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Biochimica et Biophysica Acta 1773 (2007) 1558–1571
Biochimica et Biophysica Acta BBA
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Intermittent hypoxia changes HIF-1α phosphorylation pattern in endothelial cells: Unravelling of a new PKA-dependent regulation of HIF-1α

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Received 21 February 2007; received in revised form 22 May 2007; accepted 6 June 2007
Available online 16 June 2007

Abstract

Vascularized tumors are exposed to intermittent hypoxia, that is, hypoxia followed by periods of reoxygenation. Abnormal structure and dysfunction of tumor blood vessels are responsible for these conditions. These repeated short periods of hypoxia concern tumor cells as well as endothelial cells. However, the effects of intermittent hypoxia are poorly understood. The aim of this study was to investigate the effects of intermittent hypoxia on endothelial cells and particularly on HIF-1α, a central actor in adaptive response to hypoxia. For that, endothelial cells were exposed to four repeated cycles of 1-h hypoxia followed by 30 min of reoxygenation. We showed that repeated cycles of hypoxia/reoxygenation induced a modification in HIF-lα phosphorylation pattern: a progressive increase in HIF-1α phosphorylated form was observed during the hypoxic periods. Activation of p42/p44, Akt and PKA was observed in parallel. PKA was shown to be involved in the phosphorylation of HIF-lα under intermittent hypoxia, while p42/p44 and Akt were not. As HIF-1 activity is often associated with enhanced cell survival, a better knowledge of the effects of intermittent hypoxia on endothelial cells and the highlight of particular mechanisms induced by intermittent hypoxia are essential to understand the behavior of endothelial cells during neo-angiogenesis.

Keywords: Intermittent hypoxia; HIF-1α; PKA; Neo-angiogenesis; Tumor

1. Introduction

Solid tumors require, for their continued growth, formation of blood vessels to insure nutrients and oxygen supply [1,2]. Indeed, beyond a critical size, simple diffusion of oxygen and nutrients becomes insufficient for tumor development [3,4]. For then on, tumors express various pro-angiogenic factors which lead to the formation of new blood vessels from pre-existing blood vessel cells and/or endothelial precursor cells from the bone marrow [4–7]. The vessels formed in these circumstances are described to be structurally and functionally abnormal [3]. Indeed, these tumor blood vessels are often tortuous, dilated, with uneven diameter, excessive branching and numerous dead ends [3,8]. Moreover, they are not organized into definitive venules, arterioles and capillaries like their normal counterparts, but share chaotic features of all of them [9–11]. This abnormal structure of tumor blood vessels and their compression by proliferating cancer cells induce an irregular blood flow in tumor which can lead to transient hypoxia [12–16]. This intermittent hypoxia concerns endothelial cells as well as tumor cells.

One of the major cell responses to poor oxygen supply is the activation of hypoxia-inducible factor-1, a central actor in adaptive response to hypoxia [17]. Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric transcription factor composed of the HIF-1α (120 kDa) and ARNT (94 kDa) (Aryl hydrocarbon Receptor Nuclear Translocator; also called HIF-1β) subunits. These two subunits belong to the Per-Aryl hydrocarbon nuclear translocator (ARNT)-Sim (PAS)-basic helix loop helix (bHLH) family [18,19]. HIF-1α and ARNT are constitutively expressed [20], but the formation of HIF-1 transcription factor in the nucleus depends on HIF-1α stabilization which is principally O_2 dependent [21]. Under normoxia, HIF-1α is hydroxylated on the proline residues 402

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doi:10.1016/j.bbamcr.2007.06.002

in the N-terminal part and 564 in the C-terminal part by prolyl-4-hydroxylases [22]. These hydroxylations allow the binding of pVHL (von Hippel–Lindau tumor suppressor protein) on the ODD domain of HIF-1α [23]. pVHL acts as a substrate recognition of the E3 ubiquitin ligase protein complex [24] and induces the ubiquitination of HIF-1α on its N- and C-terminal parts (aa 390–417 and 549–582, respectively) [22]. This ubiquitination targets HIF-1α for proteasomal degradation. In the other hand, under hypoxic conditions, the prolyl hydroxylase activity decreases and the degradation pathway described above is interrupted [25]. HIF-1α thus accumulates rapidly and translocates in the nucleus where, after dimerisation with ARNT, it induces target gene transcription involved notably in glycolysis (e.g. GAPDH) and angiogenesis (e.g. VEGF) [26], which allows cells to adapt to hypoxia [27].

Effects of intermittent hypoxia on endothelial cells are poorly known and in a general way, intermittent hypoxia is poorly understood. Some data indicates that transient decreases in blood flow have significant implication for delivery of chemotherapeutic agents, cellular responsiveness to those agents, and the regrowth potential of the surviving tumor cells [28]. Moreover, transient hypoxia could also be able to modify the sensitivity of cells to radiations [29]. Consequently, intermittent hypoxia plays an important role in tumor response to therapies and more largely to cell survival. Unfortunately, intracellular mechanisms induced by intermittent hypoxia in tumor cells as well as in endothelial cells are poorly described in the literature.

So, the aim of this work was to study the effects of intermittent hypoxia on endothelial cells and particularly on HIF-1, the central regulator of cell adaptation to hypoxia.

Intermittent hypoxia is described in the literature as a recurrent phenomenon inside vascularized tumours and composed of relatively short periods of hypoxia and reoxygenation [14,30–33]. Therefore, considering these data as well as the work of Martinive et al. [34], the effect of intermittent hypoxia was studied exposing endothelial cells to cycles of 1 h hypoxia followed by 30 min reoxygenation.

2. Materials and methods

2.1. Cell culture

EAhy926 endothelial cells [35] were maintained in Dulbecco's Modified Eagle Medium (DMEM) (4.5 g/L D-glucose) without L-glutamine and sodium pyruvate (Invitrogen) containing 10% (v/v) calf bovine serum (Invitrogen) and penicillin (50,000 units/l)–streptomycin (50,000 μg/l) (Invitrogen).

HMEC-1 endothelial cells were maintained in MCDB-131 Medium (Invitrogen) containing 10 ng/ml endothelial cell growth supplement (Sigma), 1 μg/ml hydrocortisone (Sigma), 10 mM L-glutamine (Sigma), and 15% (v/v) calf bovine serum (Invitrogen).

Hypoxia (1% O_2) incubations were performed in serum-free CO_2-independent medium (Invitrogen) supplemented with 50,000 units/l of penicillin, 50,000 μg/l streptomycin, and 10 mM L-glutamine (Sigma) by exposing the cells to an atmosphere containing 99% N_2 and 1% O_2. Control normoxic cells were maintained in normal atmosphere. The level of hypoxia in the medium in our experimental conditions is 10 mm Hg of dissolved oxygen within the medium. This level of hypoxia is achieved after about 10 min of incubation. On the other hand, during reoxygenation, the concentration of dissolved oxygen reaches 120 mm Hg within 2 min. Two incubation schedules were performed: chronic or intermittent hypoxia. Under chronic hypoxia, cells were incubated for a continued period from 1 to 6 h at 1% O_2. Under intermittent hypoxia, cells were subjected to repeated hypoxia (1 h, 1% O_2)/reoxygenation (30 min, 20% O_2) cycles with a maximum of 5 consecutive cycles.

2.2. Western blot analysis

Total cell extracts were prepared from EAhy926 or HMEC-1 cells grown in T25 flasks. Cell medium was replaced by CO_2-independent medium, and cells were incubated in normoxia (20% O_2) or Hypoxia (1% O_2) with or without inhibitors [KN93 (Biomol) at 15 μM, SP00125 (Calbiochem) at 20 μM, Staurosporine (Sigma) at 1 μM, SB203580 (Alexis) at 20 μM, Genistein (Sigma) at 10 μM, PD98059 (Biomol) at 20 μM, 2-Aminopurine (Sigma) at 10 mM, BAY11-70852 (Calbiochem) at 25 μM, LY294002 (Calbiochem) at 50 μM, H-89 (Biomol) at 20 μM]; or agonist [Iloprost (Cayman) at 100 nM]. After the incubation, cells were lysed using the extraction buffer [Tris 80 mM pH 7.5 (Merck), KCl 300 mM (Merck), EDTA 2 mM (Merck), Triton X-100 1% (v/v) (Sigma), containing a protease inhibitor mixture (≪ Complete ≫ from Roche Molecular Biochemicals, 1 tablet in 2 ml H_2O, added at a 1:25 dilution) and phosphatase inhibitors (NaVO3 25 mM, PNPP 250 mM, β-glycerophosphate 250 mM and NaF 125 mM, at a 1: 25 dilution)]. The lysate was collected in pre-cooled micro-tube, centrifuged 5 min at 13,000×*g*, 4 °C and the supernatant was collected. Extracts were separated on 10% sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) gels and then transferred to a polyvinylidene difluoride membrane (Amersham Biosciences). After blocking in phosphate saline buffer containing 0.1% (v/v) Tween and 5% (w/v) dried milk, the blot was probed with anti-HIF-1α antibodies (BD-Transduction Laboratories; diluted 1:2000; secondary antibody was an anti-mouse horseradish peroxidase-conjugated (Amersham) diluted 1:50,000), anti-phospho-p42 and p44 MAPKs antibodies (Cell Signaling; diluted 1:20,000; secondary antibody was an anti-rabbit horseradish peroxidase-conjugated (Amersham) diluted 1:200,000); anti-ERK-1 antibodies (Transduction Laboratories; diluted 1:20,000; secondary antibody was an anti-mouse horseradish peroxidase-conjugated (Amersham) diluted 1:200,000); anti-ERK-2 antibodies (Transduction Laboratories; diluted 1:20,000; secondary antibody was an anti-mouse horseradish peroxidase-conjugated (Amersham) diluted 1:200,000); anti-α-tubulin antibodies (Sigma; diluted 1:40,000; secondary antibody was an anti-mouse horseradish peroxidase-conjugated (Amersham) diluted 1:200,000); anti-phospho-AKT (ser 473) antibodies (Cell Signaling; diluted 1:10,000; secondary antibody was an anti-mouse horseradish peroxidase-conjugated (Amersham) diluted 1:50,000); anti-AKT antibodies (Cell Signaling; diluted 1:10,000; secondary antibody was an anti-rabbit horseradish peroxidase-conjugated (Amersham) diluted 1:50,000); anti-phospho-CREB antibodies (Upstate; diluted 1:10,000; secondary antibody was an anti-rabbit horseradish peroxidase-conjugated (Amersham) diluted 1:50,000); anti-CREB antibodies (Cell Signaling; diluted 1:5000; secondary antibody was an anti-rabbit horseradish peroxidase-conjugated (Amersham) diluted 1:50,000); anti-Aldolase antibodies (Chemicon international; diluted 1:500; secondary antibody was an anti-goat horseradish peroxidase-conjugated (Amersham) diluted 1:50,000). Chemiluminescent detection was performed with ECL Advance™ western blotting detection kit (Amersham Biosciences). All the experiments have been performed 2 or 3 times. One representation is shown in each figure.

2.3. λ-protein phosphatase (λ-PPase) treatment

40 μg proteins of total cell extracts for western blot were incubated at 30 °C during 60 min in the presence or not of 100 units of lambda protein phosphatase (New England Biolabs) and 1× λ-PPase reaction buffer supplemented with 2 mM $MnCl_2$ (New England Biolabs). These samples were used immediately to perform a western blot analysis, as described here above.

2.4. PKA kinase activity assay

EAhy926 cells were grown in T75 flasks. Cell medium was replaced by CO_2-independent medium and cells were incubated under intermittent hypoxia with or without H-89 (Biomol) at 20 μM. After the incubation, the medium was

removed and cells were washed 1X with ice-cold PBS. 1 ml of fresh lysis buffer [20 mM MOPS (Merck), 50 mM β-glycerophosphate (Prolabo), 50 mM sodium fluoride (Merck), 1 mM sodium vanadate (Sigma), 5 mM EGTA (Sigma), 2 mM EDTA (Merck), 1% (v/v) NP40 (Sigma), 1 mM dithiothreitol (DTT) (Sigma), 1 mM benzamidine (Sigma), 1 mM phenylmethanesulphonylfluoride (PMSF) (Sigma) and protease inhibitors 4% (v/v) (Complete®; Roche)] was added per T75 flask and the flasks were put on ice. After 10 min incubation period, cells were scraped using a rubber policeman/cell scraper and cell lysates were collected in a pre-chilled 1.5 ml microtube kept on ice and then sonicated 3×20 s. Microtubes were centrifuged at 13,000×*g* for 15 min at 4 °C. Clear supernatant was transferred to a pre-chilled 1.5 ml microtube and protein concentration was determined using Bradford method. The assay was performed immediately after cell lysis using a PKA Kinase Activity Assay Kit (Non-Radioactive) (Stressgen BIOREAGENT) with 40 μg of fresh lysate proteins for each condition.

2.5. Total RNA extraction

EAhy926 endothelial cells were grown in T75 flasks. Cell medium was replaced by CO_2 -independent medium and cells were incubated under intermittent or chronic hypoxia for periods of time from 1 to 7 h. After the

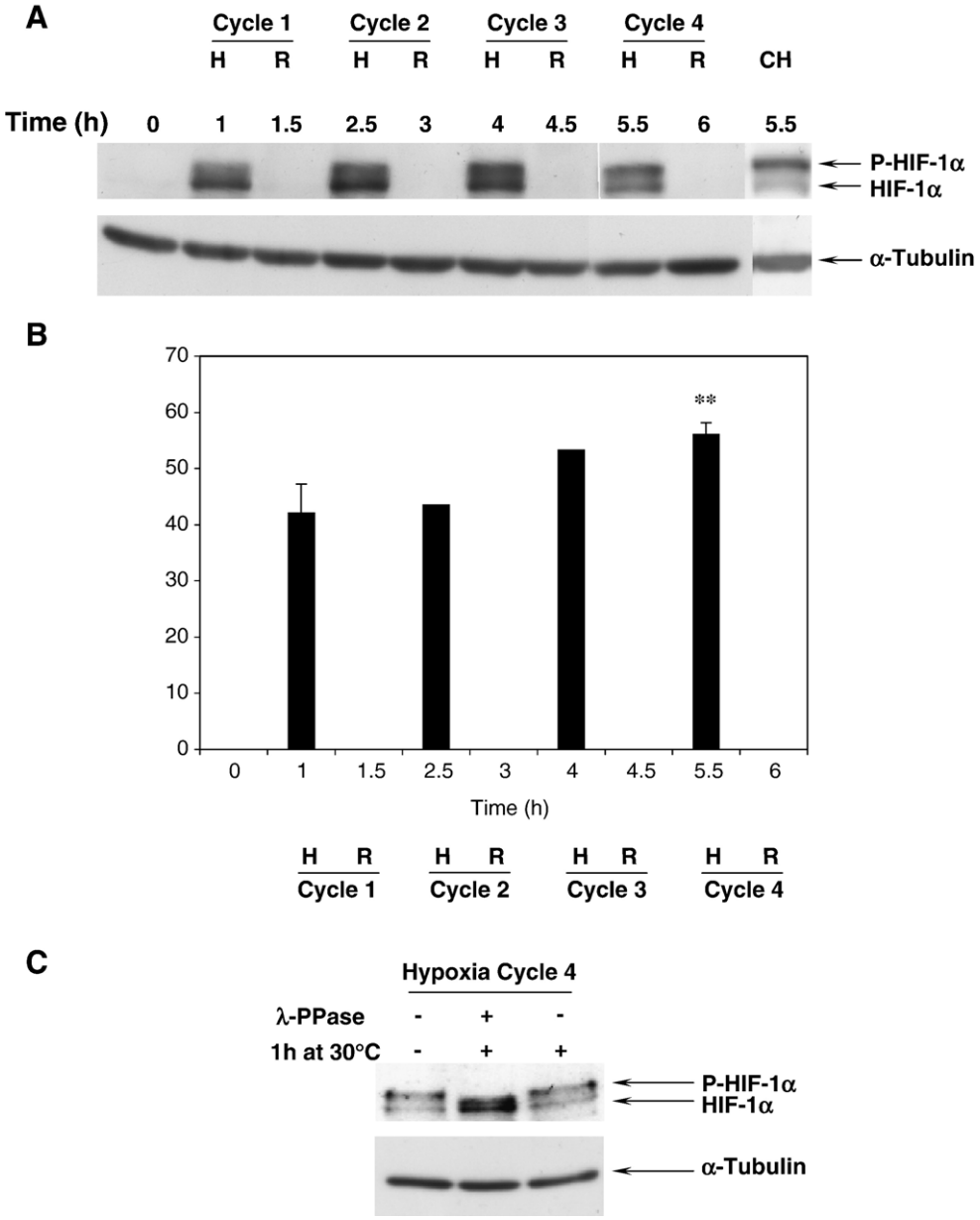

Fig. 1. Effect of hypoxia–reoxygenation (H–R) cycles on the abundance of the HIF-1α phosphorylated (P-HIF-1α) and non-phosphorylated (HIF-1α) forms. (A) EAhy926 cells were incubated during increasing times under cycles of hypoxia (H, 1 h)–reoxygenation (R, 30 min.) or during 5.5 h under normoxia (N) or chronic hypoxia (CH). After the incubation, total cell lysates were recovered for western blotting with antibodies against HIF-1α and α-tubulin used as loading control. This blot is representative of three independent experiments. (B) The optical density (O.D.) for each band on western blot analysis was determined. Ratios between the O.D. of the upper band and the O.D. of the corresponding lower band were reported on graph ($n=3$). **$p<0.01$ vs. H cycle 1 (Student's *t* test). (C) Cells were incubated under intermittent hypoxia and total cell lysates were treated or not with a λ-protein phosphatase and incubated or not during 1 h30 at 30 °C. These samples were then analysed by western blotting with antibodies against HIF-1α and α-tubulin used as loading control.

incubation, the medium was removed and total RNA extraction was performed using RNAgents kit according to the manufacturer's instructions (Promega).

2.6. Reverse transcription

For each condition, 2 μg of total RNA were mixed with 2 μl oligo (dT) (12–18) (500 ng/μl) (Gibco). The volume was then brought up to 9 μl with nuclease-free water (Promega). This mix was incubated 10 min at 70 °C and then put on ice for 5 min. 9 μl of reaction mix [4 μl Buffer RT 5X (Promega); 2 μl DTT 0,1 M (Promega); 1 μl RNAsin (40U/μl)(Promega); 2 μl dNTP mix (Eurogentec)] were added and the samples were incubated 5 min at room temperature. After addition of 1.5 μl SuperScriptRII (200U/μl) (Invitrogen), the samples were incubated 90 min at 42 °C and then 15 min at 70 °C. Finally, 1 μl of Ribonuclease H (2U/μl) (Gibco) was added and the samples were incubated 20 min at 37 °C before to be stored at −20 °C.

2.7. Realtime RT-PCR

The levels of aldolase transcripts were determined by real time reverse transcriptase (RT)-PCR. cDNA (5 μl) previously obtained by reverse transcription of total RNA was mixed to SYBR Green Master Mix PCR [2.5 μl distilled water, 1.7 μl of primer Reverse at 9 μM; 1.7 μl of primer Forward at 9 μM; 12.5 μl of SYBR green]. PCRs were carried out in a real time PCR cycler (ABI PRISM 7700 Sequence Detector, PE Applied Biosystems). Thermal cycling conditions were: initial incubation of 10 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 1 min at 57 °C annealing temperature, and 30 s at 72 °C. Samples were compared using the relative Ct method. To normalize for input load of cDNA between samples, α-tubulin was used as an endogenous standard. Specific primers were used: aldolase forward 5′-TGCGCAGGAGGAGTATGTCA-3′; aldolase reverse 5′-AGGCGTGGTTAGAGACGAAGAG-3′; α-tubulin forward 5′-CCCGAGGGCACTACACCAT-3′; α-tubulin reverse 5′-CAGGGAGGTGAACCCAGAAC-3′.

2.8. Transfection

To assay HIF-1 transcriptional activity, we used the pGL3-(PGK-HRE$_6$)-tk-luc reporter vector, which contains 6 HRE cis-elements from the PGK gene linked to the thymidine kinase basal promoter and to the firefly luciferase gene [36]. A Renilla luciferase plasmid (pRL, Promega) was used as the transfection normalization vector. For transfection, 1 μg of plasmid (5/6 pGL3-(PGK-HRE$_6$)-tk-luc + 1/6 pRL) was mixed with 60 μl of OptiMEM medium (Gibco). 0.6 μl of Superfect (QIAGEN) was then added and the solution was incubated 15 min at RT. Finally, the transfection solution was completed with 360 μl of OptiMEM medium containing 5.9% (v/v) FBS. 420 μl of this mixture was dispensed on cells cultured in a 24-well dish and previously rinsed with PBS. Cells were incubated 3 h at 37 °C, 5% CO_2 with the transfection solution and then rinsed with DHG medium. After transfection, cells were incubated 24 h at 37 °C, 5% CO_2 with DHG medium containing 10% (v/v) FBS. Before incubation under hypoxia, the medium was replaced by CO_2 independent medium as described previously.

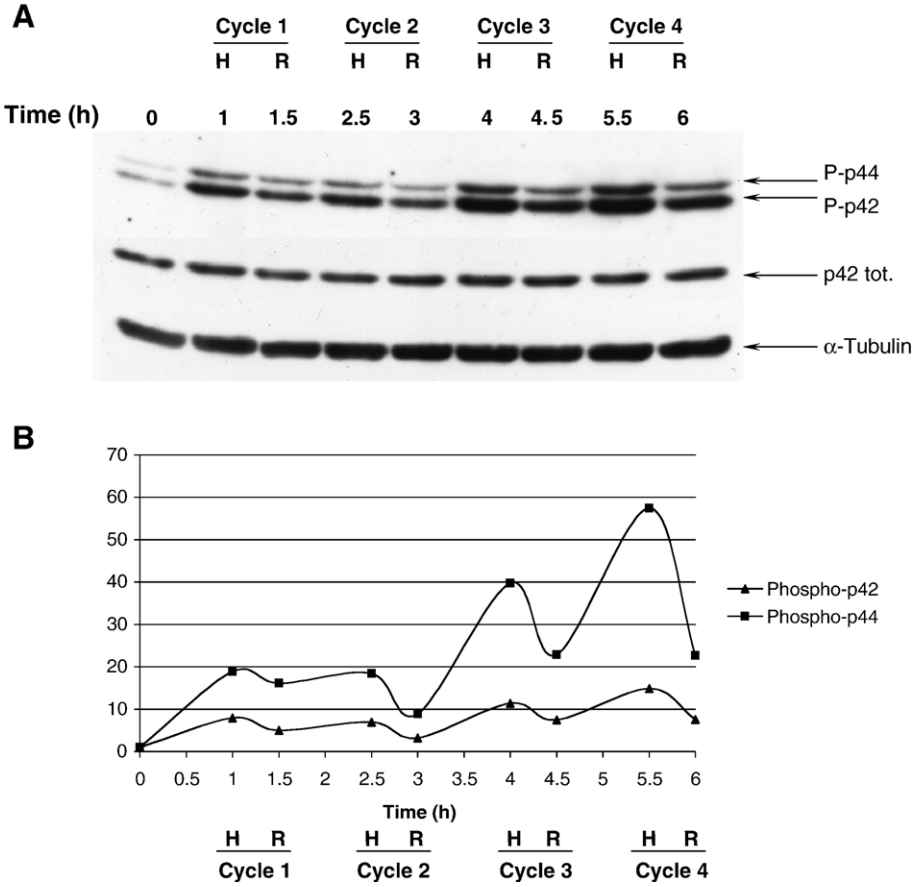

Fig. 2. Effect of Hypoxia–Reoxygenation cycles on p42 and p44 MAPK phosphorylation. (A) EAhy926 endothelial cells were exposed to four repeated hypoxia (H, 1 h)–reoxygenation (R, 30 min) cycles. After each step of the kinetics, total cell lysates were recovered for western blotting with antibodies against phospho-p42 MAPK (P-p42), phospho-p44 MAPK (P-p44), p42 MAPK total, and α-tubulin used as loading control. (B) The optical density (O.D.) of P-p42, P-p44 and α-tubulin bands on the western blot analysis from A were determined. The O.D. values for both P-p42 and P-p44 were normalized by the α-tubulin O.D. values.

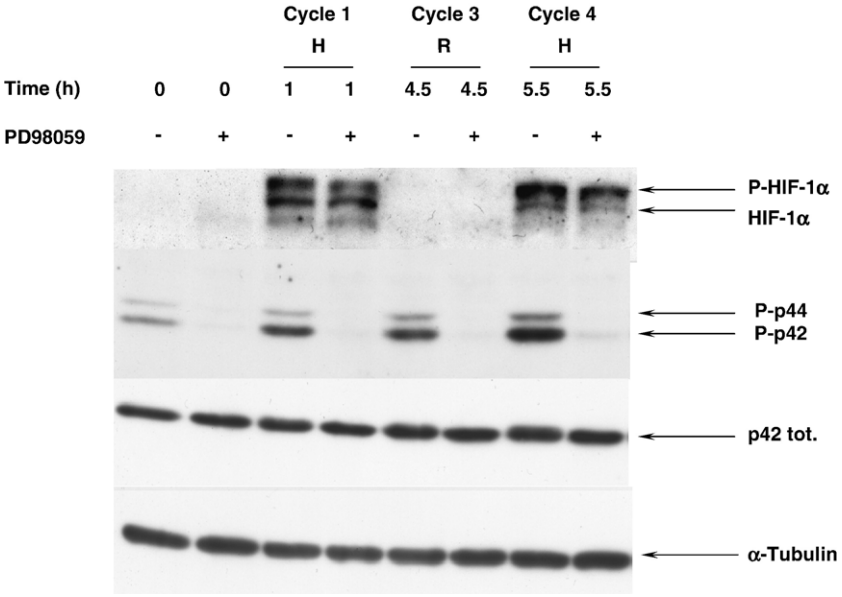

Fig. 3. Effect of p42 and p44 MAPK inhibition on HIF-1α phosphorylation during hypoxia–reoxygenation cycles. EAhy926 endothelial cells were incubated in the presence or not of PD98059 at 20 μM under intermittent hypoxia. Cell lysates at times 0, 1 h, 4.5 h and 5.5 h were recovered for western blotting with antibodies against HIF-1α, phospho-p42 MAPK (P-p42), phospho-p44 MAPK (P-p44), p42 MAPK total, and α-tubulin used as loading control. This blot is representative of two independent experiments.

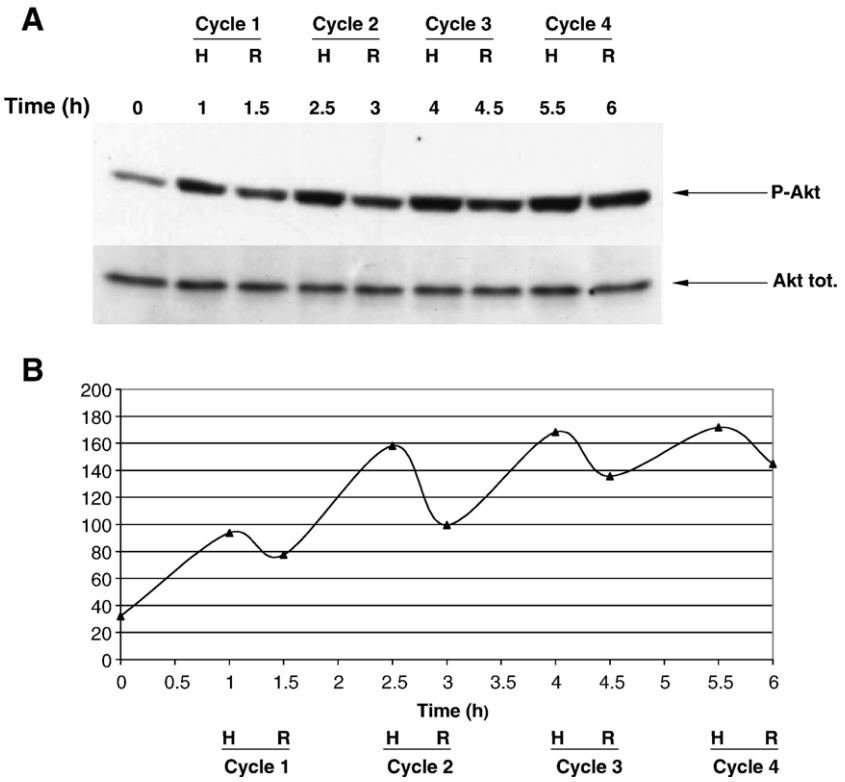

Fig. 4. (A) Effect of hypoxia–reoxygenation cycles on Akt phosphorylation. EAhy926 endothelial cells were exposed to four repeated hypoxia (H, 1 h)–reoxygenation (R, 30 min) cycles. After each step of the kinetics, total cell lysates were recovered for western blotting with antibodies against phospho-Akt (P-Akt), and Akt total. (B) The optical density (O.D.) for both P-Akt and Akt-total bands on the western blot analysis from A was determined. The O.D. values for P-Akt were normalized by the Akt-total values.

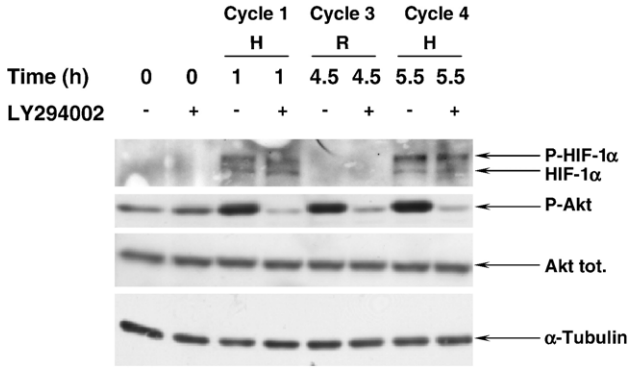

Fig. 5. Effect of AKT inhibition on HIF-1α phosphorylation during hypoxia–reoxygenation cycles. EAhy926 endothelial cells were incubated in the presence or not of LY294002 at 50 μM under intermittent hypoxia. Cell lysates at times 0, 1 h, 4.5 h and 5.5 h were recovered for western blotting with antibodies against HIF-1α, phospho-Akt (P-Akt), Akt total, and α-tubulin used as loading control. This blot is representative of two independent experiments.

The luciferase activity was quantified in a luminometer using the Dual Luciferase Assay System (Promega).

3. Results

3.1. Effect of hypoxia–reoxygenation cycles on HIF-1 alpha phosphorylation

EAhy926 endothelial cells were exposed to intermittent hypoxia and western blotting analysis was performed for HIF-1α. Two bands appeared on the western blot; the slower migrating line being a phosphorylated form of HIF-1α. Indeed, treatment with a λ-protein phosphatase (λ-PPase) of samples exposed to four hypoxia–reoxygenation cycles, induced the shift of the upper band to the lower band on western blotting analysis. No modification was observed for the lower band (Fig. 1C).

Cells exposure to hypoxia/reoxygenation repeated cycles induced an increase in the abundance of HIF-1α protein during the first three cycles as well as an increase in the abundance of the HIF-1α-phosphorylated form with respect to its non-phosphorylated form (Fig. 1A and B). After the first hour of hypoxia (cycle 1, time 1 h), HIF-1α-non-phosphorylated form is more abundant (about 60%). On the other hand, after the fourth hour of hypoxia (cycle 4, time 5.5 h), HIF-1α-phosphorylated form was predominant (about 70%). Changes in the respective abundance of these two forms of HIF-1α were made in a progressive way in the course of hypoxia/reoxygenation cycles. The abundance of the HIF-1α-phosphorylated form with respect to its non-phosphorylated form, after the fourth hypoxia–reoxygenation cycle, nearly reached the level observed for incubation under chronic hypoxia during 5.5 h. No HIF-1α was observed under normoxia and a complete disappearance of HIF-1α occurred at each reoxygenation step.

Viability was checked directly after the incubations using the ethidium bromide/acridine orange staining : about 10% of the cells were orange stained in all conditions: 5.5 h normoxia, 5.5 h chronic hypoxia and 4 cycles of intermittent hypoxia (data not shown). Long range recovery under normoxia has also been checked by a clonogenic assay: no difference was observed for cells incubated under normoxia or intermittent hypoxia after 7 days. The proliferative capacity of cells that have been incubated 5.5 h under chronic hypoxia was slightly higher (data not shown). All these results indicate that intermittent hypoxia did not affect cell viability in our experiment conditions.

3.2. Activation of MAPK p42 and p44 under intermittent hypoxia

Both p42 and p44 MAPKs (also respectively called ERK-2 and ERK-1) have been shown to be activated under hypoxia. It is also well established that ERK-1 can directly phosphorylate HIF-1α in its carboxyterminal domain [37]. Considering these data, we wanted to know whether these kinases could be responsible for the increase in the phosphorylation of HIF-1α observed under intermittent hypoxia. First, the MAPKs activation was followed in the course of hypoxia/reoxygenation cycles. Their phosphorylation state was assessed by western blot analysis (Fig. 2). A progressive increase in the phosphorylated form of both kinases was observed after each hypoxia step

Table 1
Effect of different kinase inhibitors on HIF-1α abundance and phosphorylation in EAhy926 endothelial cells under intermittent hypoxia

Inhibitors	Targeted Kinases	HIF-1α total abundance (with respect to the control hypoxia without kinase inhibitor)	Modification in HIF-1α phosphorylation pattern
KN93	Calmodulin Kinases	Decrease	No
SP00125	JNK	No modification	No
Staurosporin	Protein Kinases	Decrease	No
SB203580	P38 MAPK	Decrease	No
PD98059+SB203580	MEK and P38 MAPK	Decrease	No
Aminopurin	Ser/Thr Kinases	Decrease	No
BAY11-7082	IKK	Decrease	No
H-89	PKA	No modification	Yes

EAhy926 endothelial cells were submitted to four repeated hypoxia (H, 1 h)–reoxygenation (R, 30 min) cycles in the presence or not of KN93 (15 μM), SP00125 (20 μM), staurosporine (1 μM), SB203580 (20 μM), genistein (10 μM), PD98059 (20 μM)+SB203580 (1 μM), aminopurine (10 mM) or BAY11-70852 (25 μM). Cell lysates were recovered for western blotting with an antibody against HIF-1α.

when compared to the control (time zero). On the other hand, reoxygenation step decreased p42/p44 phosphorylation. However, the initial low phosphorylation state was never reached again. The parallelism between the increase in p42/p44 phosphorylation and the increase in HIF-1α phosphorylation was striking.

In order to study the possible implication of MAPKs in the phosphorylation of HIF-1α in the course of hypoxia/reoxygenation cycles, cells were incubated in the presence or not of PD98059, a MAPKKs inhibitor. MAPKKs phosphorylate and activate both p42 and p44. Two extreme points of the hypoxia/reoxygenation cycle kinetics (hypoxia cycle 1 and hypoxia

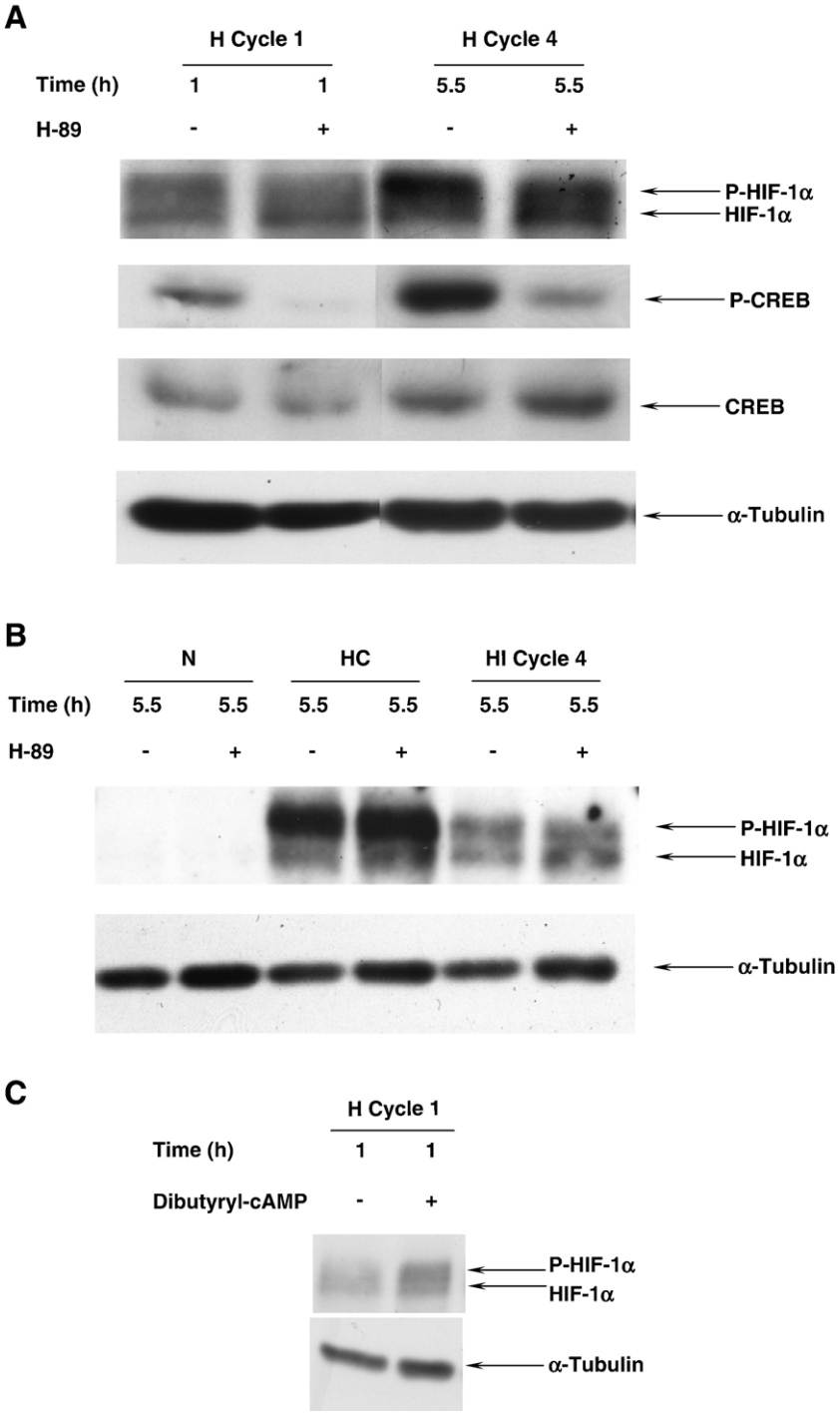

Fig. 6. Effect of PKA inhibition (A, B) or activation (C) on HIF-1α phosphorylation during hypoxia–reoxygenation cycles or under chronic hypoxia. EAhy926 endothelial cells were incubated in the presence or not of H-89 at 20 μM or iloprost at 100 nM under intermittent hypoxia, chronic hypoxia (HC) or normoxia (N). Cell lysates at both times 1 h and 5.5 h were recovered for western blotting with antibodies against HIF-1α, phospho-CREB (P-CREB), CREB and α-tubulin used as loading control. These blots are representative of three independent experiments.

cycle 4) were studied since they are the times for which the ratios between the abundance of HIF-1α-phosphorylated form and HIF-1α non-phosphorylated form were opposite. PD98059 at 20 μM induced a complete inhibition of p42/p44 phosphorylation at all the time points, as observed on the western blot. However, we did not notice any difference in the ratio of HIF-1α phosphorylated form versus its non-phosphorylated form in the presence of this inhibitor (Fig. 3). These results indicate that p42 and p44 were not responsible for HIF-1α phosphorylation under intermittent hypoxia.

3.3. Activation of Akt under intermittent hypoxia

In addition the MAPKs pathway, it is also well established that the PI3K/Akt pathway can influence HIF-1α stabilization [38] and HIF-1α activity [39]. In order to study the implication of this pathway in the modification of HIF-1α phosphorylation pattern under intermittent hypoxia, Akt phosphorylation was followed throughout the hypoxia/reoxygenation cycles kinetics. An increase in Akt phosphorylation under hypoxia was observed while a decrease occurred when cells were reoxygenated (Fig. 4). Akt phosphorylation gradually increased after each hypoxia/reoxygenation cycle under hypoxia as well as after reoxygenation. Indeed, phosphorylation level never returned to the one observed under normoxia (time zero). As for p42/p44 phosphorylation, this modification of Akt phosphorylation in the course of hypoxia/reoxygenation cycle kinetics paralleled the changes observed for HIF-1α-phosphorylation. In order to test whether Akt could be responsible for the phosphorylation of HIF-1α in these conditions, cells were incubated in the presence or not of LY294002, a PI3K inhibitor. As for MAPKs, this experiment was limited to the end points of the hypoxia/reoxygenation cycles kinetics. As previously observed, Akt was phosphorylated under hypoxia with an increase in phosphorylation after 5.5 h with respect to 1 h (Fig. 5). As expected, Akt phosphorylation decreased in the presence of LY294002 at all time points.

With regard to HIF-1α, the phosphorylation shift in the presence of LY294002 between the time 1 h and 5.5 h was again observed. No difference in HIF-1α phosphorylation with respect to the conditions without inhibitor could be evidenced. These results suggest that Akt was not involved in the modification of HIF-1α phosphorylation pattern in the course of hypoxia/reoxygenation cycles.

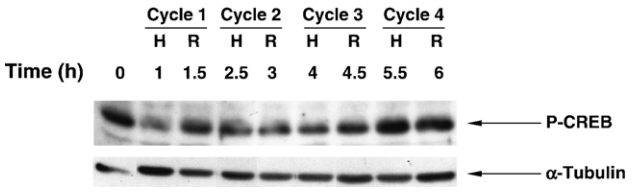

Fig. 7. Effect of Hypoxia–Reoxygenation cycles on CREB phosphorylation. EAhy926 endothelial cells were exposed to four repeated hypoxia (H, 1 h)–reoxygenation (R, 30 min) cycles. After each step of the kinetics, total cell lysates were recovered for western blotting with antibodies against phospho-CREB (P-CREB) and α-tubulin used as loading control.

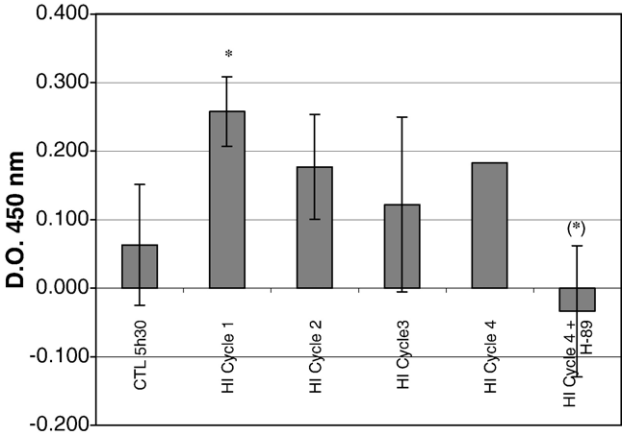

Fig. 8. Effect of Hypoxia–Reoxygenation cycles on PKA activity. EAhy926 endothelial cells were exposed to four repeated hypoxia (H, 1 h)–reoxygenation (R, 30 min) cycles in the presence or not of H89 at 20 μM. After each step of hypoxia, total cell lysates were recovered for a PKA Kinase Activity Assay (Stressgen Bioreagents, Canada). Three separate experiments were performed and the results are expressed as means ± 1 SD. *$p<0.05$ vs. normoxia; (*) $p<0.05$ vs. H cycle 4 (Student's t test).

3.4. Implication of different kinases

In order to try to identify the kinase(s) involved in HIF-1α phosphorylation in the course of hypoxia/reoxygenation cycles, different inhibitors targeting the "main" cellular kinases currently described in the literature were tested. These inhibitors and their target kinases are listed in Table 1. In this experiment, we focused our attention on the fourth hypoxia cycle for which, in our experimental conditions, the HIF-1α phosphorylated form was predominant with respect to its non-phosphorylated form. Our aim was to observe, in the presence of a particular inhibitor, a decrease in the proportion of HIF-1α-phosphorylated form with respect to its non-phosphorylated form.

An inhibition of HIF-1α stabilization was observed when PKC or JNK+p42/p44 were inhibited. Whether or not a decrease of the total abundance of HIF-1α level was observed, an identical pattern of phosphorylation to what is observed for the control was still observed in the presence of all these inhibitors, indicating that these kinases were probably not involved in the increase in HIF-1α phosphorylation under repeated hypoxia/reoxygenation cycles. These results are summarized in Table 1.

3.5. Involvement of PKA

The role of the PKA in HIF-1α phosphorylation during intermittent hypoxia was then studied. The HIF-1α-phosphorylated state was followed in the presence or not of H-89, an inhibitor of PKA, at cycles one and four. As observed in Fig. 6A, the phosphorylation shift was observed as previously in the absence of inhibitor. On the other hand, in the presence of H-89, the phosphorylation shift did no longer occur and a decrease in the abundance of the HIF-1α-phosphorylated form was observed both at 1 h and 5.5 h. Conversely, iloprost, a

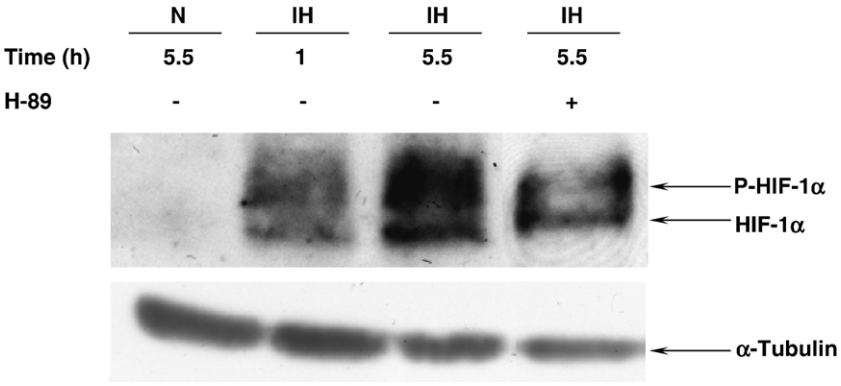

Fig. 9. Effect of hypoxia–reoxygenation (H–R) cycles and PKA inhibition on the abundance of the HIF-1α phosphorylated (P-HIF-1α) and non-phosphorylated (HIF-1α) form in HMEC-1 endothelial cells. HMEC-1 endothelial cells were incubated under normoxia or intermittent hypoxia in the presence or not of H-89 at 40 μM. Cell lysates at times 1 h and 5.5 h were recovered for western blotting with antibodies against HIF-1α and α-tubulin used as loading control. This blot is representative of two independent experiments.

PKA activator, enhanced the abundance of the phosphorylated form of HIF-1α (Fig. 6C). Interestingly, under chronic hypoxia, the inhibition of PKA by the H-89 did not modify the ratio observed between the abundance of the phosphorylated and non-phosphorylated form of HIF-1α (Fig. 6B). These results suggest that PKA may be involved in the phosphorylation of HIF-1α under intermittent hypoxia but not under chronic hypoxia.

In parallel, the phosphorylation of one of the many PKA substrates, the protein CREB was studied. CREB phosphorylation state after each stage of hypoxia and reoxygenation of hypoxia/reoxygenation cycles kinetics was followed by western blotting. In resting conditions, in EAhy926 cells, CREB was already strongly phosphorylated (Fig. 7). The first hypoxia induced a marked decrease in CREB phosphorylation. Then, its phosphorylation gradually increased after each hypoxia period and reached its maximal level in hypoxia/reoxygenation cycles kinetics after the fourth cycle, when the HIF-1α-phosphorylated form was the most abundant. Incubation in the presence of H-89 induced a strong decrease in CREB phosphorylation under intermittent hypoxia (Fig. 6A).

In order to study the actual PKA activity in the course of hypoxia–reoxygenation cycles, a PKA kinase assay was performed. No significant PKA activity was detected in normoxia. After the first hypoxia step, PKA activity was markedly increased with respect to normoxia. This increase in activity was also observed for the three other hypoxia steps. In the presence of H-89, after the fourth hypoxia incubation, the PKA activity was undetectable; indicating that the activity measured was specific for PKA (Fig. 8). These results indicate that an increase in PKA activity was observed during the four periods of intermittent hypoxia, which would be then responsible for the progressive increase in CREB phosphorylation as well as in HIF-1α phosphorylation.

HIF-1α phosphorylation under intermittent hypoxia was also studied in HMEC-1 (human microvascular endothelial cells). Four cycles of hypoxia–reoxygenation induced an increased in HIF-1α phosphorylation, as observed in EAhy926 endothelial cells. Moreover, in the presence of H-89, a decrease in HIF-1α phosphorylation was observed (Fig. 9). These results indicate that intermittent hypoxia also modifies HIF-1α phosphorylation pattern in another line of endothelial cells and that PKA is also involved in these modifications.

3.6. HIF-1α is transcriptionally active during intermittent hypoxia

To complete our study, we wanted to investigate whether this increase in the proportion of the HIF-1α-phosphorylated form modified HIF-1 transcriptional activity. Indeed, reports in the literature describe that HIF-1α phosphorylation increases HIF-1 transcriptional activity [37,40].

To be transcriptionally active, HIF-1α needs to localize in the cell nucleus. Considering this fact, the subcellular localization of HIF-1α during both intermittent and chronic hypoxia was investigated by immunofluorescence analysis. The results showed that HIF-1α was localized in the nucleus after each hypoxia cycle. However, after the second and the third cycles, HIF-1α was also present in the cytosol. Under chronic hypoxia, HIF-1α was exclusively nuclear (data not shown).

HIF-1 transcriptional activity was followed by measuring the quantity of one HIF-1 target gene mRNA by real-time RT-PCR. Under intermittent hypoxia, a progressive increase in aldolase mRNA level was observed at the third, fourth and fifth periods of hypoxia (Fig. 10A). On the other hand, in the presence of H-89, a decrease in aldolase mRNA level was observed (Fig. 10B) while H-89 did not decrease the increase in aldolase mRNA levels under chronic hypoxia (data not shown). These results indicate that phosphorylation of HIF-1α by PKA is important for HIF-1 transcriptional activity under intermittent hypoxia but not chronic hypoxia.

HIF-1 transcriptional activity was also studied under hypoxia using a reporter gene assay (Fig. 11A). A significant increase in the luciferase activity was observed after incubation of cells under chronic and intermittent hypoxia with respect to the normoxic control. The effect of PKA activity inhibition by H-89

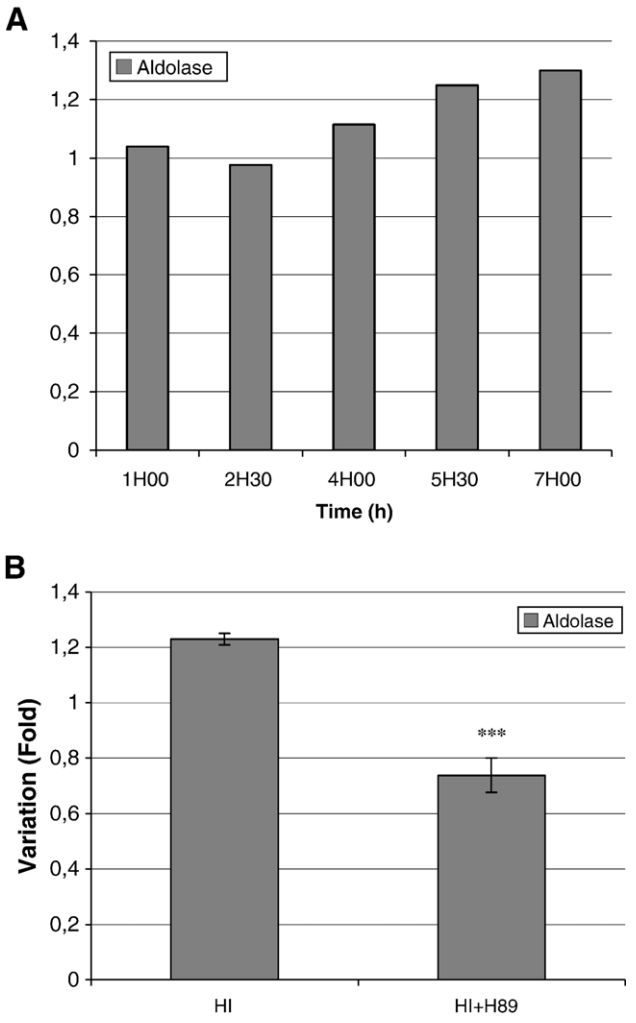

Fig. 10. Effect of Hypoxia–Reoxygenation cycles on Aldolase mRNA expression. (A) EAhy926 endothelial cells were exposed to 4 repeated hypoxia (1 h)–reoxygenation (30 min) cycles ended by a step of 1 h of hypoxia. mRNA was extracted after each hypoxia step and retrotranscribed into cDNA which was used to process a real time RT-PCR for Aldolase and α-tubulin using specific primers. Results are means from two independent experiments. (B) EAhy926 endothelial cells were submitted, in the presence or not of H-89 (at 20 μM), to 5 repeated hypoxia (1 h)–reoxygenation (30 min) cycles ended by a step of 1 h of hypoxia. mRNA was extracted at the end of the kinetics and retrotranscribed into cDNA which was used to process a real time RT-PCR for Aldolase and α-tubulin using specific primers. Results are means from three independent experiments. ***$p<0.001$ vs. HI (Student's t test).

on HIF-1 transcriptional activity could not be tested using the reporter system because of interference with the reporter luciferase expression. These results indicate that HIF-1 is transcriptionally active under intermittent hypoxia, as already suggested by the increase in aldolase mRNA level.

In parallel with the reporter gene assay, HIF-1 transcriptional activity was studied by western blot analysis by following the abundance of aldolase protein under hypoxia. An increase in the abundance of aldolase was observed after four cycles of hypoxia–reoxygenation as well as under chronic hypoxia (Fig. 11B). All together, these results indicate that HIF-1 is transcriptionally active under intermittent hypoxia.

4. Discussion

This study evidenced a modification in HIF-1α phosphorylation pattern under intermittent hypoxia in EAhy926 endothelial cells. Indeed, an increase in the abundance of HIF-1α phosphorylated form with respect to its non-phosphorylated form was observed along with the number of hypoxia/reoxygenation cycles. This modification in HIF-1α phosphorylation pattern was also observed in HMEC-1 endothelial cells.

A modification in total abundance of HIF-1α was also evidenced under intermittent hypoxia at least during the first three hypoxia/reoxygenation cycles. This increase was observed in western blot and immunofluorescence analysis. The gradual increase in the abundance of HIF-1α in the course of the three first hypoxia/reoxygenation cycles was not an accumulation of HIF-1α in the course of time. Indeed, during each reoxygenation step, HIF-1α was completely degraded. These results suggest an enhanced HIF-1α stabilization at each of hypoxia period. This observation has also been made in HUVEC [34].

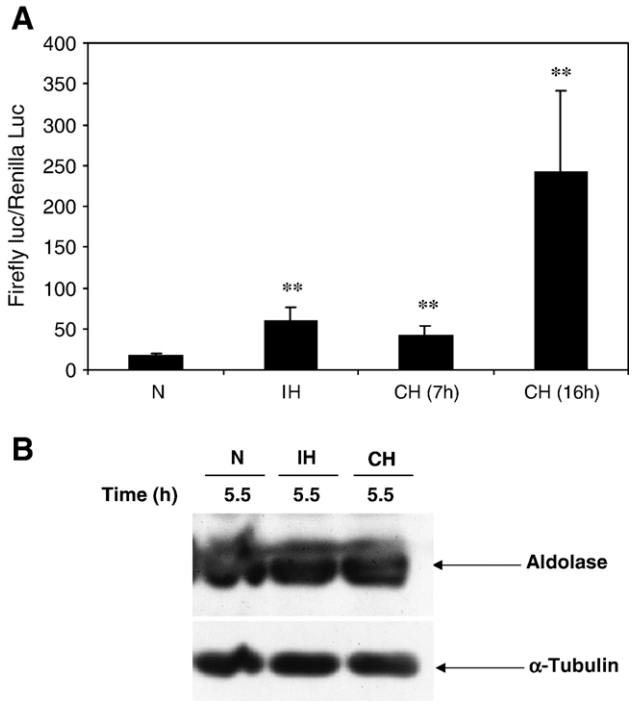

Fig. 11. Effect of Hypoxia–Reoxygenation cycles on HIF-1 transcriptional activity. (A) EAhy926 endothelial cells were transfected with the pGL3-(PGK-HRE6)-tk-Luc and pRL normalization vectors. Then the cells were exposed to 5 cycles of hypoxia (1 h)–reoxygenation (30 min) (HI), 7 h or 16 h under chronic hypoxia (CH) or 7 h under normoxia (N). Thereafter, the cells were incubated 16 h in normoxia in order to allow the synthesis of proteins. Data represent the ratio between test firefly luciferase activity normalized with the renilla luciferase activity. Results are presented as means±S.D. for triplicates. **$p<0.01$ vs. normoxia (Student's t test). (B) EAhy926 endothelial cells were incubated during 5.5 h under normoxia (N), intermittent hypoxia (IH) (4 cycles of hypoxia (1 h)–reoxygenation (30 min)), or chronic hypoxia (CH). After the incubation, total cell lysates were recovered for western blotting with antibodies against aldolase and α-tubulin used as loading control.

HIF-1α phosphorylation under intermittent hypoxia is poorly described in the literature, contrary to HIF-1α phosphorylation evidenced under chronic hypoxia. Similarly, the relevance of this phosphorylation under intermittent hypoxia remains poorly understood.

Several kinases are known to be activated by chronic hypoxia and at least one of them has been shown to directly phosphorylate HIF-1α [37,40,41]. We thus wanted to determine the kinase involved in HIF-1α phosphorylation under intermittent hypoxia. To this aim, we studied the activation of different kinases and determined their implication in HIF-1α phosphorylation under intermittent hypoxia using specific inhibitors.

p42 and p44 MAPKs activation was evidenced in our experimental conditions. Indeed, after each hypoxia step, an increase in the abundance of phospho-p42 and phospho-p44 was observed. This increase was gradual in the course of hypoxia/reoxygenation cycles and reached its maximum at the last hypoxia step. In the other hand, after each reoxygenation, the phosphorylation decreased, but it never returned to the basal level.

The inhibition of p42 and p44 MAPKs activation by PD98059 did not affect HIF-1α phosphorylation in our experimental conditions. These results indicate that p42 and p44 MAPKs are NOT involved in HIF-1α phosphorylation under intermittent hypoxia and thus indicate that the pathways involved in HIF-1α phosphorylation under chronic and intermittent hypoxia are different. Indeed, Mylonis et al. [41] showed that p42/p44 MAPKs phosphorylate HIF-1α on serine 641 and serine 643 and that these phosphorylation sites are needed for HIF-1 transcriptional activity under chronic hypoxia. The physiological relevance of p42/p44 activation under intermittent hypoxia, for example for cell survival and/or proliferation, remains to be elucidated.

PI3K/Akt pathway activation under chronic hypoxia has also been described in the literature. Moreover, inhibition of this pathway prevents HIF-1α stabilization under chronic hypoxia [38,42]. Activation of this pathway was evidenced by Akt. In the course of hypoxia/reoxygenation cycles, a gradual increase in the phosphorylation of Akt was observed during the hypoxia periods. The abundance of phospho-Akt increased after each hypoxia step and reached its maximum level at the last step of hypoxia, as observed for phospho-p42 and phospho-p44 MAPKs. On the other hand, each reoxygenation step induced a partial dephosphorylation of Akt.

Inhibition of this pathway with LY294002 did not prevent HIF-1α stabilization in our experimental conditions. Moreover, no modification in HIF-1α phosphorylation pattern was observed. This discrepancy could be explained either by difference in cell type, or by different mechanisms switched on during intermittent hypoxia in comparison to chronic hypoxia. Indeed, Mottet et al. showed HIF-1α destabilization under hypoxia in the presence of LY294002 in hepatoma HepG2 cells [38], while Arsham et al. did not observe such an effect in glioblastoma cells [43]. As mentioned before, activation of Akt during the hypoxia period may play a role in the increase in HIF-1α stabilization. It certainly has also consequences for cell survival pathways.

Using chemical inhibitors of several kinases, we evidenced the implication of the PKA in HIF-1α phosphorylation under intermittent hypoxia. Indeed, a modification of HIF-1α phosphorylation pattern could be observed in the course of hypoxia/reoxygenation cycles when cells were incubated in the presence of H-89 (a PKA inhibitor). In the presence of H-89, the non-phosphorylated form of HIF-1α became predominant with respect to the phosphorylated form, while without H-89 the phosphorylated form of HIF-1α was predominant. The reverse was observed in the presence of iloprost, a PKA activator. This modification in HIF-1α phosphorylation pattern in the presence of H-89 indicates that PKA is involved in HIF-1α phosphorylation under intermittent hypoxia. On the other hand, under chronic hypoxia, H-89 did not affect the HIF-1α phosphorylation pattern. Therefore, HIF-1α phosphorylation by PKA would seem to be a specific response under intermittent hypoxia, but not under chronic hypoxia. This is coherent with the results of Batmunkh et al. [44] that showed dbAMP did not enhance HIF-1α protein level under chronic hypoxia.

It is well established that under hypoxia, intracellular cAMP concentration can increase [45,46], hence driving PKA activation. In our experimental conditions, PKA activation was evidenced. Indeed, a direct kinase assay showed enhanced PKA activity under intermittent hypoxia in comparison to normoxic cells. An increased PKA activity has also recently been observed in murine macrophages exposed to chronic hypoxia [47]. In parallel, a gradual increase in the phosphorylation of CREB, a target of PKA, was observed in the course of hypoxia/reoxygenation cycles while its phosphorylation decreased when cells were reoxygenated. CREB phosphorylation under intermittent hypoxia was PKA dependent, because it was inhibited by H-89. An increase in CREB phosphorylation was also evidenced after repeated hypoxic exposure in mice brain [48]. At least two non-exclusive hypotheses can be proposed to explain the increase in PKA activity observed under intermittent hypoxia. (i) The activation of the PKA under intermittent hypoxia could be dependent on adenosine A_2 receptor (A_2R). Indeed, it was evidenced that hypoxia could induce the activation of the cAMP-PKA pathway by the A_2R in retinal endothelial cells [49,50]. Hypoxia is known to lead to a release of endogenous nucleoside adenosine [51]. It was evidenced recently in HUVEC, that this increase in concentration of extracellular adenosine under hypoxia is due to a reduced transport of adenosine by the human Equilibrative Nucleoside Transporters 1 (hENT1). Indeed, hypoxia can reduce the adenosine transport velocity of hENT1, as its protein and mRNA levels [52]. Extracellular adenosine interacts with the A_2R [53] which is a members of the G protein-coupled receptor family [54]. This receptor is positively linked to adenylate cyclase [55]. Therefore, A_2R activation by adenosine leads to an increase in cAMP level in PKA activation.

(ii) Another autocrine pathway could also be switched on under hypoxia, leading to PKA activation. We have previously shown that when submitted to hypoxic conditions, HUVEC rapidly release high amount of prostaglandins (PG), amongst which is prostacyclin [56]. This synthesis is triggered by an activation of the soluble PLA_2 due to an increase in intracellular calcium concentration [57]. Similarly to adenosine, PGI_2 can

interact with its specific receptor (IP) which is also a G protein coupled receptor positively linked to the adenylate cyclase [58]. It has to be mentioned that both adenosine and prostaglandin pathways are coupled since under hypoxia, it has been shown that adenosine, through interaction with its receptor, increase PG synthesis, thereby generating cAMP by an autocrine loop [59,60]. An accumulation of nucleoside adenosine and/or prostacyclin in the culture medium during the repeated cycles of hypoxia/reoxygenation could thus lead to a continuous increase in PKA activity during these cycles and hence to a parallel increase in HIF-1α phosphorylation. These hypotheses remain however to be confirmed.

We also investigated if an oxidative stress could be responsible for the activation during the following hypoxia period. Based on the articles from Prabhakar's laboratory, we decided to study the effect of the presence of an antioxidant during the hypoxia/reoxygenation cycles, on HIF-1alpha phosphorylation. We used MnTMPyP, a SOD mimetic, at 25 μM as in Yuan et al. [61]. The results show that MnTMPyP did not affect the increase in HIF-1alpha phosphorylation observed after 4 cycles of hypoxia/reoxygenation (data not shown). The experimental conditions used by Yuan et al. were very different from ours: the length of the hypoxia and the reoxygenation periods was much smaller : 15 s hypoxia+4 min reoxygenation 60 or 120 folds in comparison to 60 hypoxia +30 min reoxygenation from 1 to 4 folds in our work. This may explain the discrepancy between the two studies.

Ryan et al. [62] reported a p38 MAPK-dependent activation of NF-kB in bovine aortic endothelial cells. NF-kB activity has been assayed in our experiment conditions but no activation could be evidenced while cells responded very well to TNF-α (data not shown). The experimental conditions used by Ryan et al. were very different from ours : the length of the hypoxia and the reoxygenation periods was much smaller : 5 min hypoxia+ 10 min reoxygenation from 2 to 16 folds in comparison to 60 hypoxia+30 min reoxygenation from 1 to 4 folds in our work. This may explain the discrepancy between the two studies. Indeed, they did observe NF-kB activation but not HIF-1 stabilization (probably because the hypoxia duration was much too short) while we did demonstrate HIF-1 stabilization. The signaling pathways initiated in both incubation protocols are thus probably very different.

HIF-1 is transcriptionally active when in the nucleus. The nuclear accumulation of HIF-1α under intermittent hypoxia could explain the increase in HIF-1 transcriptional activity observed during the hypoxia/reoxygenation cycles, as observed by the increase in target gene mRNA levels which seem to be influenced by the PKA activity. The phosphorylation of HIF-1α can also induce an increase in HIF-1 transcriptional activity [40,63]. Therefore, the increase in HIF-1α phosphorylated form with respect to the non-phosphorylated form could synergize with HIF-1α nuclear accumulation to increase the HIF-1 transcriptional activity under intermittent hypoxia.

We run the HIF-1alpha protein sequence in the NetPhosK algorithm and found 10 putative PKA phosphorylation sites. One of them (serine 475) is located in the ODD domain between the two prolines that can be hydroxylated: phosphorylation of this serine may modulate pVHL-ODD domain as it has been shown for the acetylation of lysine 532 by ARD [64] or the nitrosylation of cysteine 520 [65], and thus influence HIF-1alpha stability. Two other putative PKA phosphorylation sites (serine 760 and serine 808) are located within the C-terminal transactivation domain : phosphorylation of these serines may modulate CBP/p300-HIF-1alpha interaction as it has been shown for the hydroxylation of the arginine 803 by FIH [66] and the nitrosylation of cysteine 800 [67], and hence influence HIF-1 transcriptional activity.

The physiologic significance of the progressive HIF-1α phosphorylation under intermittent hypoxia is not known. On the other hand, numerous data exist about chronic hypoxia and the role of HIF-1α phosphorylation. Suzuki et al. [68] proposed a model, which explains the implication of HIF-1α phosphorylation status for the cell behavior under chronic hypoxia. In this model, HIF-1α would have a dual function distinguished by its phosphorylation status. Indeed, HIF-1α phosphorylated form will lead to cell survival through the binding of HIF-1α to ARNT (Aryl hydrocarbon Receptor Nuclear Translocator) and the increase in the transcription of genes involved in the adaptation of cells to hypoxia. This process would occur under mild hypoxia. On the other hand, under prolonged or severe hypoxia, HIF-1α non-phosphorylated form interacts with p53, thus leading to p53 stabilization, which would then induce apoptosis through enhanced Bax expression. In this case, HIF-1 is no longer transcriptionally active.

According to this model, the progressive increase in the abundance of HIF-1α phosphorylated form observed here would indicate that intermittent hypoxia would lead, as non-severe chronic hypoxia, to cell survival. Indeed, we did not observe any increase in the abundance of p53, nor in cell death under intermittent hypoxia (data not shown). Moreover, HIF-1α is transcriptionally active as evidenced by the increase in HIF-target gene mRNA expression and when using the HRE-dependent reporter system. Further investigations are needed to reinforce this hypothesis. In a study run in parallel to this one, the effect of intermittent hypoxia was studied on the sensitivity of endothelial cells to radiations. Martinive et al. [34] showed that intermittent hypoxia reduced radiation-induced cell death in comparison to cells incubated under normoxia, thus demonstrating that pre-conditioning is effective in these conditions. Moreover, this survival effect was HIF-1-dependent.

In summary, we showed that repeated cycles of hypoxia/reoxygenation induced a modification in HIF-1α-phosphorylation pattern in EAhy926 endothelial cells. Activation of p42/p44 MAPKs, Akt and PKA was observed in these conditions. The use of inhibitors indicates that PKA was shown to be involved in the phosphorylation of HIF-1α under intermittent hypoxia, while p42/p44 MAPKs and Akt were not, and would seem to influence the HIF-1α transcriptional activity. Some differences between intermittent hypoxia and chronic hypoxia were also highlighted regarding phosphorylation pattern as well as transcriptional activity.

In vivo, tumor endothelial cells exposed to intermittent hypoxia undergo different stresses. Hypoxia *per se* reduces cell

metabolism but it also induces a decrease in pH [69], both generating difficult survival conditions for cells. The recurrence of these stresses by repeated hypoxia/reoxygenation cycles would induce an increase in HIF-1α phosphorylation in endothelial cells as shown here, which, according to the Suzuki's model, would lead to cell adaptation to these difficult conditions. It would thus be more favorable to maintain the new blood vessel network. Moreover, regarding treatment benefit, the maintenance of blood vessels in vascularized solid tumor seems to be important for the efficacy of chemotherapeutic treatments [3], but their destruction or inhibition of their formation by angiogenic agents could also induce the regression of tumors [70]. To maintain or to destroy blood vessels is thus an important issue in cancer treatment. A better knowledge of the effects of intermittent hypoxia on endothelial cells and the highlight of particular mechanisms induced by intermittent hypoxia are thus essential to understand the behavior of endothelial cells during neo-angiogenesis and to point important mechanisms in the maintenance or destruction of tumor vascular network.

Acknowledgments

Sébastien Toffoli is recipient of a FNRS-Télévie grant. Carine Michiels and Olivier Feron are senior research associates of FNRS (Fonds National de la Recherche Scientifique, Belgium). This article presents results of the Belgian Program on Interuniversity Poles of Attraction initiated by the Belgian State, Prime Minister's Office, Science Policy Programming. The responsibility is assumed by its authors. We are grateful to Prof. C. Edgell (Pathology Department, University of North Carolina) for kindly donating the EAhy926 cells and to Prof. P. Ratcliffe (Institute of Molecular Medicine, John Radcliffe Hospital, Oxford) for giving us the pGL3(PGK-HRE6)-tk-Luc plasmid.

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