

# Impact of cyclic hypoxia on HIF-1 $\alpha$ regulation in endothelial cells – new insights for anti-tumor treatments

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Heterogeneities in tumor blood flow are associated with cyclic changes in pO<sub>2</sub> or cyclic hypoxia. A major difference from O<sub>2</sub> diffusion-limited or chronic hypoxia is that the tumor vasculature itself may be directly influenced by the fluctuating hypoxic environment, and the reoxygenation phases complicate the usual hypoxia-induced phenotypic pattern. Here, we determined the cyclic hypoxia-driven pathways that modulate hypoxia inducible factor (HIF)-1 $\alpha$  abundance in endothelial cells to identify possible therapeutic targets. We found that exposure of endothelial cells to cycles of hypoxia/reoxygenation led to accumulation of HIF-1 $\alpha$  during the hypoxic periods and the phosphorylation of protein kinase B (Akt), extracellular regulated kinase (ERK) and endothelial nitric oxide synthase (eNOS) during the reoxygenation phases. We identified stimulation of mitochondrial respiration and activation of the phosphoinositide-3 kinase (PI3K)/Akt pathway during intervening reoxygenation periods as major triggers of the stabilization of HIF-1 $\alpha$ . We also found that the NOS inhibitor nitro-L-arginine methyl ester further stimulated the cyclic hypoxia-driven HIF-1 $\alpha$  accumulation and the associated gain in endothelial cell survival, thereby mirroring the effects of a PI3K/Akt inhibitor. However, combination of both drugs resulted in a net reduction in HIF-1 $\alpha$  and a dramatic decrease in endothelial cell survival. In conclusion, this study identified cyclic hypoxia, as reported in many tumor types, as a unique biological challenge for endothelial cells that promotes their survival in a HIF-1 $\alpha$ -dependent manner through phenotypic alterations occurring during the reoxygenation periods. These observations also indicate the potential of combining Akt-targeting drugs with anti-angiogenic drugs, in particular those interfering with the NO pathway.

The transcription factor hypoxia inducible factor (HIF)-1 is a key regulator of the cellular response to hypoxia. HIF-1 consists of a constitutively expressed HIF-1 $\beta$  subunit and an inducible HIF-1 $\alpha$  subunit [1–4]. The main mechanism responsible for stabilization of HIF-1 $\alpha$  is the inhibition of prolyl 4-hydroxylase

domain (PHD) proteins, which hydroxylate the HIF-1 $\alpha$  subunit in the presence of oxygen, leading to its subsequent ubiquitination and degradation [5]. Growth factors, in particular when their expression is driven by oncogenes, iron chelators and reactive oxygen species, are also reported to increase HIF-1 $\alpha$  transcription

## Abbreviations

Akt, protein kinase B; CyH, cyclic hypoxia; ERK, extracellular regulated kinase; eNOS, endothelial nitric oxide synthase; H3, third period of hypoxia in the CyH protocol; HIF, hypoxia inducible factor; L-NAME, nitro-L-arginine methyl ester; PI3K, phosphoinositide-3 kinase; R3, third period of reoxygenation in the CyH protocol.

and/or its stabilization [6]. Conversely, inhibitors of mitochondrial respiration, including nitric oxide, may prevent the stabilization of HIF-1 $\alpha$  during hypoxia [7]. However, the impact of nitric oxide on HIF-1 $\alpha$  is not easy to assess, as NO has been shown to stabilize HIF-1 $\alpha$  at O<sub>2</sub> concentrations above those usually considered hypoxic and even in ambient air [8–10].

The HIF-1 $\alpha$ -dependent cellular response also appears to depend on the nature of the cells. Vascular endothelial cells were recently documented to induce HIF-1 $\alpha$  at lower O<sub>2</sub> concentrations than smooth muscle cells, fibroblasts or tumor cells [11]. At a first glance, the concept of hypoxic endothelial cells may appear biologically irrelevant considering the unique location of the endothelium at the interface with O<sub>2</sub>-transporting cells in the blood. However, intermittent blood flow and cyclic hypoxia in tumors [12–23] are examples of conditions where endothelial cells are exposed to very low levels of O<sub>2</sub>. We recently reported that cyclic hypoxia (i.e. several cycles of hypoxia/reoxygenation) promoted the survival of endothelial cells through an HIF-1 $\alpha$ -dependent mechanism [24]. However, key questions remained unaddressed in that study. For instance, does the accumulation of HIF-1 $\alpha$  during cyclic hypoxia result from the lack of degradation during the reoxygenation phases, or are some signaling cascades activated during the reoxygenation phase that may influence the expression of HIF-1 $\alpha$  during hypoxia? This is of crucial importance as dissection of these mechanisms may lead to new therapeutic strategies to sensitize endothelial cells to anti-angiogenic and conventional anti-tumor treatments.

In this study, we therefore exposed endothelial cells to cyclic hypoxia (CyH), and examined the impact of cycles of hypoxia/reoxygenation on the extent of activation of known regulators of HIF-1 $\alpha$ , namely phosphoinositide-3 kinase (PI3K)/protein kinase B (Akt), extracellular regulated kinase (ERK) and endothelial nitric oxide synthase (eNOS). This allowed us to identify the critical role of reoxygenation periods on the Akt pathway and mitochondrial activity, which both participate in HIF-1 $\alpha$  stabilization. Incidentally, this study indicated that PI3K/Akt and eNOS activation have opposite effects on HIF-1 $\alpha$  during cyclic hypoxia; caution is therefore required in the use of NOS inhibitors as single anti-tumor treatments. More generally, by providing new insights into the regulation of HIF-1 $\alpha$  in the context of tumor O<sub>2</sub> fluctuations, this study integrates the apparently paradoxical modes of regulation of HIF-1 $\alpha$  by hypoxia and oxidative stress.

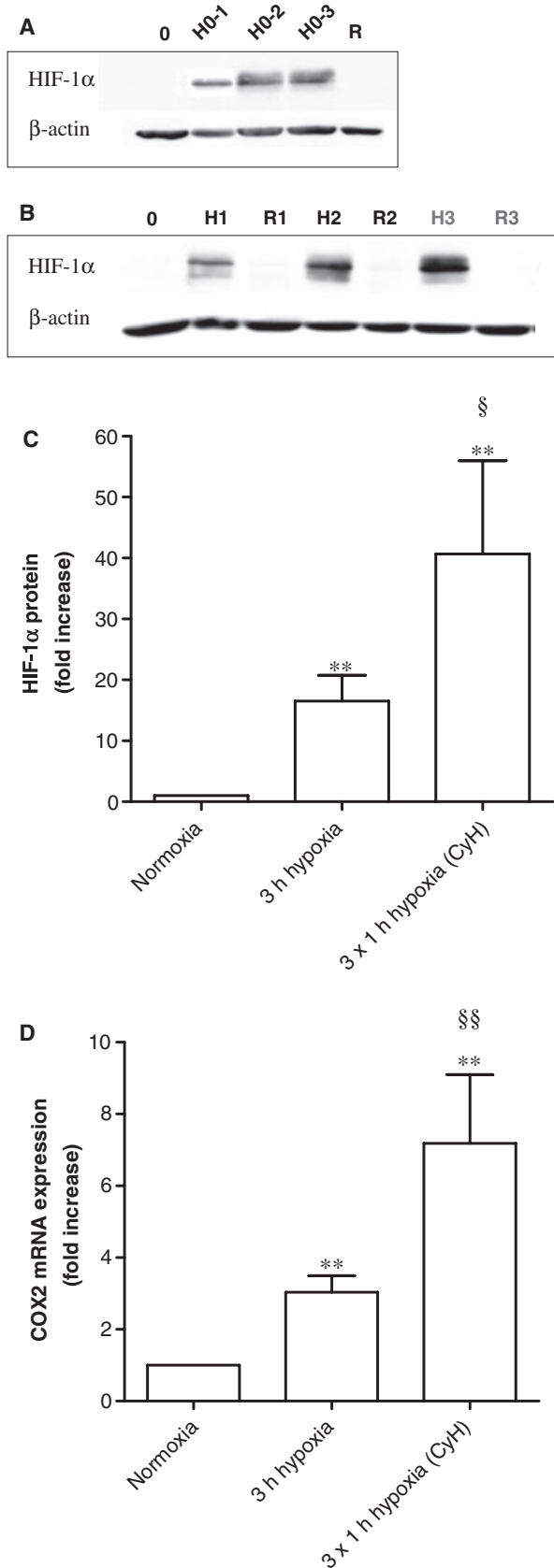
## Results

### HIF-1 $\alpha$ accumulates in response to cyclic hypoxia despite degradation during reoxygenation

We examined the impact of three cycles of 1 h hypoxia/30 min reoxygenation (versus 1, 2 and 3 h of continuous hypoxia) on the abundance of HIF-1 $\alpha$ . This protocol of cyclic hypoxia (1 h hypoxia/30 min reoxygenation) was based on previous measurements of fluctuations in the tumor vasculature occurring at the frequency of 0.5–1 cycle per hour [19,25,26]. We found that both continuous and cyclic hypoxia (CyH) induced HIF-1 $\alpha$  accumulation (Fig. 1A,B). Interestingly, HIF-1 $\alpha$  progressively accumulated at each new hypoxic cycle during the CyH protocol (i.e. H1, H2 and H3), despite degradation during the intervening reoxygenation steps (i.e. R1, R2 and R3). As shown in Fig. 1C, the level of HIF-1 $\alpha$  was significantly higher after three 1 h periods of hypoxia than after three continuous hours of hypoxia. An increase in HIF-1 $\alpha$  stabilization (versus transcription) was confirmed by the failure of actinomycin D to block HIF-1 $\alpha$  accumulation during the CyH protocol (data not shown). To confirm the functional relevance of the observed HIF-1 $\alpha$  stabilization, expression of the endothelial hypoxia-responsive element-regulated gene *COX-2* was examined. Figure 1D shows that *COX-2* expression was 7.2-fold increased after CyH, but continuous hypoxia only led to a threefold increase (versus normoxic conditions). The HIF dependency of the *COX-2* induction was shown using echinomycin, a pharmacological hypoxia-responsive element-interfering drug [27], which completely prevented the increase in *COX-2* transcript abundance (data not shown).

### Cyclic hypoxia activates a variety of signaling cascades during the reoxygenation periods

We evaluated the activation of known regulators of HIF-1 $\alpha$  activity/expression, namely Akt, ERK and eNOS [28,29], under continuous (Fig. 2A) and cyclic (Fig. 2B) hypoxia conditions. We found that activation of Akt and ERK, as determined by the extent of phosphorylation of these proteins, presented an opposite pattern to that of HIF-1 $\alpha$ . Phospho-Akt and phospho-ERK signals were increased during reoxygenation, either after the 3 h continuous hypoxia (Fig. 2A) or during the periods of reoxygenation after each hypoxic cycle (Fig. 2B,C). Figure 2 also shows that phosphorylation of eNOS on serine 1177, a hallmark of eNOS activation, was similarly influenced by reoxygenation,



but to a slightly lower extent (see Fig. 2C for quantification).

### The PI3K/Akt and eNOS pathways oppositely modulate the CyH-driven induction of HIF-1 $\alpha$

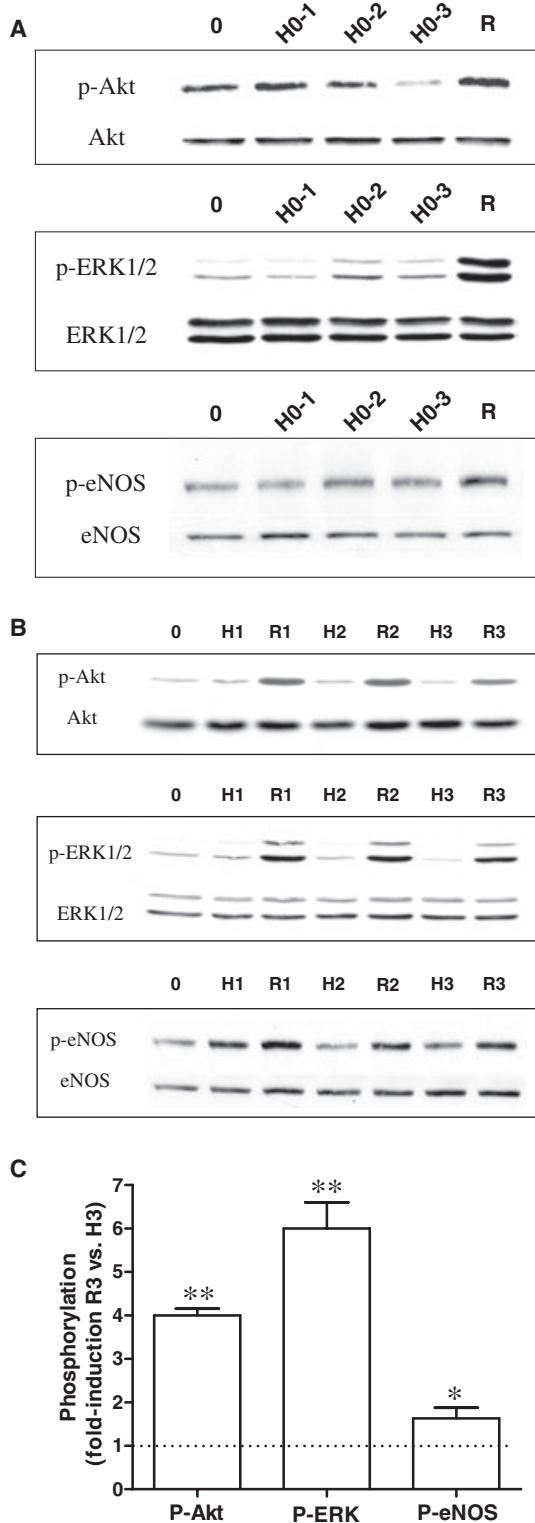
To determine the potential influence of the hypoxia/reoxygenation-dependent activation of Akt, ERK and eNOS on HIF-1 $\alpha$  upregulation, we used pharmacological inhibitors of each specific pathway. Figure 3A shows that LY294002, an inhibitor of the activity of PI3K (a kinase known to act upstream of Akt), completely prevented activation of Akt and precluded the accumulation of HIF-1 $\alpha$  throughout cyclic hypoxia (see Fig. 3D for quantification). By contrast, PD98059, which reduced the extent of ERK phosphorylation to approximately 20% of the control signal during reoxygenation, failed to prevent progressive accumulation of HIF-1 $\alpha$  during the hypoxic periods (Fig. 3B). Note that the HIF-1 $\alpha$  signal detected after the third hypoxic period (i.e. H3) and the phospho-signal detected after the third reoxygenation period (i.e. R3) in the absence of treatments are shown on the immunoblots as internal standards.

In contrast to the two other inhibitors, the NOS inhibitor nitro-L-arginine methyl ester (L-NAME) stimulated HIF-1 $\alpha$  accumulation to higher levels than the maximal signal in the absence of L-NAME (i.e. at H3) (see Fig. 3C,D for quantification).

### Cyclic hypoxia stimulates the O<sub>2</sub> consumption rate

As NO has previously been reported to inhibit mitochondrial O<sub>2</sub> consumption [11], the L-NAME-stimulated increase in the HIF-1 $\alpha$  signal suggested that

**Fig. 1.** HIF-1 $\alpha$  accumulates in response to cyclic hypoxia despite degradation during the reoxygenation periods. (A, B) Representative HIF-1 $\alpha$  immunoblots from endothelial cells collected at various time points during the continuous and cyclic hypoxia protocols. (A) Endothelial cells were exposed to hypoxia (< 1% O<sub>2</sub>) for the indicated time periods, i.e. 1, 2 or 3 continuous hours (H0-1, H0-2 and H0-3, respectively); after the 3-h hypoxia, cells were reoxygenated (R) for 30 min. (B) Endothelial cells were exposed to three cycles of 1 h hypoxia (H1, H2 and H3) interrupted (or followed) by 30 min reoxygenation (R1, R2 and R3). For both (A) and (B),  $\beta$ -actin expression is shown as a gel loading control. These experiments were repeated three times with similar results. (C, D) Influence of normoxia, 3 h continuous hypoxia and cyclic hypoxia (CyH; 3 x 1 h) on (C) HIF-1 $\alpha$  protein accumulation (at H3) and (D) COX-2 mRNA expression (at R3) in endothelial cells (\*\* $P$  < 0.01 versus normoxia; § $P$  < 0.05 and §§ $P$  < 0.01 versus 3 h continuous hypoxia,  $n$  = 5–8).



changes in cell respiration could be involved in the modulation of HIF-1 $\alpha$  abundance observed throughout cyclic hypoxia. We first evaluated the O<sub>2</sub> consump-

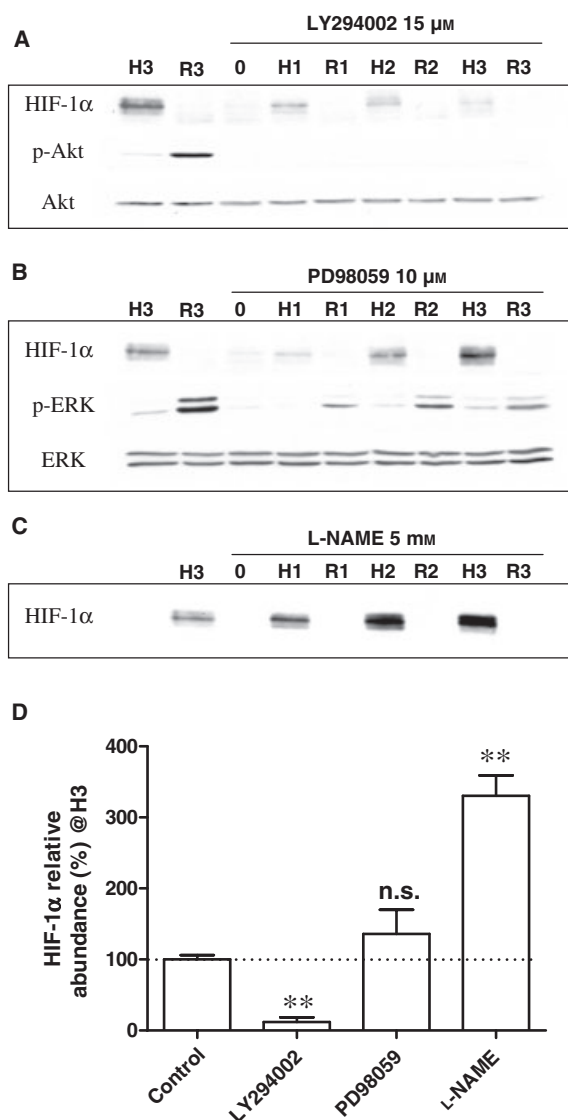
**Fig. 2.** Post-hypoxic reoxygenation stimulates Akt, ERK and eNOS phosphorylation. Representative immunoblots for the detection of phospho-Akt (Ser473), phospho-ERK (Thr185/Tyr187) and phospho-eNOS (Ser1177) in endothelial cells exposed to the continuous (A) and cyclic (B) hypoxia protocols described in the legend to Fig. 1. Immunoblots for total Akt, ERK and eNOS are also shown and were used for signal normalization. These experiments were repeated two or three times with similar results. (C) Extent of Akt, ERK and eNOS phosphorylation measured after the third period of reoxygenation (R3). Data are presented as fold induction versus H3 conditions (third period of hypoxia): \*\* $P < 0.01$ , \* $P < 0.05$  ( $n = 3-4$ ).

tion rate in endothelial cells exposed to the CyH protocol described above. We found that the CyH pre-challenge significantly stimulated the respiratory metabolism of endothelial cells ( $P < 0.01$ ,  $n = 5$ ) versus cells exposed to 3 h continuous hypoxia or maintained in normoxia (Fig. 4A). This metabolic adaptation was progressive, with the O<sub>2</sub> consumption rate increasing after each new hypoxia/reoxygenation cycle (see Fig. 4B).

We then used rotenone, an inhibitor of mitochondrial chain respiration, and found that it could prevent HIF-1 $\alpha$  accumulation following three cycles of 1 h hypoxia (Fig. 4C). Addition of rotenone had no effect on the induction of HIF-1 $\alpha$  after uninterrupted 1 or 3 h hypoxia, indicating that, under our experimental conditions, acceleration of respiration was a major trigger of HIF-1 $\alpha$  stabilization in response to CyH. Furthermore, when we used of combined treatment with L-NAME with rotenone, the NOS inhibitor failed to induce accumulation of HIF-1 $\alpha$  (Fig. 4D), confirming that, in our CyH protocol, the L-NAME-mediated increase in HIF-1 $\alpha$  (see Fig. 3C,D) very probably resulted from NO-dependent inhibition of the respiratory chain.

#### PI3K/Akt and eNOS inhibitors exert opposite effects on cyclic hypoxia-driven cell survival

We then sought to determine whether the PI3K inhibitor LY294002 could prevent L-NAME-driven amplification of the HIF-1 $\alpha$  response in endothelial cells and how the combination of both inhibitors could influence the fate of cells exposed to CyH. Figure 5A shows that the L-NAME-driven increased abundance of HIF-1 $\alpha$  was largely prevented by co-administration of LY294002 (see Fig. 5B for quantitative analysis). We next used a clonogenic assay to evaluate the effects of both inhibitors. We observed a dramatic gain in endothelial cell survival when first pre-challenged by cyclic hypoxia (versus cells maintained in normoxia, which modestly survive the assay procedure) (Fig. 5C). Inter-



**Fig. 3.** CyH-driven activations of Akt, ERK and eNOS influence HIF-1 $\alpha$  accumulation differently. (A–C) Representative HIF-1 $\alpha$  immunoblots from endothelial cells exposed to the cyclic hypoxia protocol (described in the legend to Fig. 1) and pre-treated with the following pharmacological inhibitors: (A) 15  $\mu$ M LY294002, (B) 10  $\mu$ M PD98059 or (C) 5 mM L-NAME. The effects of LY294002 (A) and PD98059 (B) treatments on the extent of Akt and ERK phosphorylation, respectively, are also shown for validation of the inhibition of the corresponding phosphorylations (L-NAME is not an eNOS phosphorylation inhibitor). Immunoblots for total Akt and ERK are also presented and were used as controls of gel loading. These experiments were repeated twice with similar results. (D) Impact of the indicated pharmacological inhibitors on the relative HIF-1 $\alpha$  abundance measured after the third period of hypoxia (H3): \*\* $P < 0.01$ , n.s., not significant ( $n = 3-4$ ).

estingly, while LY294002 dose-dependently inhibited the CyH-driven protection of endothelial cells, the NOS inhibitor L-NAME significantly increased the

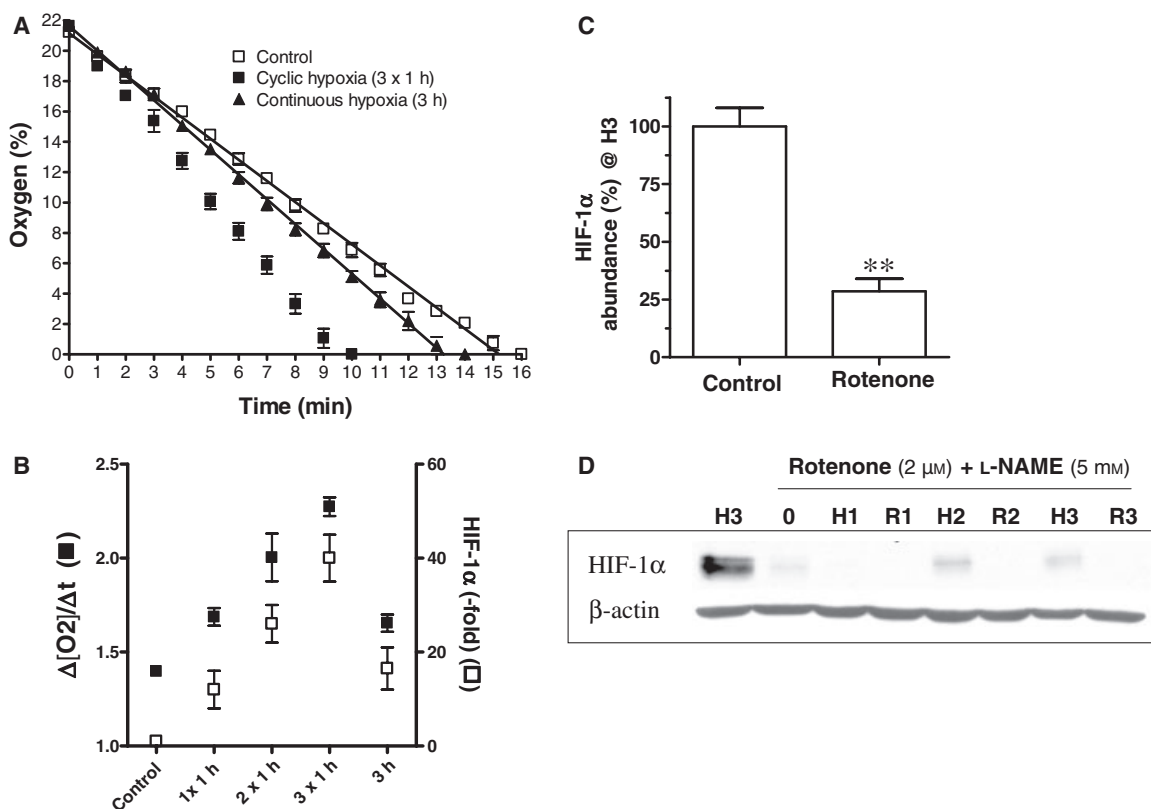
survival advantages conferred by CyH (Fig. 5C), in agreement with the net increase in the HIF-1 $\alpha$  immunoblot signal (Fig. 5A,B). Importantly, when we combined the PI3K and NOS inhibitors, we found that the reduction in endothelial cell survival was similar to that obtained with LY294002 alone, suggesting that the pro-survival effects of L-NAME could be eliminated by use of LY294002 (Fig. 5C).

## Discussion

The major findings of this study are that (a) cyclic hypoxia, an increasingly recognized hallmark of many tumor types [23], leads to a unique activation pattern of key signaling enzymes including Akt and eNOS, which tune the accumulation of HIF-1 $\alpha$  in endothelial cells, (b) the PI3K/Akt activation occurring during the reoxygenation phases accounts for the observed CyH-driven HIF-1 $\alpha$  stabilization, a phenomenon further exacerbated by the increase in O<sub>2</sub> consumption in CyH-exposed endothelial cells, (c) the eNOS activation (also triggered by CyH) partly attenuates the HIF-1 $\alpha$  increase by interfering with cell respiration, and (d) the HIF-1 $\alpha$ -driven increase in the survival of endothelial cells exposed to CyH is further increased by a NOS inhibitor but may be combated by (co-) administration of a PI3K/Akt inhibitor.

The origins of cyclic exposure of cells within tumors to various pO<sub>2</sub> levels are multiple as described above. Here we focused on the effects of CyH on endothelial cells, a cell type that is not directly concerned by hypoxia in healthy tissues. The location of the endothelium at the interface between O<sub>2</sub>-transporting blood cells and perfused tissues normally protects them from any major influence of hypoxia. However, in tumors, although so-called chronic hypoxia is dependent on the diffusion of O<sub>2</sub> and therefore does not influence endothelial cells located at the beginning of the O<sub>2</sub> gradient, heterogeneities in tumor blood flow directly influence the endothelium of tumor vessels.

Here, we provide mechanistic insights that account for the accumulation of HIF-1 $\alpha$  in endothelial cells exposed to CyH. Cyclic fluctuations of pO<sub>2</sub> lead to a unique combination of parameters with direct and indirect impacts on HIF-1 $\alpha$  accumulation. First, the reoxygenation phases are associated with activation of signaling enzymes, including Akt, ERK and eNOS. Using pharmacological inhibitors, we identified the key role for the reoxygenation-driven PI3K/Akt pathway in stabilization of HIF-1 $\alpha$  during consecutive hypoxic periods. The prevention of HIF-1 $\alpha$  accumulation in the presence of a PI3K/Akt inhibitor (as observed in

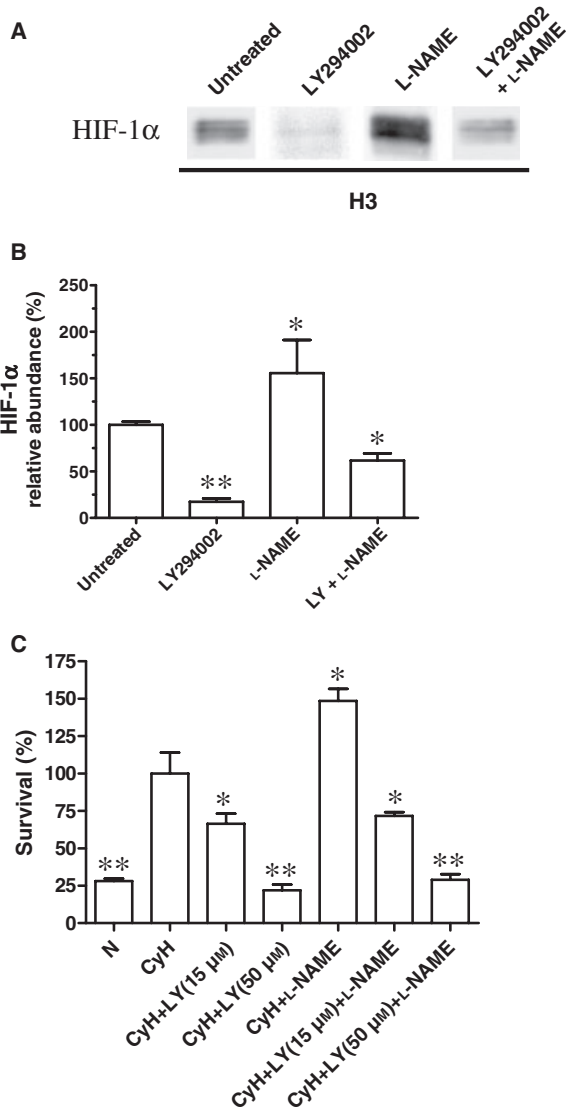


**Fig. 4.** CyH increases the oxygen consumption rate in endothelial cells. (A) Endothelial cell oxygen consumption measured by electron paramagnetic resonance at baseline (open square;  $n = 5$ ) as well as after 3 h continuous hypoxia (closed triangle,  $n = 5$ ) and cyclic (3  $\times$  1 h) hypoxia (closed square,  $n = 4$ ), as described in the legend to Fig. 1; note that the measurements were performed after 30 min reoxygenation at the end of both protocols. (B) Slope values derived from the corresponding  $O_2$  consumption rate ( $\mu\text{M}\cdot\text{min}^{-1}$ ) as observed in Fig. 4A (left y axis) and the corresponding HIF-1 $\alpha$  expression values (right y axis) determined as in Fig. 1; note the parallel increases in the slope values and HIF-1 $\alpha$  accumulation with the number of hypoxia/reoxygenation cycles. (C) Relative abundance of HIF-1 $\alpha$  after the third period of hypoxia (i.e. H3) in endothelial cells exposed or not to 2  $\mu\text{M}$  rotenone; these experiments were repeated three times with similar results.  $***P < 0.01$  versus control conditions (D) Representative HIF-1 $\alpha$  immunoblots from endothelial cells exposed to cyclic hypoxia after pre-treatment with 5 mM L-NAME and 2  $\mu\text{M}$  rotenone; the immunoblot signal at H3 in the absence of pharmacological treatment is shown as a control. This experiment was repeated twice with similar results.

Fig. 3A) was previously reported to involve a reduction in steady-state concentrations of Hsp90 and/or Hsp70 [30]. Interestingly, the phosphorylation of Akt observed during the reoxygenation phases did not increase proportionally to the accumulation of HIF-1 $\alpha$  (see Figs 1B and 2B). Together, these data indicate that Akt activation is necessary but not sufficient to support the CyH-triggered accumulation of HIF-1 $\alpha$ . This led us to identify the acceleration of the endothelial cell respiration as a secondary mechanism driven by cyclic hypoxia and promoting HIF-1 $\alpha$  accumulation. The decrease in intracellular  $O_2$  bioavailability parallels the progressive accumulation of HIF-1 $\alpha$  at each new hypoxic cycle (see Figs 1B and 4B). These data indicate that CyH-induced stimulation of the mitochondrial respiratory chain (i.e. the increase in  $O_2$

consumption) and the concomitant activation of Akt concur to support the accumulation of HIF-1 $\alpha$  during CyH.

Of note, in the immunoblotting data corresponding to the various hypoxic and reoxygenation phases, cells were collected at the end of the 60 min hypoxia or 30 min reoxygenation periods, respectively. This may have led an underestimation of the ability of CyH to both favor phosphorylation of signaling enzymes such as Akt during hypoxia and support induction of HIF-1 $\alpha$  during at least part of the reoxygenation period. Alterations in cell respiration (as reported in Fig. 4A) and thus cell metabolism could also account for a reduction in the extent of Akt, ERK and eNOS phosphorylation during the hypoxia periods. However, given the long-term fluctuations of  $pO_2$  values reported

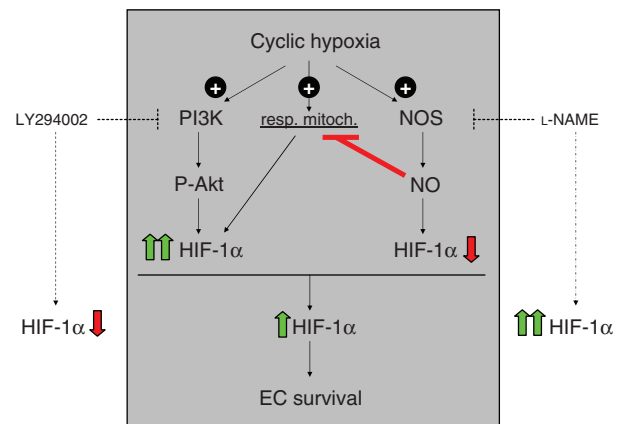


**Fig. 5.** PI3K/Akt inhibition and NO blockade oppositely influence CyH-driven survival of endothelial cells. Endothelial cells were exposed to cyclic hypoxia (as described in the legend to Fig. 1) after pre-treatment (or not) with 15  $\mu$ M LY294002, 5 mM L-NAME or a combination of both. (A) Representative HIF-1 $\alpha$  immunoblots from endothelial cells collected at the end of the third hypoxic cycle (H3). (B) Relative HIF-1 $\alpha$  abundance after the third period of hypoxia (i.e. H3) in endothelial cells pre-treated as indicated (\* $P$  < 0.05, \*\* $P$  < 0.01 versus untreated conditions,  $n$  = 5–6). (C) Clonogenic survival of endothelial cells maintained in normoxia (N) or after exposure to cyclic hypoxia (CyH) in the presence of the indicated pharmacological treatments. Results are expressed as a percentage of the survival obtained after CyH (\* $P$  < 0.05, \*\* $P$  < 0.01 versus CyH,  $n$  = 3–4).

to occur *in vivo* (instead of the three cycles used in our experimental protocol) and/or a yet higher rate of pO<sub>2</sub> alternation as recently reported [18,31], permanent

instabilities in tumor blood flow and oxygenation may instead favor continuous Akt activation and HIF-1 $\alpha$  expression in tumor endothelial cells.

Our study also showed opposite effects of PI3K/Akt and eNOS inhibitors on the CyH-driven survival of endothelial cells (see Fig. 5C), thereby confirming the differential effects of these drugs on HIF-1 $\alpha$  abundance (Fig. 5A,B). In particular, exacerbation of HIF-1 $\alpha$  induction by L-NAME indicates that the stimulatory effect of CyH on HIF-1 $\alpha$  was dampened by eNOS activation/phosphorylation. Furthermore, the failure of the NOS inhibitor to maintain the induction of HIF-1 $\alpha$  in the presence of rotenone (Fig. 4D) strongly suggests that NO exerts these effects through inhibition of the mitochondrial respiratory chain. This is in agreement with the previously reported redistribution of oxygen toward prolyl hydroxylases observed upon inhibition of mitochondrial respiration by NO under hypoxia [7]. Importantly, co-administration of a PI3K/Akt inhibitor obliterated the stimulatory effects of the NOS inhibitor on HIF-1 $\alpha$ . Therefore, from a therapeutic perspective, our study provides a new rationale for the use of Akt inhibitors to abrogate the pro-survival effects of CyH, and also provides evidence that use of NOS inhibitors (in particular for their anti-angiogenic potential) may benefit from the co-administration of Akt-targeting drugs. The interest in such a combination is further increased by the capacity of



**Fig. 6.** Schematic representation of the interplay between the multiple factors regulating HIF-1 $\alpha$  abundance in endothelial cells exposed to CyH. The stimulatory effects of CyH on both the PI3K/Akt pathway and the cell respiration rate lead to an increase in HIF-1 $\alpha$  stabilization, thereby promoting endothelial cell survival. These effects are partly attenuated by the concomitant eNOS activation through probable inhibition of the mitochondrial chain. Consequently, the drugs targeting these enzymes have opposite effects: a PI3K/Akt inhibitor will leave the effects of NO unbridled, promoting a decrease in HIF-1 $\alpha$  abundance, whereas a NOS inhibitor will accentuate the induction of HIF-1 $\alpha$  in response to CyH.

PI3K/Akt inhibitors to prevent eNOS activation (through phosphorylation on serine 1177) and the consequent NO-mediated angiogenesis [32,33].

In conclusion, this study offers new insights into the impact of cyclic hypoxia on vascular cells, an underestimated component of the tumor stroma in terms of phenotypic alterations by hypoxia. The scheme shown in Fig. 6 summarizes the interplay between the major signaling events elicited by cyclic hypoxia in endothelial cells. The accumulation of HIF-1 $\alpha$  in response to cyclic hypoxia is largely promoted by Akt activation during the periods of higher pO<sub>2</sub>, favored by a concomitant increase in the oxygenation consumption rate of endothelial cells and further increased by pharmacological inhibition of NOS activity. Our study underlines the therapeutic relevance of combining emerging strategies that block the PI3K/Akt pathway [34] with other anti-cancer modalities (especially drugs interfering with the eNOS or COX-2 pro-survival pathways, both of which are found to be activated in response to cyclic hypoxia) to take full advantage of a reduction in the resistance threshold of endothelial cells lining tumor blood vessels.

## Experimental procedures

### Cell culture

Human umbilical vein endothelial cells were routinely cultured in 60 mm dishes in endothelial cell growth medium (Clonetics, Walkersville, MD, USA). Two hours before starting the treatments, cells were serum-starved; for long-term survival studies, culture medium was re-supplemented with serum. To achieve and control hypoxia conditions, cells were placed in a modular incubator chamber (Billups Rothenberg Inc., Del Mar, CA, USA) and flushed for 10 min with a gas mixture of 5% CO<sub>2</sub>/95% N<sub>2</sub>; the final pO<sub>2</sub> value measured in the extracellular medium was consistently below 1%. The chamber was then sealed and placed at 37 °C in conventional cell incubator. The cyclic hypoxia protocol consisted of three periods of 1 h hypoxia interrupted by 30 min reoxygenation; 1, 2 or 3 h of uninterrupted exposure to hypoxia were used for the continuous hypoxia protocol. In some experiments, cells were treated with rotenone (2  $\mu$ M), L-NAME (5 mM), LY294002 (15 or 50  $\mu$ M) or PD98059 (10  $\mu$ M); all these drugs were obtained from Sigma (Bornem, Belgium).

### Immunoblotting

Endothelial cells were collected and homogenized in a buffer containing protease and phosphatase inhibitors. Total lysates were immunoblotted with HIF-1 $\alpha$  antibodies

and antibodies directed against phospho- and non-modified Akt, eNOS and ERK, as previously described [24,35]. All the antibodies were purchased from BD Pharmingen (Lexington, KY, USA), except the  $\beta$ -actin antibody that was used to normalize gel loading, which was obtained from Sigma.

### Real-time PCR

COX-2 mRNA expression was determined after reverse transcription from total RNA isolated from endothelial cells exposed or not to hypoxia protocols. Real-time quantitative PCR analyses were performed in triplicate using SYBR Green PCR Master Mix (Bio-Rad, Nazareth, Belgium) and the primers COX-2 sense (5'-CAGCCATACAGCAAATCCTTG-3') and COX-2 antisense (5'-AATCCGTGCCGGGTACAATC-3'). The C<sub>t</sub> value (number of cycles require to generate a fluorescent signal above a pre-defined threshold) was determined for each sample, and the relative mRNA expression was calculated using the formula 2<sup>- $\Delta\Delta$ C<sub>t</sub></sup> formula after normalization to RPL19 ( $\Delta$ C<sub>t</sub>) and determination of the difference in C<sub>t</sub> ( $\Delta\Delta$ C<sub>t</sub>) between the various conditions tested.

### Clonogenic assay

To assess the effects of cyclic hypoxia on endothelial cell survival, clonogenic cell survival assays were performed as previously described [24]. This test (generally reserved for tumor cells) entails a pro-apoptotic stress for endothelial cells, which need to recover from an important dilution at the time of plating. After a 7-day incubation period, cells were stained with crystal violet and colonies (> 50 cells) were counted.

### O<sub>2</sub> consumption assay

Electron paramagnetic resonance oximetry was used to track the O<sub>2</sub> consumption rate in endothelial cells pre-challenged or not by CyH, according to a method developed by P. James [36] and further validated by us [37,38]. A neutral nitroxide, <sup>15</sup>N-PDT (4-oxo-2,2,6,6-tetramethylpiperidine-d16-<sup>15</sup>N-1-oxyl) (CDN Isotopes, Quebec, Canada), was added to cells, which were then drawn into glass capillary tubes. They were then rapidly placed into quartz electron spin resonance tubes and maintained at 37 °C during recording on a Bruker EMX electron paramagnetic resonance spectrometer (Bruker, Brussels, Belgium) operating at 9 GHz.

### Statistical analyses

Data are reported as means  $\pm$  SEM. Student's *t*-test and one- or two-way ANOVA were used where appropriate.



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