Fig. 4.** Hypoxia-dependent PA accumulation via DGK is a general cellular response to low oxygen tension and PA elevation is sustained during the hypoxic exposure. A. \(^{[32P]}\)orthophosphate metabolically labeled 293-T or Hep3B cells were preincubated with vehicle (–) (0.4% Me₂SO (v/v)) or (+) R59949 (20 \(\mu\)M) for 15 min before exposure to hypoxia (HP) or normoxia (N) for an additional 6 h. Cellular phospholipids were then extracted, and the \(^{32P}\)-radiolabeled PA was visualized by TLC (top panel) and quantified (bottom panel). Each bar represents the PA fold induction (mean ± S.D.) of a representative experiment performed in duplicate. Similar results were obtained from two independent experiments. B, \(^{[32P]}\)orthophosphate metabolically labeled 293-T cells were exposed to hypoxia or normoxia for 1, 3, 6, or 15 h. Thereafter, \(^{32P}\)-radiolabeled PA was quantified. Each bar represents the PA fold induction (mean ± S.D.) of a representative experiment performed in duplicate.
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PA (36, 37). As we show in HeLa cells, the hypoxia-dependent elevation of PA via DGK occurred in parallel with a marked accumulation of DAG (Figs. 1 and 3). The parallel accumulation of DAG and PA seems to be not only restricted to HeLa cells, because it has been reported that hypoxia induced DAG accumulation in different cells types, including Hep3B (44), in which we have also detected a marked hypoxia-dependent PA accumulation. This hypoxia-dependent DAG accumulation was prevented with the previously recognized inhibitor of phosphatidylethanolamine-phospholipase C and sphingomyelin synthase activities D609 (51, 62) (data not shown), in agreement with Goldberg et al. (43). Interestingly, we observed that the pretreatment with the same doses of D609 that reduced the hypoxia-dependent DAG accumulation resulted in a complete inhibition of HIF-1-dependent transcription.2 Therefore, we proposed that the involvement of hypoxia-dependent generation of DAG in the regulation of HIF-1 depends on its conversion to PA through DGK due to the coordinated activity of phosphatidylethanolamine phospholipase C or sphingomyelin synthase with DGK enzymes.

In addition, the hypoxia-induced sustained PA accumulation occurred in parallel with the previously reported sustained induction of HIF-1 protein (53). These time course experiments and the fact that the pharmacological elimination of hypoxia-dependent PA accumulation inhibits HIF-1 induction led us to strongly suggest that the accumulation of PA via DGK plays an essential role in the regulation of HIF-1.

Nine different isoforms of mammalian DGKs have been identified to date, and they have been classified in five different families (35–37). All the enzymes contain a C-terminal catalytic domain and two or three cysteine-rich domains. The different families are characterized by having different N-terminal regulatory domains, suggesting different mechanisms of regulation for the different isoforms. To date, we have not identified the DGK isoform(s) that controls the activity of HIF-1, but we have shown that this transcriptional activity is sensitive to the DGK inhibitor R59949. This compound has been recognized as a inhibitor of the type I isoforms of DGKs (35, 39, 63). Previous data have shown that R59949 does not affect DGKζ activity (64), although whether this compound affects other DGK isoforms has not been fully determined. Because our data strongly suggest that the hypoxia-dependent PA accumulation through an R59949-sensitive DGK is a general response of cellular cultures to hypoxia, it is possible to speculate that a common R59949-sensitive DGK isoform(s) is exclusively involved in the regulation of this transcription factor or that

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every cell type regulates HIF-1 through its particular set of R59949-sensitive DGK isoforms. It has been previously reported that every DGK isoform shows a specific intracellular localization that could be required to control specific cellular functions (65). In this regard, it has been shown that the regulation of the cell cycle by DGKz required its nuclear localization (38). These published data lead us to consider that the activation of HIF-1 could require the local accumulation of PA in particular cellular compartments by a specific DGK isoform(s). Further analysis of the role of DGK in the regulation of HIF-1 will require the identification of the DGK isotypes expressed in different cell types, including HeLa, Hep3B, and 293-T cells, as well as the future design of “loss of function” and/or dominant-negative versions of the DGK isoforms identified.

It has been previously proposed that the accumulation of PA regulates important processes, such as cell cycle progression and cytoskeletal organization (32–34). Here, we propose a novel role of PA generated during hypoxia through a DGK-dependent mechanism in the hypoxia-induced accumulation of HIF-1α as well as in its transactivational function. By itself, PA is a presumed second messenger that has been shown to be involved in the activation a number of enzymes, including raf kinase (66), type I phosphatidylinositol 4-phosphate 5-kinases (67), protein phosphatase 1 (68), n-chimaerin (69), the ζ isoform of PKC (70), an unidentified protein kinase that phosphorylates the NADPH oxidase protein p47phox (71, 72), and the activation of the SHP-1 protein phosphatase (73). It has been previously proposed that the dephosphorylation of residues 551 and 552 of HIF-1α could prevent its ubiquitinization and subsequent degradation by the proteasome pathway in hypoxic conditions (74), as well as that redox modification of the cysteine residue (C800) of HIF-1α is essential for the recruitment of transcriptional coactivators to its C-terminal transactivation domain (75). Therefore, future experiments will be designed to explore whether hypoxia-dependent PA elevation and some of previously reported PA-dependent intracellular events are required to connect the accumulation of PA and the posttranslational modifications of HIF-1α required for its activation.

Herein we propose that the DGK activity is essential in the regulation of HIF-1-dependent transcription by low oxygen tension. Recently, it has been shown that the generation of mitochondrial ROIs in Hep3B and 293 cells lines (22, 23) and the activation of the Ser-Thr kinase AKT in PTEN mutant glioblastoma cell lines are intracellular mechanisms involved in the activation of HIF-1α in response to hypoxia (26). Further work will be required to establish whether the accumulation of PA through DGK functions independently of ROIs and/or AKT or whether they act together in a single transduction pathway leading to HIF-1 activation. In this regard, studies in our own laboratory indicate that the regulation of cell cycle entry in T-lymphocytes exerted by DGK-mediated PA accumulation is independent of the activation of AKT (40). Therefore, further studies will be necessary to investigate whether the generation of PA during hypoxia is related to the activation of AKT.

Our results also show that although hypoxia raises the level of PA primarily through DGK, there is also a moderate activation of PLD upon cellular exposure to hypoxia (Fig. 2). This suggests the existence of more than one mechanism responsible for PA generation. This hypoxia-dependent activation of PLD activity has also been detected in hypoxia-exposed sheep pulmonary artery cultured smooth muscle cells (76). This PLD activation is probably due to the activation of the DAG-dependent PKC ζ isoform by hypoxia (43). Because the accumulation of PA through the action of DGK appears to be important in the regulation of HIF-1, it will be of interest to explore whether the hypoxia-induced PA generation derived from PLD is also important for the activation of HIF-1.

Taken together, these data suggest that the sustained conversion of DAG into PA through the action of DGK serves to connect oxygen sensing mechanisms to the activation of HIF-1-dependent transcription.

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