Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 315 (2004) 44–50
BBRC
www.elsevier.com/locate/ybbrc

Role of diacylglycerol induced by hypoxia in the regulation of HIF-1α activity

Elisa Temes,[a,1] Silvia Martín-Puig,[a,1] Julián Aragonés,[a,2] David R. Jones,[b,3] Gemma Olmos,[a] Isabel Mérida,[b] and Manuel O. Landázuri[a,*]

[a] *Servicio de Inmunología, Hospital de la Princesa, Universidad Autónoma de Madrid (UAM), c/Diego de León 62, 28006 Madrid, Spain*
[b] *Departamento de Inmunología y Oncología, Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas, Cantoblanco, 28049 Madrid, Spain*

Received 21 October 2003

Abstract

Hypoxia-inducible factor 1 (HIF-1) is a critical transcription factor for the adaptation to lowered oxygen environments. We have previously reported that hypoxia induced phosphatidic acid (PA) accumulation through diacylglycerol kinase (DGK) activity and provided evidence that this PA production regulated HIF-1 expression. Here we report that hypoxia also produces a marked intracellular accumulation of diacylglycerol (DAG) in different cell types. The previously proposed inhibitor of phosphatidylcholine phospholipase C (PC-PLC)/sphingomyelin synthase (SMS) activities, D609, specifically abrogates both hypoxia-dependent DAG accumulation and hypoxia-induced HIF-1 expression. We show that DAG-dependent protein kinase C (PKC) isoforms do not play an essential role in the regulation of HIF-1 expression. D609 inhibits PA accumulation triggered by hypoxia, suggesting that DAG could act as substrate for its conversion into PA by DGK upon these conditions. Therefore, this work provides novel evidence for the existence of DAG/PA-dependent intracellular mechanisms involved in the regulation of HIF-1 expression.

Keywords: HIF-1; Hypoxia; Diacylglycerol; Phosphatidic acid; Signal transduction; Protein regulation; Lipid second messengers

Cells respond to low oxygen tension by inducing a gene expression program to overcome this situation. It has been demonstrated that hypoxia-inducible factor 1 (HIF-1) is the transcription factor that controls the oxygen-dependent response [1]. HIF-1 regulates various genes including the vascular endothelial growth factor (VEGF), erythropoietin (EPO), and glycolytic enzymes among others [2].

HIF-1 is a heterodimer composed of α and β subunits that belongs to the basic helix–loop–helix PAS (Per, Arnt, and Sim) family of transcription factors [3]. In normoxia, the tumor suppressor Von Hippel–Lindau (VHL), which is a component of the E3 ubiquitin ligase complex, interacts with the α subunit of HIF-1 (HIF-1α) and this recognition leads to HIF-1α ubiquitinization and subsequent proteasome-dependent degradation [4,5]. It has been recently demonstrated that hydroxylation of proline residues 402 and 564 of HIF-1α by a novel class of prolyl hydroxylases (PHDs) is required for the interaction of VHL with HIF-1α [6,7]. Exposure of cells to hypoxia induces stabilization of the HIF-1α subunit due to the inhibition of PHDs activity, leading to the formation of the HIF-1α/β heterodimers that bind to DNA hypoxia-responsive elements (HRE) to drive transcription [8]. PHDs have been recently involved in a HIF-dependent feedback regulatory mechanism, supporting their role as key molecules in oxygen sensing [9,10].

Cells respond to multiple extracellular stimuli by acutely generating lipid second messengers. We have previously shown that hypoxia leads to an increase in

* Corresponding author. Fax: +34-91-520-23-74.
E-mail address: mortiz.hlpr@salud.madrid.org (M.O. Landázuri).
[1] Equal contribution.
[2] Present address: Center for Transgene Technology and Gene Therapy, VIB, KULeuven Campus Gasthuisberg O&N, Herestraat 49, B-3000 Leuven, Belgium.
[3] Present address: Division of Cellular Biochemistry, The Netherlands Cancer Institute Plesmanlaan 121.1066 CX Amsterdam, The Netherlands.

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.01.015

the intracellular level of phosphatidic acid (PA) through the action of diacylglycerol kinase (DGK) [11]. Another second lipid messenger is diacylglycerol (DAG) that could arise from several sources. The best established is the hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$) by phospholipase C (PI-PLC) [12,13]. An alternative mechanism is the breakdown of PC by a putative eukaryotic phosphatidylcholine phospholipase C (PC-PLC)-like activity [14,15]. Although protein/s responsible for this activity have not yet been cloned in mammalian cells, the existence of this alternative pathway relies on the use of D609, a previously proposed inhibitor of PC-PLC-like activity [16–18]. In addition, it has been suggested that cellular sphingomyelin synthase (SMS) activity could account for the putative PC-PLC activity, since first this enzymatic activity converts simultaneously PC ceramide into DAG and sphingomyelin and second it is inhibited by D609 [19]. One of the well-known intracellular functions of DAG is to serve as an allosteric activator of classical and novel protein kinase C (PKC) isoforms that mediate many cellular responses including cell growth and differentiation [20,21]. In the present work we report that hypoxia causes DAG accumulation. Elimination of this hypoxia-induced DAG with pharmacological inhibition of PC-PLC-like activity or SMS activity specifically abrogates HIF-1-dependent transcription. In contrast, the PI-PLC inhibitor, U73211, does not affect the HIF-1 transcriptional activity. Furthermore, here we discard the role of DAG-dependent PKCs upon HIF-1 activation by hypoxia. All these data led us to suggest that hypoxia-induced DAG accumulation serves as substrate for DGK to generate PA that regulates HIF-1 activation.

Materials and methods

Reagents. [^{32}P]Orthophosphate (carrier free) and [γ-^{32}P]ATP (sp.act. 3000 Ci/mmol) were purchased from Amersham–Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). Silica gel thin layer chromatography (TLC) plates (60 Å, LK6D) were from Whatman (Clifton, NJ). D609 was purchased from Sigma (St. Louis, MO). Ro31-8220 and U73211 were from Calbiochem (La Jolla, CA). The authentic phospholipid standard for 1,2-dioleoyl phosphatidic acid and phorbol ester (PMA) were from Sigma (St. Louis, MO). Analytic grade organic solvents for TLC were from Merck (Darmstadt, Germany).

Cell culture and cellular treatments. HeLa cells were grown in RPMI 1640 with GLUTAMAX-I (Life Technologies, Paisley, UK) whereas 293-T cells were grown in Dulbecco's MEM (Biochrom KG, Berlin, Germany) in the presence of 10% (v/v) foetal calf serum (FCS) (Labtech International, Woodside, UK). Cells were routinely cultured in 95% air/5% CO_2 (normoxic conditions) at 37 °C. Cellular exposure to hypoxia was performed as previously described with a mixture of 1% O_2, 5% CO_2, and 94% N_2 [11].

Determination of total cell DAG. Lipids were extracted by the method of Bligh and Dyer [22] from cells exposed to normoxia or hypoxia for 5–6 h in the presence or absence of D609. The amount of DAG was determined by its conversion into [^{32}P]PA by *Escherichia coli* DGK in the presence of [γ-^{32}P]ATP as previously described [23]. DAG levels were corrected to the total phospholipid phosphate content. The method of Bartlett [24] was used for the assay of total phosphate.

Measurement of PA. Cells were cultured in phosphate-free medium supplemented with 10% (v/v) foetal calf serum (extensively dialyzed against 0.9% (w/v) NaCl) for 90 min before the addition of [^{32}P]orthophosphate (100 μCi/ml) for an additional 90 min. Then, the cells were exposed to normoxia or hypoxia. Phospholipids were then extracted by the method of Bligh and Dyer [22] and analyzed as previously described [11]. Quantification of the band corresponding to [^{32}P]PA was performed using the Bio-Rad Molecular Analyst Software.

Determination of calcium levels. Serum-starved cells for 20 h were resuspended in Hanks' Balanced Salt Solution (HBBS) at 4×10^6 cells/ml. Thereafter they were labeled adding fluo-3, AM (4 μg/ml) (Molecular Probes, Leiden, The Netherlands), for 30 min at 37 °C. Then cells were washed twice with HBBS. Thereafter, the cells were pretreated with the PI-PLC inhibitor, U-73122, or vehicle for 1 h before serum stimulation. Then, fluorescence intensity was analyzed by flow cytometry in a FACScalibur apparatus (Beckton–Dickinson).

Recombinant plasmids and transfections. The HIF-1-responsive plasmid p9HIF1-Luc, EGR-responsive plasmid pEGR-Lu, and pCMV-LUC have been previously described [11,25]. Confluent cell cultures growing in 100 mm culture dishes were transfected in Dulbecco's MEM containing 10% (v/v) FCS with 1 μg p9HIF-1 Luc or 1 μg pCMV Luc in combination with 19 μg of empty plasmid pCDNA3 in the case of 293-T cells; and 20 μg of p9HIF-1 Luc or 20 μg of pCMV Luc in the case of HeLa cells by using a standard calcium phosphate method [26]. After 10 h of transfection, cells were pooled and plated onto 24-well cell plates. After 14–16 h cells were pretreated or not with D609, Ro 31-8220 or U-73122 before incubating in normoxic or hypoxic conditions for an additional 6 h. Thereafter the cells were lysed and luciferase activity was measured (Promega, Madison, WI) with a Lumat LB9501 luminometer (Berthold, Wildbad, Germany).

Immunoblotting. Whole cellular lysates were resolved on 8% polyacrylamide–SDS gels and transferred to nitrocellulose membranes. The levels of HIF-1α subunit or Sp-1 protein were detected with specific antibodies (Transduction Laboratories and Santa Cruz Biotechnology, respectively) by Western blotting.

Results

Hypoxia induces DAG accumulation through a PC-PLC/SMS activity

We have previously reported that the cellular exposure to low oxygen tension produces an increase in the total cell DAG content of HeLa cells [11]. Here we analyzed whether this phenomenon was present in another cell type. The exposure of 293-T cells to hypoxia induced a significant increase in total cell DAG, similar to HeLa cells (Fig. 1). In agreement with our data, hypoxia has also been found to increase the total DAG content in neonatal rat ventricular myocytes and in Hep3B cells [27,28]. The magnitude of the total cell DAG increase observed in hypoxia is similar to that observed in response to other stimuli [14,15]. Therefore, it could be proposed that the accumulation of DAG is a common mechanism in the cellular response to hypoxia.

A PC-PLC-like activity as well as SMS activity has been reported to generate DAG in response to several stimuli. The effect of the previously described inhibitor

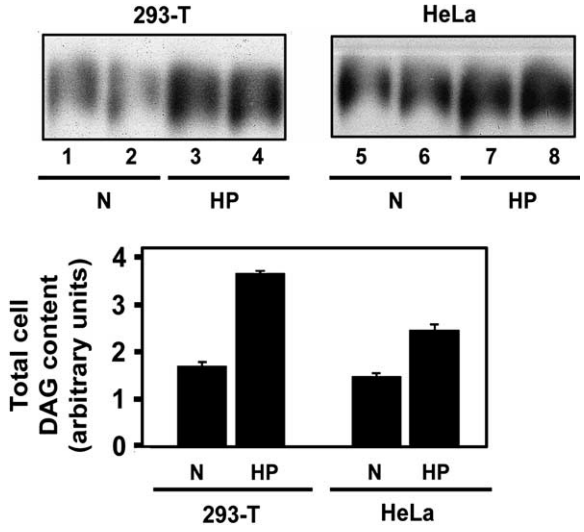

Fig. 1. Hypoxia induces DAG production. HeLa and 293-T cell cultures were incubated for 6 h in normoxia (N) or hypoxia (HP). Cellular lipids were extracted and DAG levels were determined by the conversion of DAG into [^{32}P]PA. [^{32}P]PA generated was separated from all other phospholipids by TLC and then visualized by autoradiography (upper panel). Quantification of the radiolabeled PA normalized to the total phospholipid phosphate content is shown (lower panel). An experiment performed in duplicate is shown. Similar results were obtained in two further experiments.

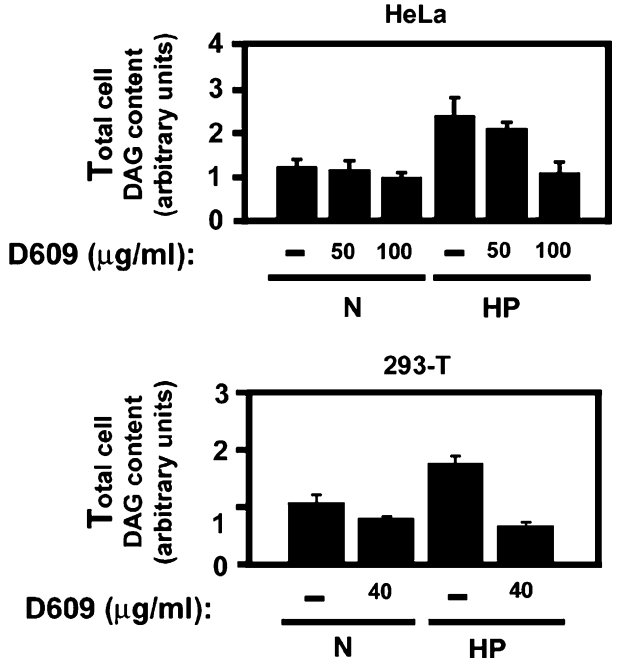

Fig. 2. The PC-PLC/SMS activity inhibitor D609 prevents hypoxia-induced DAG accumulation. HeLa cells (upper panel) and 293-T cells (lower panel) were incubated with vehicle (water 2% v/v) or D609 at the indicated doses during exposure to normoxia (N) or hypoxia (HP) for 6 h. Cellular lipids were extracted and DAG levels were determined as indicated above. An experiment performed in duplicate is shown. Similar results were obtained in two further experiments.

of these enzymatic activities, D609, [17,29] was analyzed on hypoxia-induced DAG accumulation. We found that pretreatment of 293-T and HeLa cells with D609 completely prevented the increase of DAG levels in response to hypoxia (Fig. 2). It is important to note that the dose of D609 required to abrogate hypoxia-induced DAG accumulation was 100 μg/ml in HeLa cells and 40 μg/ml in 293-T cells (Fig. 2), indicating a differential sensitivity to D609 between these two cell types. These doses were similar to those required to prevent DAG accumulation observed in response to other stimuli [16] as well as to inhibit the putative PC-PLC/SMS activity in vivo and in vitro [19]. The basal level of DAG was also slightly affected (Fig. 2), suggesting that the generation of DAG through a PC-PLC/SMS activity is operative under normoxic conditions and that oxygen deprivation enhances this pathway of DAG accumulation.

Effect of pharmacological inhibition of hypoxia-induced DAG accumulation on HIF-1-dependent transcription

We also analyzed whether the inhibition of hypoxia-induced DAG accumulation affected the activation of HIF-1. As a first approach, 293-T cells were transfected with the HIF-1-responsive reporter plasmid p9HIF-1 Luc (see Materials and methods). Pretreatment of transfected 293-T cells with D609, at doses identical to those that inhibited hypoxia-inducible total cell DAG accumulation (Fig. 2), inhibited the hypoxia-inducible transcription promoted by p9HIF-1 Luc (Fig. 3A, upper panel), without affecting pCMV-Luc-dependent transcription (Fig. 3A, lower panel). These results led us to conclude that pharmacological inhibition of hypoxia-triggered DAG accumulation with D609 impaired HIF-dependent transcriptional activity.

To further investigate hypoxia-induced DAG origin we analyzed the possible role of PI-PLC in HIF-1 activation. Therefore, HIF-1-dependent transcription was measured in the presence of the previously recognized PI-PLC inhibitor, U73122 [30,31]. First of all we determined the doses of U73122 required to abolish cellular PI-PLC activity. For this purpose, we analyzed the intracellular calcium accumulation in response to serum stimulation, a well-known effect mediated by PI-PLC in response to mitogens [32]. We found that a dose of 30 μM was sufficient to abolish the intracellular calcium peak observed in response to serum in 293-T cells (Fig. 3B, upper panel). In parallel experiments we found that 30 μM of U-73122 did not affect significantly the p9HIF-1 Luc-driven transcription (Fig. 3B, lower panel). These data ruled out an essential role of PI-PLC activity in the transcriptional activity regulation of HIF-1-dependent transcription.

One of the events required to promote HIF-1-dependent transcription is the hypoxia-induced stabilization and subsequent accumulation of the HIF-1α subunit [5–7]. Therefore, we analyzed whether

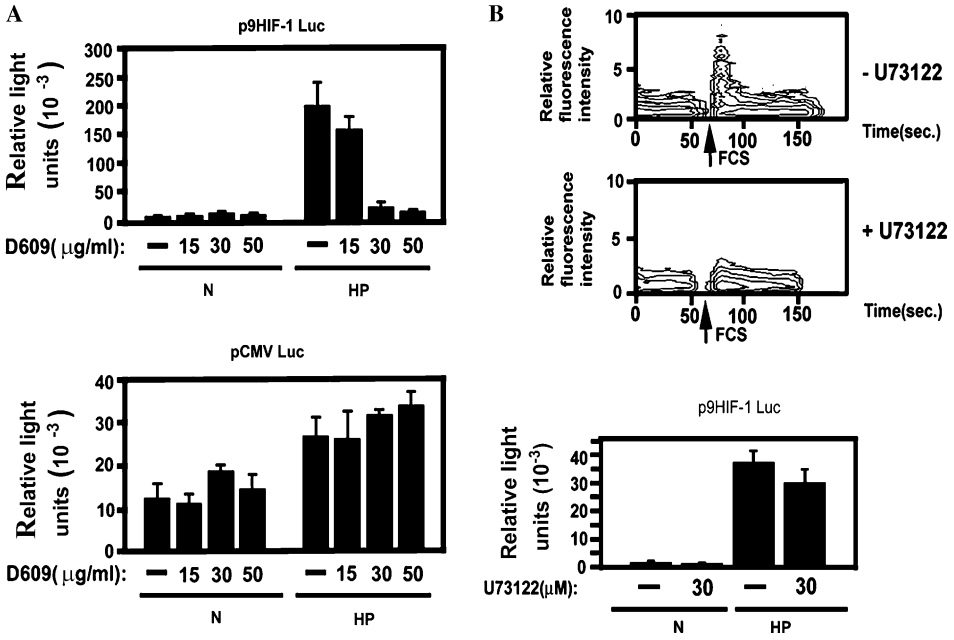

Fig. 3. The pharmacological inhibition of PC-PLC/SMS activities but not of PI-PLC prevents HIF-1-dependent transcription. (A) 293-T cells transiently transfected with the p9HIF-1 Luc (upper panel) or pCMVLuc (lower panel) were incubated with vehicle (water 2% v/v) or the indicated doses of D609 during exposure to normoxia (N) or hypoxia (HP) for 6 h. After such incubation luciferase activity was determined in cell lysates. Each bar represents the luciferase activity (means ± SD) of duplicate cell lysates from one representative experiment. Similar results were observed in five independent experiments. Normoxic values corresponding to p9HIF-1 Luc were magnified 10×. (B) 293-T cells loaded with fluo-3, AM were preincubated with vehicle (Me_2SO, 0.3% v/v) or U73122 at 30 μM for 30 min before determination of intracellular calcium level in response to FCS 10% by FACS analysis (upper panel). A representative result from three more is shown. 293-T cells transiently transfected with the p9HIF-1 Luc (lower panel) were preincubated with vehicle (Me_2SO, 0.3% v/v) or in the presence of U73122 (30 μM) for 1 h and then exposed to normoxia (N) or hypoxia (HP) for an additional 6 h. Luciferase activity was determined in cell lysates.

D609-mediated inhibition of the hypoxia-induced increase in total cell DAG affected the hypoxia-induced accumulation of the HIF-1α subunit. We found that D609 reduced the generation of the hypoxia-induced HIF-1α subunit at the same doses that were able to interfere with the hypoxia-triggered DAG production in both, HeLa and 293-T cells (Figs. 4A and B). These data indicate that there is a close correlation between DAG production and HIF-1α expression upon hypoxic stimulation. As a control for specificity, we also analyzed the protein level of the Sp-1 transcription factor in the same lysates by Western blotting (Fig. 4). Taken together all these results suggest that the hypoxia-triggered DAG accumulation, probably through the action of PC-PLC/SMS activity, plays a role in the activation of HIF-dependent transcription due to its involvement in the hypoxia-induced stabilization of HIF-1α subunit.

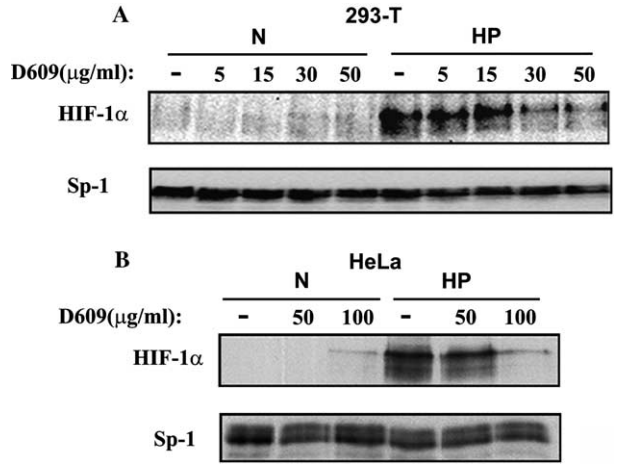

Fig. 4. D609 interferes with the hypoxia-dependent accumulation of HIF-1α subunit. 293-T cells (A) and HeLa cells (B) treated with vehicle (−) (water 2%) or D609 at the indicated doses were exposed to normoxia (N) or hypoxia (HP) for 6 h. HIF-1α and Sp-1 protein levels were analyzed by immunoblotting from whole cell lysates. A representative experiment from four performed is shown.

PKC activity is not essential for hypoxia-induced HIF-1α accumulation. Role of PA

Since we had observed that DAG was important for HIF-dependent transcription activity, we decided to investigate through which mechanisms was DAG mediating this effect. One of the well-known DAG-dependent intracellular effects is the activation of various PKC isoforms [20,21]. Therefore, we asked whether PKC activity was necessary for the regulation of HIF-1 expression. To further investigate this possibility we used the previously recognized PKC inhibitor Ro 31-8220

that very efficiently inhibits the effects induced by PMA, an activator of DAG-dependent PKC isoforms [33]. We tested the inhibitory effect of Ro 31-8220 on the previously described PMA-dependent transcription driven by the early growth response factor 1 (EGR 1) [34]. As shown in the lower panel of Fig. 5A, Ro 31-8220 at doses of 0.5 and 1 μM was enough to abrogate the PMA-induced transcription driven by EGR transcription factor. In contrast, the same doses of Ro 31-8220 did not inhibit the HIF-1-dependent transcription (Fig. 5A, upper panel). In agreement with these data we found that Ro31-8220 at 1 μM did not affect the hypoxia-induced HIF-1α subunit accumulation neither in HeLa nor in 293-T cell lines (Fig. 5B). Therefore, we conclude from these experiments that cellular DAG-dependent PKC activity is not essential for the regulation of HIF-1 expression by hypoxia.

We have previously reported that hypoxia induces significant PA accumulation most likely via the action of DGK [11]. Furthermore, evidence has been provided for a role of DGK in the regulation of HIF-1 not only by low oxygen tension [11] but also by hypoxia mimetics such as nickel chloride [35]. We have also shown the importance of this PA upon HIF-1 activation [11]. Since we have ruled out the role of PKCs in HIF-1 activation, we next asked whether the hypoxia-induced DAG could

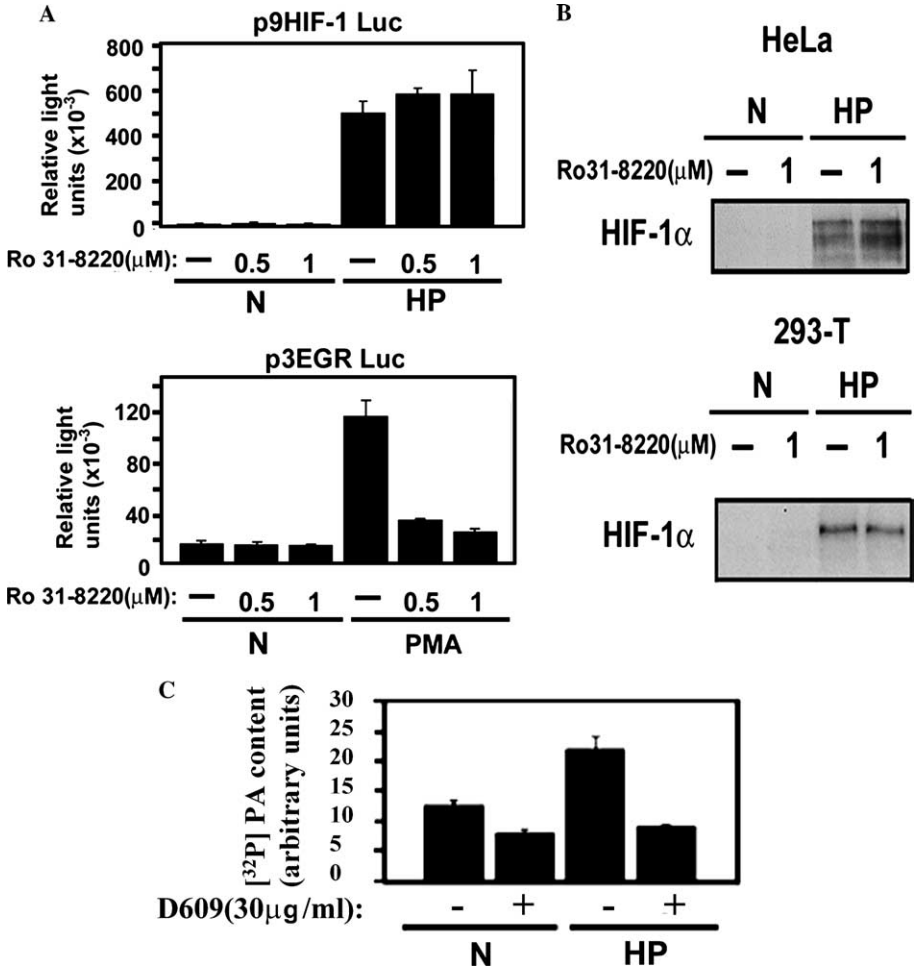

Fig. 5. The inhibition of cellular PKC activity did not prevent hypoxia-induced HIF-1α accumulation. D609 inhibits phosphatidic acid accumulation triggered by hypoxia. (A) HeLa cells transiently transfected with the p9HIF-1 Luc (upper panel) or p3EGR Luc (lower panel) were preincubated with vehicle (−) (Me_2SO, 0.1% v/v) or the indicated doses of Ro 31-8220 for 15 min before exposure to normoxia (N), hypoxia (HP) or PMA (80 ng/ml) for an additional 6 h. After such incubation luciferase activity was determined in cell lysates. Each bar represents the luciferase activity (means ± SD) of duplicate cell lysates from one representative experiment. Similar results were observed in five independent experiments. Normoxic values corresponding to p9HIF-1 Luc were magnified 10×. (B) HIF-1α protein level was analyzed by immunoblotting of whole lysates of HeLa and 293-T cells pretreated with vehicle (−) (Me_2SO, 0.1%) or Ro 31-8220 for 15 min before exposure to normoxia (N) or hypoxia (HP) for an additional 6 h. A representative experiment from three performed is shown. (C) [^{32}P]Orthophosphate metabolically labeled 293-T cells were incubated with D609 (30 μg/ml) or vehicle (water 2%) during exposure to hypoxia (HP) or normoxia (N) for 6 h. Cellular phospholipids were then extracted and the PA content was determined as described (see Materials and methods). An experiment performed in duplicate is shown. Three more experiments gave an identical result.

be converted to PA through DGK activity. Here we found that inhibition of DAG production with D609 resulted in the inhibition of hypoxia-induced PA accumulation (Fig. 5C). D609 also slightly reduced normoxic PA levels, indicating that conversion of DAG into PA is occurring in normoxic cells and is markedly enhanced after hypoxia exposure. Therefore, these data lead us to suggest that hypoxia-induced elevation of cellular PA through DGK is a direct consequence of hypoxia-induced DAG accumulation.

Discussion

In the present work we propose that generation of DAG in response to hypoxia could be due to a PC-PLC/SMS activity, since D609 inhibits such DAG accumulation. In agreement with these data, Goldberg et al. [27] found that exposure of neonatal rat cardiomyocytes to hypoxia led to a D609-sensitive DAG accumulation. Moreover, it has been found in in vivo studies that DAG accumulation, probably from breakdown of PC, occurs after ischemic/hypoxic conditions [36]. All these data together indicate that DAG accumulation can be considered a common molecular marker of cellular exposure to oxygen deprivation and suggest a role of a PC-PLC/SMS activity in cellular response to hypoxia. Furthermore, we have also provided evidence for the involvement of PC-PLC/SMS activity in the regulation of HIF-1 since D609, at the same doses that affect hypoxia-induced DAG accumulation, prevents HIF-1α stabilization as well as HIF-1-dependent transcription. Further analysis of the role of this PC-PLC/SMS activity in hypoxia responses will require the cloning of these putative mammalian enzymes.

Goldberg et al. [27] have also reported that hypoxia-induced DAG accumulation led to the translocation to membranes and activation of DAG-dependent PKC isoforms [27]. However, we have found that effective doses of Ro 31-8220 did affect neither HIF-1α accumulation nor HIF-1-dependent transcription. In agreement with our data, Yan et al. [37] reported that hypoxia-dependent induction of glucose transporter Glut-1 by HIF-1 is not affected in pKCβ null (−/−) mice. Altogether these results indicate that DAG-dependent PKC activity does not play an essential role in the hypoxia-induced HIF-1α activation. However, DAG-sensitive-PKCs have been involved in HIF-1α gene transcription in vascular smooth muscle cells in response to angiotensin II [38]. Therefore, DAG-sensitive PKCs could play a role in HIF-1α expression depending on the stimuli and in some cases cell type.

We have previously reported that PA accumulation in response to hypoxia is generated primarily by DGK activity [11]. Now we hypothesize that DAG increments observed upon hypoxia could serve as substrate for this enzyme. The fact that the same doses of D609 that inhibited the hypoxia-induced DAG level were able to prevent PA production supports our previous data. Furthermore, since our previous work proposes a role of PA in HIF-1 activation upon hypoxia, we can assume that the effect observed upon HIF-1 by DAG inhibition with D609 may be due to the subsequent decrease in PA level. In this regard ,we are currently investigating in more detail the role of PA in HIF activation and we have obtained data suggesting that PA could be involved in the VHL-HIF-1 recognition process (manuscript in preparation). However, further experiments will be required to completely support our hypothesis

Our previous evidence for the role of DGK in the regulation of HIF-1 [11] and currently presented data strongly suggest that the role of DAG in the regulation of HIF-1 depends on its conversion to PA through DGK but not on DAG-dependent PKC activity, proposing a coordinated action of PC-PLC/SMS and DGK enzymatic activities.

References

[1] C.W. Pugh, P.J. Ratcliffe, Regulation of angiogenesis by hypoxia: role of the HIF system, Nat. Med. 9 (2003) 677–684.
[2] R.H. Wenger, Mammalian oxygen sensing, signalling and gene regulation, J. Exp. Biol. 203 (2000) 1253–1263.
[3] G.L. Wang, G.L. Semenza, Purification and characterization of hypoxia-inducible factor 1, J. Biol. Chem. 270 (1995) 1230–1237.
[4] W.G.J. Kaelin, Molecular basis of the VHL hereditary cancer syndrome, Nat. Rev. Cancer 2 (2002) 673–682.
[5] P.H. Maxwell, M.S. Wiesener, G.W. Chang, S.C. Clifford, E.C. Vaux, M.E. Cockman, C.C. Wykoff, C.W. Pugh, E.R. Maher, P.J. Ratcliffe, The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis (see comments), Nature 399 (1999) 271–275.
[6] P. Jaakkola, D.R. Mole, Y.M. Tian, M.I. Wilson, J. Gielbert, S.J. Gaskell, A. Kriegsheim, H.F. Hebestreit, M. Mukherji, C.J. Schofield, P.H. Maxwell, C.W. Pugh, P.J. Ratcliffe, Targeting of HIF-alpha to the von Hippel–Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation, Science 292 (2001) 468–472.
[7] M. Ivan, K. Kondo, H. Yang, W. Kim, J. Valiando, M. Ohh, A. Salic, J.M. Asara, W.S. Lane, W.G. Kaelin Jr., HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing, Science 292 (2001) 464–468.
[8] L.E. Huang, Z. Arany, D.M. Livingston, H.F. Bunn, Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit, J. Biol. Chem. 271 (1996) 32253–32259.
[9] E. Berra, E. Benizri, A. Ginouves, V. Volmat, D. Roux, J. Pouyssegur, HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia, EMBO J. 22 (2003) 4082–4090.
[10] L. Del Peso, M.C. Castellanos, E. Temes, S. Martin-Puig, Y. Cuevas, G. Olmos, M.O. Landazuri, The von Hippel–Lindau/hypoxia inducible factor (HIF) pathway regulates the transcription of the HIF-proline hydroxylase genes in response to low oxygen, J. Biol. Chem. 12 (2003) 123–128.
[11] J. Aragones, D.R. Jones, S. Martin, M.A. San Juan, A. Alfranca, F. Vidal, A. Vara, I. Merida, M.O. Landazuri, Evidence for the involvement of diacylglycerol kinase in the activation of

hypoxia-inducible transcription factor 1 by low oxygen tension, J. Biol. Chem. 276 (2001) 10548–10555.
[12] M. Katan, Families of phosphoinositide-specific phospholipase C: structure and function, Biochim. Biophys. Acta 1436 (1998) 5–17.
[13] W.D. Singer, H.A. Brown, P.C. Sternweis, Regulation of eukaryotic phosphatidylinositol-specific phospholipase C and phospholipase D, Annu. Rev. Biochem. 66 (1997) 475–509.
[14] O. Kozawa, A. Suzuki, T. Kaida, H. Tokuda, T. Uematsu, Tumor necrosis factor-alpha autoregulates interleukin-6 synthesis via activation of protein kinase C. Function of sphingosine 1-phosphate and phosphatidylcholine-specific phospholipase C, J. Biol. Chem. 272 (1997) 25099–25104.
[15] J. Zamorano, M.D. Rivas, A. Garcia-Trinidad, C.K. Qu, A.D. Keegan, Phosphatidylcholine-specific phospholipase C activity is necessary for the activation of STAT6, J. Immunol. 171 (2003) 4203–4209.
[16] S. Schutze, K. Potthoff, T. Machleidt, D. Berkovic, K. Wiegmann, M. Kronke, TNF activates NF-kappa B by phosphatidylcholine-specific phospholipase C-induced 'acidic' sphingomyelin breakdown, Cell 71 (1992) 765–776.
[17] I. Preuss, I. Kaiser, U. Gehring, Molecular characterization of a phosphatidylcholine-hydrolyzing phospholipase C, Eur. J. Biochem. 268 (2001) 5081–5091.
[18] H. Ito, S. Adachi, M. Tamamori, H. Fujisaki, M. Tanaka, M. Lin, H. Akimoto, F. Marumo, M. Hiroe, Mild hypoxia induces hypertrophy of cultured neonatal rat cardiomyocytes: a possible endogenous endothelin-1-mediated mechanism, J. Mol. Cell. Cardiol. 28 (1996) 1271–1277.
[19] C. Luberto, Y.A. Hannun, Sphingomyelin synthase, a potential regulator of intracellular levels of ceramide and diacylglycerol during SV40 transformation. Does sphingomyelin synthase account for the putative phosphatidylcholine-specific phospholipase C? J. Biol. Chem. 273 (1998) 14550–14559.
[20] Y. Shirai, N. Saito, Activation mechanisms of protein kinase C: maturation, catalytic activation, and targeting, J. Biochem. 132 (2002) 663–668.
[21] R.H. Michell, The multiplying roles of inositol lipids and phosphates in cell control processes, Essays Biochem. 32 (1997) 31–47.
[22] E. Bligh, W. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917.
[23] I. Flores, A.C. Martinez, Y.A. Hannun, I. Merida, Dual role of ceramide in the control of apoptosis following IL-2 withdrawal, J. Immunol. 160 (1998) 3528–3533.
[24] G. Bartlett, Phosphorous assay in column chromatography, J. Biol. Chem. 234 (1958) 466–468.
[25] A. Nueda, M. Lopez-Cabrera, A. Vara, A.L. Corbi, Characterization of the CD11a (alpha L, LFA-1 alpha) integrin gene promoter, J. Biol. Chem. 268 (1993) 19305–19311.
[26] J. Sambrook, E. Fritsch, T. Maniatis, Molecular cloning. A Laboratory Manual, second ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
[27] M. Goldberg, H.L. Zhang, S.F. Steinberg, Hypoxia alters the subcellular distribution of protein kinase C isoforms in neonatal rat ventricular myocytes, J. Clin. Invest. 99 (1997) 55–61.
[28] K. Yoshioka, S. Clejan, J.W. Fisher, Activation of protein kinase C in human hepatocellular carcinoma (HEP3B) cells increases erythropoietin production, Life Sci. 63 (1998) 523–535.
[29] J. Ito, Y. Nagayasu, S. Ueno, S. Yokoyama, Apolipoprotein-mediated cellular lipid release requires replenishment of sphingomyelin in a phosphatidylcholine-specific phospholipase C-dependent manner, J. Biol. Chem. 277 (2002) 44709–44714.
[30] R.J. Smith, L.M. Sam, J.M. Justen, G.L. Bundy, G.A. Bala, J.E. Bleasdale, Receptor-coupled signal transduction in human polymorphonuclear neutrophils: effects of a novel inhibitor of phospholipase C-dependent processes on cell responsiveness, J. Pharmacol. Exp. Ther. 253 (1990) 688–697.
[31] L.M. Broad, T.R. Cannon, C.W. Taylor, A non-capacitative pathway activated by arachidonic acid is the major Ca^{2+} entry mechanism in rat A7r5 smooth muscle cells stimulated with low concentrations of vasopressin, J. Physiol. 517 (1999) 121–134.
[32] L.M. Broad, F.J. Braun, J.P. Lievremont, G.S. Bird, T. Kurosaki, J.W.J. Putney, Role of the phospholipase C-inositol 1,4,5-trisphosphate pathway in calcium release-activated calcium current and capacitative calcium entry, J. Biol. Chem. 276 (2001) 15945–15952.
[33] E.J. Yeo, J.H. Exton, Stimulation of phospholipase D by epidermal growth factor requires protein kinase C activation in Swiss 3T3 cells, J. Biol. Chem. 270 (1995) 3980–3988.
[34] A. Gashler, V.P. Sukhatme, Early growth response protein 1 (Egr-1): prototype of a zinc-finger family of transcription factors, Prog. Nucleic. Acid Res. Mol. Biol. 50 (1995) 191–224.
[35] A.S. Andrew, L.R. Klei, A. Barchowsky, Nickel requires hypoxia-inducible factor-1a, not redox signaling, to induce plasminogen activator inhibitor-1, Am. J. Physiol. Lung Cell. Mol. Physiol. 281 (2001) L607–L615.
[36] A. Gysembergh, A. Zakaroff-Girard, J. Loufoua, L. Meunier, X. Andre-Fouet, M. Lagarde, A.F. Prigent, M. Ovize, Brief preconditioning ischemia alters diacylglycerol content and composition in rabbit heart, Basic Res. Cardiol. 95 (2000) 457–465.
[37] S.F. Yan, J. Lu, Y.S. Zou, W. Kisiel, N. Mackman, M. Leitges, S. Steinberg, D. Pinsky, D. Stern, Protein kinase C-beta and oxygen deprivation. A novel Egr-1-dependent pathway for fibrin deposition in hypoxemic vasculature, J. Biol. Chem. 275 (2000) 11921–11928.
[38] E.L. Page, G.A. Robitaille, J. Pouyssegur, D.E. Richard, Induction of hypoxia-inducible factor 1alpha by transcriptional and translational mechanisms, J. Biol. Chem. 277 (2002) 48403–48409.