Regulation of hypoxia inducible factor-1 by nitric oxide in contrast to hypoxia in microvascular endothelium

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Abstract Hypoxia activates the transcription factor, hypoxia inducible factor-1 (HIF-1). Besides hypoxia, HIF-1 can be activated under normoxic conditions by nitric oxide. The signal transduction pathways involved in HIF-1α stabilization, HIF-1 DNA binding and transactivation by NO and hypoxia in microvascular endothelium remains unknown. We report that protein phosphorylation is involved in HIF-1 activation during hypoxia and NO. The phosphatidylinositol 3-kinase (PI-3K)/Akt pathway has differential effects on HIF-1 activation by hypoxia and NO. Our data indicate that the PI-3K/Akt pathway is insufficient for HIF-1α induction by hypoxia. The lipid and protein phosphatase activities of PTEN also appear to be involved in regulation of HIF-1α by NO.

Key words: Hypoxia inducible factor-1; Nitric oxide; Signal transduction; PI-3K/Akt; PTEN

1. Introduction

Mammalian cells adapt to situations of reduced oxygen availability (hypoxia) by altering glucose metabolism, angiogenesis, vasodilation and erythropoiesis [1]. This is achieved by regulating proteins such as erythropoietin, vascular endothelial growth factor, glycolytic enzymes and nitric oxide synthase. These proteins are under control of the transcription factor hypoxia inducible factor-1 (HIF-1). Functional HIF-1 is a heterodimeric unit composed of a HIF-1α and a HIF-1β subunit. The HIF-1 subunits are constitutively transcribed and translated, but HIF-1α protein levels are regulated by ubiquitination and proteosomal degradation [2,3]. Under normoxic conditions, HIF-1α is a cytosolic protein with a short half-life. During hypoxia, HIF-1α degradation is progressively inhibited. Stabilized HIF-1α translocates to the nucleus, dimerizes with HIF-1β and regulates gene transcription by binding to hypoxia response elements on genes [4]. The mechanisms of HIF-1α modification and destruction, including the involvement of prolyl hydroxylases, E3 ubiquiting ligase and von Hippel–Lindau tumor suppressor protein, have recently been determined [5–8]. In addition to prolyl hydroxylation, control of hypoxia-induced HIF-1α levels involves regulatory pathways such as ROS [9], MAPK cascades [10–12], diacylglycerol kinase [13], and PI-3K (phosphatidylinositol-3 kinase) [14,15].

Besides hypoxia, HIF-1 can be activated under normoxic conditions by compounds that chelate iron such as desferroxamine and transition metals. Certain cytokines, growth factors and hormones have also been shown to activate HIF-1 under normoxic conditions [15–17]. Recently, we and others have shown that chemically diverse nitric oxide (NO) donors and endogenous NO evoke HIF-1α stabilization, HIF-1 DNA binding and downstream target gene expression under normoxic conditions [18–21]. However, little information exists on signal transduction pathways involved in this NO-mediated response. In LLC-PK$_i$ proximal tubular cells, Sandau et al. [22] initially noticed that the general kinase inhibitor, genistein, and the PI-3K inhibitors, wortmannin and LY294002, blocked HIF-1α accumulation upon exposure to NO. They concluded that NO-evoked HIF-1 induction occurred in a PI-3K-dependent manner that closely correlated with Akt phosphorylation. Further MAPK pathways played no role in this induction. More recently, Sandau et al. [18] showed that HIF-1α stabilization was blocked by interrupting the PI-3K/Akt pathway following exposure to both hypoxia and NO. In contradistinction, by using a variety of different cell types, Arsham et al. [23] showed that inhibitors of the PI-K/Akt pathway did not block the hypoxic induction of HIF-1α. Alvarez-Tejado et al. [24] also demonstrated that PI-3K/Akt activity is not sufficient for HIF induction, nor is it essential for its regulation by hypoxia. Thus it appears that although the mechanisms involved in HIF-1α degradation are becoming increasingly clear, the signaling pathways, especially the PI-3K/Akt pathway, that determines HIF-1α stabilization, nuclear translocation and HIF-1 transactivational ability under hypoxic or normoxic conditions, remain confusing and controversial.

PI-3K catalyzes the conversion of phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-biphosphate to phosphatidylinositol-3,4-biphosphate and phosphatidylinositol-3,4,5-triphosphate. These products activate phosphatidylinositol-dependent kinase 1 (PDK1), which then phosphorylates and activates the proto-oncogenic serine/threonine kinase Akt (protein kinase B). Akt has downstream pro-growth, anti-apoptotic effects when activated by growth factors through PI-3K (reviewed in [25]). This pathway is reversed by PTEN (phosphatase and tensin homolog deleted on chromosome 10), a tumor suppressor gene that encodes a dual-

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Abbreviations: HIF-1, hypoxia inducible factor-1; PI-3K, phosphatidylinositol 3-kinase; HMEC-1, human microvascular endothelial cells-1; ERK, extracellular signal-regulated kinase; PKC, protein kinase C; GSNO, *S*-nitrosoglutathione

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specificity phosphatase that can dephosphorylate tyrosine-containing peptides, Shc, focal adhesion kinase and phosphoinositide substrates [26–28]. While several lines of evidence indicate that the lipid phosphatase activity of PTEN is required for tumor suppression [29,30], the physiological roles of its protein phosphatase activity regarding suppression of the PI-3K/Akt signaling pathway remain unclear.

In this study, we examined the signaling mechanisms involved in HIF-1α stabilization, nuclear translocation and HIF-1 transactivational ability after exposure of human microvascular endothelial cells (HMEC-1) to NO. We compared our results to hypoxic exposure of HMEC-1. We show that HIF-1 is activated in HMEC-1 under normoxic conditions by NO. Protein phosphorylation events are involved in HIF-1 activation during hypoxia and NO exposure. Interestingly, the PI-3K pathway has differential effects on HIF-1 activation by hypoxia and NO. Our data indicate that the PI-3K/Akt pathway is insufficient for HIF-1α induction by hypoxia. In addition, both the lipid phosphatase and protein phosphatase activities of PTEN appear to be involved in regulation of HIF-1α by NO.

2. Materials and methods

2.1. Endothelial cell culture

The HMEC-1 cell line utilized for this study was obtained from the Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA. The cell line was immortalized by Dr. Edwin Ades, Mr. Fransisco J. Candal of the CDC and Dr. Thomas Lawly of Emory University and was designated HMEC-1. HMEC-1 cells were cultured as previously described [31].

2.2. Establishing hypoxic environments

Hypoxic conditions (3% O_2) were generated as previously described [31]. Viability of HMEC-1 subjected to hypoxia was also assessed as described previously [31] using a two-color fluorescence (LIVE/DEAD®, Molecular Probes) assay.

2.3. Plasmids

Plasmids used for transient transfections were hypoxia response element luciferase reporter vector pEpo3′Glut1-Luc (designated pHRE-luc), which contains a trimer of murine Epo 3′ enhancer and the Glut-1 promoter [32] (kind gift of Dr. Paul Schumaker), the expression plasmids encoding wild-type PTEN (pcDNA3-PTENwt) [33], mutant PTEN deficient in lipid phosphatase activity, pcDNA3-PTEN(G129E) [34] and mutant PTEN deficient in both lipid and protein phosphates activities, pcDNA3-PTEN(C124S) [35] (kind gifts of Dr. Marty W. Mayo), and pHRL-null (Promega), which contains a synthetic Renilla gene sequence (hRluc) cleared of transcription factor binding sites, thus enabling accurate control for transfection efficiency and indexing of luciferase activity.

2.4. Western blot analysis

Nuclear extracts (20 μg) and whole cell extracts (50 μg) were prepared at the indicated times as described previously [36], resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (4–20%) and electrophoretically transferred to polyvinylidene fluoride membranes (PVDF, 0.45 μm pore size). Immunodetection was performed using HIF-1α (Santa Cruz Biotechnology), phospho-Akt (ser473) and Akt (Cell Signaling Technology) antibodies and the Renaissance® Western Blot Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences). All membranes were stained with Ponceau S solution (0.2% wt/vol in 1% acetic acid; Sigma) to ensure equal loading and transfer of proteins [37].

2.5. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared at the indicated times and 10 μg used for EMSA with a consensus HIF-1 oligonucleotide (Santa Cruz Biotechnology) as described previously [31]. Specific HIF-1α binding was previously demonstrated by a competitive EMSA with 100-fold excess unlabeled double-stranded HIF-1 oligonucleotide in the binding reaction prior to the addition of the end-labeled oligonucleotide and by super-shift assays using a primary HIF-1α antibody [21]. Densitometry of the shifted bands was performed using Kodak Digital Sciences EDAS 120 system, with 1D Image analysis software, and results obtained were expressed as relative densitometric units.

2.6. Transient transfections and dual luciferase reporter assay

HMEC-1 cells were co-transfected with vector pHRL-null (Promega) for transfection efficiency and indexing of luciferase activity. Cells were transfected using Effectene® optimized according to the manufacturer's instructions. 24 h post-transfection, cells were pre-incubated with the inhibitor or vehicle for 30 min. Cells were then incubated under normoxic conditions or exposed to hypoxia for a further 24 h in growth medium containing 2% fetal bovine serum (FBS). Alternately, the NO donor *S*-nitrosoglutathione (GSNO) was added and cultures incubated for 24 h in growth medium containing 2% FBS and 10 mM HEPES. In experiments involving the PTEN expression plasmids, all cell groups that received PTEN were also co-transfected with pCMV-LacZ for control purposes. Dual luciferase output (Dual-Luciferase™ Reporter Assay System) was quantified with a luminometer and results expressed as an index of relative light units (RLU).

2.7. Statistical analysis

Mean values were calculated from data obtained from three or more separate experiments. The significance was assessed by Student's *t*-test. Statistical significance was confirmed at a P value < 0.01. A minimum of three independent experiments was used to confirm observations.

3. Results and discussion

3.1. Effect of inhibitors of protein kinase on HIF-1 in hypoxic HMEC-1 cells

HIF-1 is a phosphorylated protein [38–40]. To investigate the role of protein phosphorylation in HIF-1α subunit stabilization, HIF-1 DNA binding, and in the regulation of HIF-1 transcriptional activity in microvascular endothelium, we pretreated HMEC-1 with vehicle alone or with different inhibitors for 30 min prior to hypoxic exposure. Nuclear extracts were prepared as described in Section 2 after exposure of HMEC-1 to hypoxia for 6 h and used for detection of HIF-1α by Western blot analysis and for HIF-1 DNA binding activity by EMSA with a labeled, double-stranded consensus HIF-1 oligonucleotide. To determine HIF-1 transcriptional activity, transfected HMEC-1 were exposed to hypoxia for 24 h and then harvested for DLR assay. As seen in Fig. 1A,B, hypoxia strongly induced stabilization of HIF-1α and HIF-1 DNA binding activity. Pre-treatment with PD98059 (50 μM), an inhibitor of the ERK (extracellular signal-regulated kinase) kinase pathway had no effect on HIF-1α stabilization or HIF-1 DNA binding by hypoxia. This result is in agreement with the results from Salceda [40], Semenza [41], and Michiels [42]. In contrast to results obtained in tumor cell lines from Giaccia [43] and Semenza [15], the PI-3K/Akt inhibitor, LY294002 (25 μM), did not affect the hypoxic stabilization of HIF-1α or HIF-1 DNA binding activity in HMEC-1. However, both PD98059 and LY294002 suppressed hypoxia-induced transcriptional activity of HIF-1 (Fig. 1C). This suggests that the PI-3K/Akt pathway and ERK signaling pathways are involved only in the regulation of the transactivation ability of hypoxia-induced HIF-1.

Pre-treatment with the tyrosine kinase inhibitors, genistein (100 μM) or herbimycin A (2 μM), significantly blocked hypoxia-induced stabilization of HIF-1α and HIF-1 DNA binding activity (Fig. 1A,B). This is in accordance to results from Semenza [44], who used genistein to show the involvement of

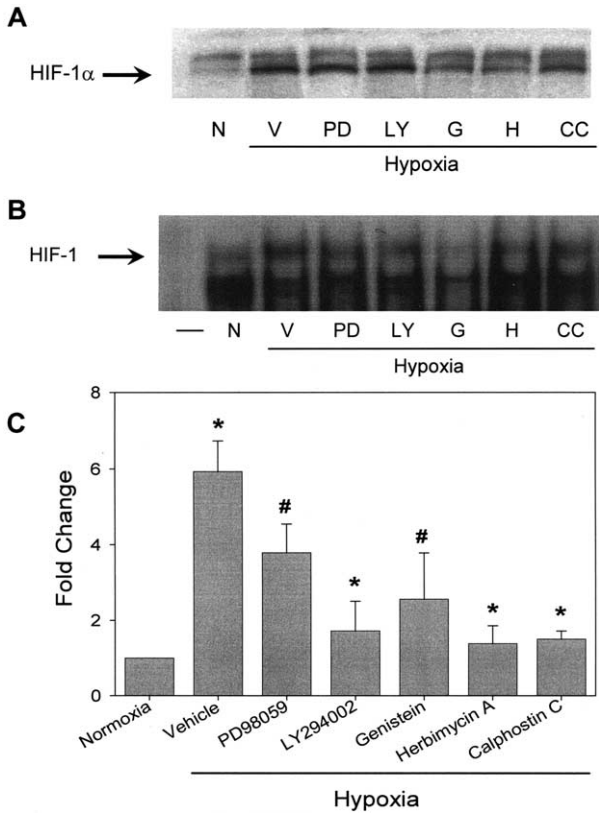

Fig. 1. Effect of inhibitors of protein kinase on HIF-1 in hypoxic HMEC-1 cells. Confluent HMEC-1 cells were exposed to vehicle alone or to different inhibitors for 30 min prior to hypoxic exposure for 6 h. Nuclear extracts were isolated and used for A: Western blot analysis (20 μg) for HIF-1α or B: EMSA analysis for HIF-1. N, normoxia (21% O_2); hypoxia (1% O_2); V, vehicle; PD, PD98059 (50 μM); LY, LY294002 (25 μM); G, genistein (100 μM); H, herbimycin A (2 μM); CC, Calphostin C (100 nM). C: HMEC-1 cultures were transiently co-transfected with pHRE-luc and pHRL-null. 24 h post-transfection, HMEC-1 cells were exposed to vehicle alone or to different inhibitors for 30 min prior to hypoxic exposure for 24 h. Dual luciferase output was quantified and the results expressed as an index of relative light units (RLU) ±S.E.M. of three independent transfections (*, $P<0.005$; #, $P<0.01$).

tyrosine phosphorylation in hypoxic induction of HIF-1α. Accordingly, HIF-1-dependent transcriptional ability was severely compromised (Fig. 1C).

The highly specific inhibitor of protein kinase C (PKC), Calphostin C (100 nM), repressed only the hypoxia-induced transcriptional ability of HIF-1 without significantly affecting either the hypoxic stabilization of HIF-1α or HIF-1 DNA binding activity in HMEC-1. While PKC has been implicated in transmitting hypoxia-induced signals to HIF-1 [45], suppression of hypoxia-induced HIF-1 transcriptional ability by a PKC inhibitor has not yet been reported. Thus protein phosphorylation events play an active role in hypoxia-induced HIF-1 activation in HMEC-1.

3.2. *Effect of inhibitors of protein kinases on HIF-1 in HMEC-1 exposed to nitric oxide*

Diverse nitric oxide donors as well as endogenously generated NO have recently been shown to induce HIF-1α stabilization, HIF-1 DNA binding and transactivation under normoxic conditions [18,20,22,46]. HIF-1α has been shown to be extensively *S*-nitrosated [47]. A possible role for tyrosine phosphorylation and involvement of the PI-3K/Akt pathway in NO-dependent stabilization of HIF-1α and HIF-1-dependent transactivation in proximal tubular LLC-PK_1 cells has been demonstrated [18,22]. However, the role of protein phosphorylation in NO-mediated HIF-1 DNA binding and transactivation under normoxic conditions in microvascular endothelium remains unidentified. We therefore pre-treated HMEC-1 with vehicle alone or with different inhibitors for 30 min prior to exposure to the NO donor, GSNO (500 μM) for 5 h. GSNO produced robust stabilization of HIF-1α in HMEC-1 (Fig. 2A) as well as strong DNA binding activity (Fig. 2B) and transcriptional activity (Fig. 2C). Similar to the hypoxic response of HIF-1, the ERK pathway inhibitor, PD98059 (50 μM), had no effect on either HIF-1α stabilization or HIF-1 DNA binding activity (Fig. 2A,B) by NO. However, pre-treatment with PD98059 produced a sharp decrease in HIF-1 transcriptional activity. This suggests that, similar to hypoxia, the ERK pathway is involved in regulation of HIF-1-dependent transcriptional activity by NO. Taken together with data shown in Fig. 1, the ERK signaling path-

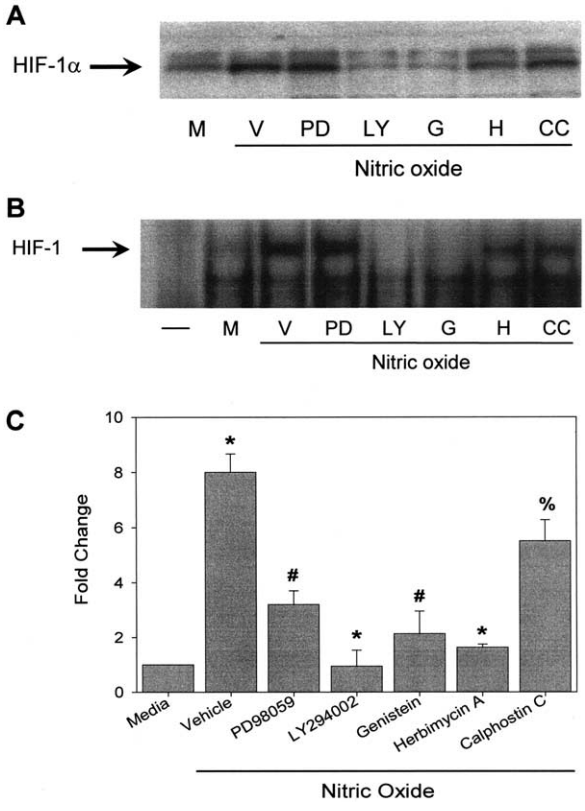

Fig. 2. Effect of inhibitors of protein kinases on HIF-1 in HMEC-1 exposed to nitric oxide. Confluent HMEC-1 cells were exposed to vehicle alone or to different inhibitors for 30 min prior to exposure to the NO donor, GSNO (500 μM), for 5 h. Nuclear extracts were isolated and used for A: Western blot analysis (20 μg) for HIF-1α or B: EMSA analysis for HIF-1. M, media; nitric oxide, GSNO (500 μM); V, vehicle; PD, PD98059 (50 μM); LY, LY294002 (25 μM); G, genistein (100 μM); H, herbimycin A (2 μM); CC, Calphostin C (100 nM). C: HMEC-1 cultures were transiently co-transfected with pHRE-luc and pHRL-null. 24 h post-transfection, HMEC-1 cells were exposed to vehicle alone or to different inhibitors for 30 min prior to exposure to GSNO (500 μM) for 5 h. Dual luciferase output was quantified and the results expressed as an index of relative light units (RLU) ±S.E.M. of three independent transfections (*, $P<0.001$; #, $P<0.005$; %, $P<0.05$).

way appears to be involved in regulation of the transactivation ability of HIF-1 following exposure to both hypoxia and NO.

Next we explored signal transmission involving PI-3K by using the specific PI-3K inhibitor LY294002. Unlike hypoxic exposure, both HIF-1α stabilization and HIF-1 DNA binding, as well as HIF-1-mediated transactivation following exposure to GSNO, were blocked by pre-treatment with LY294002 (50 μM) (Fig. 2), implying an essential role of the PI-3K pathway for NO-triggered HIF-1 activation. Thus data from Figs. 1 and 2 suggest that PI-3K pathway involvement of HIF-1-dependent gene expression is differentially regulated following exposure to hypoxia and NO.

Involvement of protein tyrosine phosphorylation was studied by pre-treatment with genistein (100 μM) and herbimycin A (2 μM). As seen in Fig. 2, both genistein and herbimycin were effective in blocking HIF-1α stabilization, HIF-1 DNA binding and transactivation following exposure to NO. While Sandau et al. [22] showed that genistein blocked the stabilization of NO-induced HIF-1α in LLC-PK$_1$ cells, the effect of these inhibitors on NO-induced HIF-1 DNA binding and transactivation is a novel finding.

PKC has been implicated in transmitting hypoxia-induced signals of HIF-1 and is reported to be upstream of PI-3K [45]. However, the involvement of PKC in transmitting NO-induced signals to HIF-1 remains unknown. HMEC-1 cells were pre-treated with the highly specific inhibitor of protein kinase C, Calphostin C (100 nM), prior to exposure to GSNO (500 μM). As seen in Fig. 2, HIF-1α stabilization by GSNO was moderately diminished in the presence of Calphostin C. Accordingly, both HIF-1 DNA binding and transactivation were moderately repressed, implying that PKC may be involved in NO-mediated HIF-1α stabilization.

3.3. *Effect of PTEN over-expression on HIF-1-dependent transactivation in hypoxic and NO-exposed HMEC-1 cells*

As demonstrated in Figs. 1 and 2, the PI-3K pathway is differentially involved in the regulation of HIF-1 following exposure to hypoxia or NO. While data from Fig. 1 showed that the PI-3K/Akt pathway is involved only in regulation of the transactivation ability of hypoxia-induced HIF-1, data from Fig. 2 suggest that PI-3K is required for HIF-1α stabilization, HIF-1 DNA binding as wel as HIF-1-mediated transactivation following exposure to NO. We therefore used molecular tools to further examine the involvement of the PI-3K pathway in HIF-1 transactivation in microvascular endothelium and performed transient transfection assays using wild-type and mutant over-expression vectors for PTEN. HMEC-1 cells were co-transfected with pHRE-luc, pHRL-null and either the expression plasmids encoding wild-type PTEN (PTEN), mutant PTEN deficient in lipid phosphatase activity, PTEN(G129E) [34] or mutant PTEN deficient in both lipid and protein phosphates activities, PTEN(C124S) [35]. 24 h post-transfection, cells were exposed to hypoxia or to GSNO for 24 h prior to harvest for DLR assay. As seen in Fig. 3, both hypoxia and GSNO produced a robust increase in pHRE-luc activity. Over-expression of PTEN decreased NO-driven luciferase activity by 50% but had no effect on the hypoxia-induced luciferase activity. This suggests that hypoxia-driven HIF-1 transactivation is PI-3K-independent and is in agreement with reports by Arsham et al. [23] and Alvarez-Tejado et al. [24]. This is in contrast to the data shown in Fig. 1C, in which the PI-3K inhibitor, LY294002, suppressed hypoxia-driven pHRE-luc activity. In this regard it has recently been reported that LY294002 inhibits casein kinase II at doses similar to those required to inhibit PI-3K [48] and could account for the decrease in HIF-1 transactivation seen in Fig. 1C. On the other hand, PI-3K appears to be essential for NO-dependent HIF-1 transactivation.

Co-transfection with the lipid phosphatase mutant PTEN(G129E) decreased NO-driven luciferase activity, suggesting that the protein phosphatase activity of PTEN was required for NO-driven HIF-1 activation. It is possible that the decrease observed here arose due to lower efficiencies of transfection. However, this appears unlikely since the PTEN-(C124S) mutant, which lacks both lipid and protein phosphatase activities, abrogated the suppression observed with the wild-type PTEN. Taken together with the data presented in Fig. 2, this suggests that the NO-dependent transactivation of HIF-1 was dependent on both the protein phosphatase activity and lipid phosphatase activity of PTEN. While the lipid phosphatase activity of PTEN has been extensively shown to be involved in its tumor suppressor function, the role of its protein phosphatase activity has remained largely unknown. The protein phosphatase activity of PTEN has been shown to be involved in the inhibition of cell migration and invasion by targeting the focal adhesion kinase [49] and also in the suppression of matrix metalloproteinase-2 gene expression in human glioma cells [50]. Our results show for the first time that the protein phosphatase activity of PTEN may be involved in the induction of HIF-1-dependent transactivation by NO.

3.4. *Involvement of Akt in HIF-1 activation by hypoxic and NO-exposed HMEC-1 cells*

Akt kinase, also known as protein kinase B, is well recognized as a downstream target of PI-3K. Therefore we examined the activation of Akt by hypoxia and NO in microvascular endothelium. First we determined if Akt could be phosphorylated by endogenous NO. Acetyl choline has been demonstrated to rapidly activate endothelial constitutive nitric oxide synthase (ecNOS) [51,52], whereas cytomix (IL-1β,

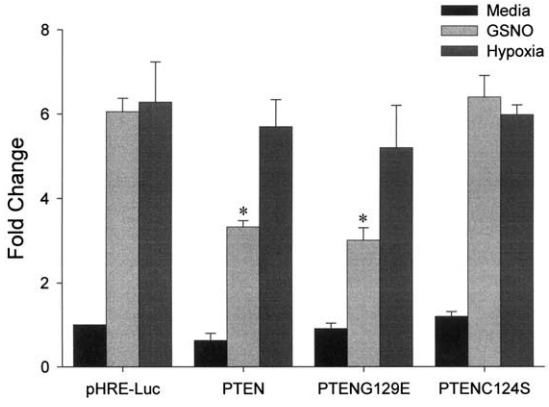

Fig. 3. Effect of PTEN over-expression on HIF-1-dependent transactivation in hypoxic and NO-exposed HMEC-1 cells. HMEC-1 cells were co-transfected with pHRE-luc, pHRL-null and either the expression plasmids encoding wild-type PTEN (PTEN) or mutant plasmids PTEN(G129E) or PTEN(C124S). 24 h post-transfection, cells were exposed to hypoxia or to GSNO (500 μM) for 24 h prior to harvest for DLR assay. Results expressed as an index of relative light units (RLU) ±S.E.M. of three independent transfections (*, $P < 0.01$).

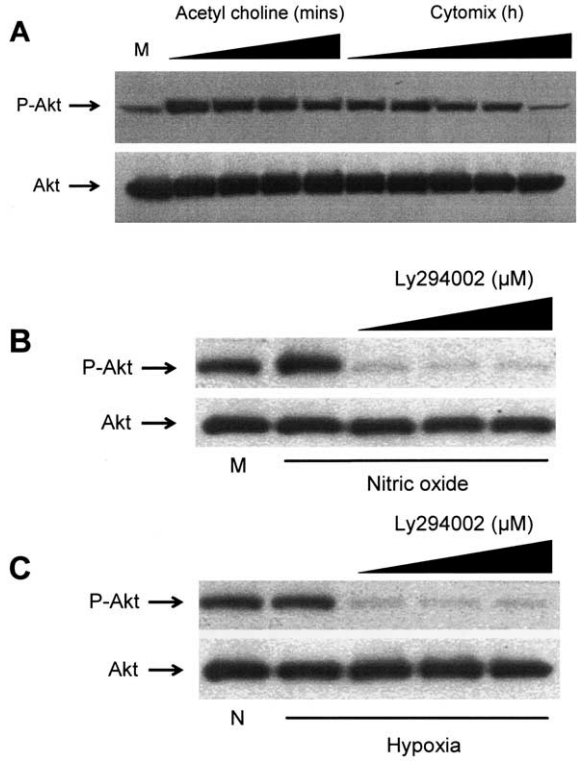

Fig. 4. Involvement of Akt in HIF-1 activation by hypoxic and NO-exposed HMEC-1 cells. A: Confluent HMEC-1 cells were exposed to media alone, acetyl choline (1 μM) for 10, 20, 30 and 60 min, or cytomix (10 pg/ml each of IL-1β, IFN-γ and TNF-α) for 1, 2, 4, 6 and 24 h. Alternately, confluent HMEC-1 cells were pre-incubated with LY294002 (10, 25 and 50 μM) for 30 min prior to exposure to GSNO (500 μM, 5 h) (panel B) or hypoxis (6 h) (panel C). Whole cell extracts were isolated and P-Akt detected via Western blot analysis using rabbit anti-human P-Akt (ser473) antibody. Membranes were stripped and re-probed for Akt using rabbit anti-human Akt antibody.

IFN-γ and TNF-α) activates inducible nitric oxide synthase (iNOS) [53]. Therefore we exposed HMEC-1 cells to acetyl choline (1 μM) for 10, 20, 30 and 60 min or cytomix (10 pg/ml each of IL-1β, IFN-γ and TNF-α) for 1, 2, 4, 6 and 24 h and examined the activation of Akt by Western blot analysis. Since phosphorylation of Ser473 on Akt reflects its active form we used a phospho-specific ser473Akt antibody in Western blots to probe for Akt activation. As seen in Fig. 4A, acetyl choline, which activates ecNOS, rapidly induced Akt phosphorylation. Akt activation was observed after only a 10 min exposure to acetyl choline and then was followed by a gradual decline in activated Akt. Cytomix, which activates iNOS at the transcriptional level, also induced Akt phosphorylation at 1 h. Levels of P-Akt gradually declined and reached basal levels after 24 h exposure to cytomix. Parallel experiments performed following transfection of HMEC-1 with pHRE-luc resulted in increased HIF-1-driven luciferase activity following exposure to either acetyl choline or cytomix (data not shown), suggesting that endogenous NO stimulates HIF-1-dependent transcription in a PI-3K/Akt-dependent manner. Next we examined the activation of Akt by hypoxia and exogenous NO. HMEC-1 cells were pre-incubated with LY294002 for 30 min prior to exposure to hypoxia (6 h) or GSNO (500 μM, 5 h). As seen in Fig. 4B, exposure of HMEC-1 to GSNO resulted in a more than two-fold increase in Akt activation that could be suppressed by LY294002. Exposure to hypoxia (Fig. 4C), however, failed to activate Akt, suggesting that Akt activation may not be required for hypoxic induction of HIF-1. These results further support our data in Fig. 3 that PI-3K is not involved in hypoxic activation of HIF-1. Western blots were stripped and re-probed with an Akt antibody that recognizes the unphosphorylated form of Akt to ensure equal loading.

Thus, in this study we showed that HIF-1 is activated under non-hypoxic conditions by NO. Protein phosphorylation events are involved in HIF-1 activation, both during hypoxia and NO exposure. Specifically, the ERK and PKC signaling pathways are involved in regulation of the transactivation ability of HIF-1 following exposure to both hypoxia and NO. The PI-3K pathway has differential effects on HIF-1 activation. The data presented here indicate that the PI-3K/Akt pathway is not required for HIF-1α induction, nor does it play an essential role in HIF-1 transactivation during hypoxia. In contradistinction, NO-dependent HIF-1α stabilization and HIF-1 transactivation potential are regulated by PI-3K/Akt. Since over-expression mut PTEN significantly decreased HIF-1-dependent transactivation but did not abolish it completely, both the lipid phosphatase and protein phosphatase activities of PTEN appear to be involved in regulation of HIF-1α by NO. Future studies of the protein phosphatase targets of PTEN would provide important information for regulation of HIF-1 under conditions associated with massive NO production such as inflammation and ischemia-reperfusion.

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