ERK activation upon hypoxia: involvement in HIF-1 activation

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Abstract Hypoxia-inducible factor-1 (HIF-1) is a transcription factor activated by hypoxia. The HIF-1 activation transduction pathway is poorly understood. In this report, we investigated the activation of extracellular regulated kinases (ERK) in hypoxia and their involvement in HIF-1 activation. We demonstrated that in human microvascular endothelial cells-1 (HMEC-1), ERK kinases are activated during hypoxia. Using dominant negative mutants, we showed that ERK1 is needed for hypoxia-induced HIF-1 transactivation activity. Moreover, using a kinase assay and Western blot experiments, we showed that HIF-1α is phosphorylated in hypoxia by an ERK-dependent pathway. These results evidence the role of mitogen-activated protein kinase in the transcriptional response to hypoxia.

Key words: Hypoxia; Hypoxia-inducible factor-1 (HIF-1); Extracellular regulated kinase 1 and 2 (ERK)

1. Introduction

Vascular injuries, ischemic cardiovascular diseases and thrombosis provoke pathological decreases in oxygen tension [1,2]. In response to these hypoxic conditions, mammalian cells increase the expression of genes encoding proteins involved in the anaerobic production of ATP (aldolase A, enolase-α, GLUT-1, etc. [1]). Hypoxic cells also secrete VEGF, an angiogenic hormone [1]. HIF-1 transcription factor is activated by hypoxia and is responsible for these modifications in gene expression.

HIF-1 is a heterodimeric protein composed of the HIF-1α and ARNT sub-units [3]. These two sub-units belong to the basic helix-loop-helix (bHLH)-PAS protein family [3,4]. HIF-1α is the sub-unit regulated by hypoxia.

In normoxia, HIF-1α is a cytosolic protein rapidly degraded by the ubiquitin-proteasome pathway [5]. This degradation is mediated by the oxygen-dependent degradation domain (ODD, residues 401–603) [6]. In hypoxia, the degradation of HIF-1α is inhibited. The protein is then translocated into the nucleus where it dimerizes with ARNT [7], a nuclear protein [8]. HIF-1 then binds to the hypoxia responsive element (HRE) [9] *cis*-elements found in promoters or enhancers.

The mechanisms of activation of HIF-1 are currently poorly understood. Phosphorylations seem to be involved for the stabilization of the HIF-1α sub-unit in hypoxia. Indeed, kinase inhibitors are able to impair the HIF-1α stabilization in hypoxia [10]. Moreover, Semenza et al. demonstrated that the carboxy terminal domain of HIF-1α is composed of three distinct domains [11]: transactivation domain-N terminal (TAD-N, residues 531–575) and TAD-C (transactivation domain-C terminal, residues 786–826) and the ID (inhibitory domain, residues 576–785) [11]. The TAD-C and the TAD-N are involved in hypoxia in the transcriptional activity induced by a GAL-4/HIF-1α chimeric protein [11]. The expression level of these fusion proteins was the same in normoxia and in hypoxia [11]. The ID is able to inhibit the normoxic transcriptional activity of the TAD-N/C without affecting the fusion protein stability [11]. These results indicate that the increase in gene expression induced by HIF-1 in hypoxia firstly requires the stabilization of the HIF-1α sub-unit and secondly the activation/derepression of the carboxy-terminal domain of HIF-1α. The activation of the TAD-N/C could occur by interaction with co-activator (CBP/p300) [12], and/or by phosphorylation(s) [11,13]. The inhibition of the ID domain in hypoxia could also occur by phosphorylations [11].

In this report, we investigated the role of the mitogen-activated protein kinases (MAPK) ERK1 and ERK2 in the activation of HIF-1 by hypoxia in endothelial cells. Firstly, we showed that ERK1 and ERK2 are activated during hypoxia. Inhibition of these kinases with PD98059 [14] did not inhibit the hypoxic stabilization of the HIF-1α sub-unit nor did it inhibit the DNA binding activity of HIF-1. However, in cells treated with PD98059, we observed the inhibition of the HIF-1 transcriptional activity induced by hypoxia. Moreover, the HIF-1-induced transcriptional activity was inhibited in hypoxic human microvascular endothelial cells-1 (HMEC-1) expressing ERK1 but not ERK2 dominant negative mutant. At last, hypoxically activated ERK1 is able to directly phosphorylate the carboxy-terminal domain of HIF-1α. These results show that ERK1 is involved in the hypoxia-induced transcriptional activity of HIF-1 but that this kinase does not participate in the stabilization of the HIF-1α protein.

2. Materials and methods

2.1. Cell culture

HMEC-1 [15] were grown in MCDB 131 (Gibco, UK) containing 15% fetal calf serum, 10 ng/ml epidermal growth factor (EGF), 1 μg/ml hydrocortisone, 10 mM glutamine, 50 U/ml penicillin G, 50 ng/ml amphotericin B. 24 h prior hypoxia, the cells were incubated in MCDB 131 containing 0.2% serum and without EGF. Normoxia (21% O_2) and hypoxia (1% O_2) incubations were performed in CO_2-independent medium (Gibco, UK) supplemented with 10 mM glutamine, 1 μg/ml hydrocortisone, 50 U/ml penicillin G, 50 ng/ml amphotericin B and 0.2% serum. COS-7 cells were grown in DMEM containing 20 mM HEPES pH 7.2, 50 U/ml penicillin G, 50 ng/ml amphotericin B and 10% fetal calf serum.

2.2. Western blot analysis

Total cell extracts were prepared from HMEC-1 grown in 25 cm^2

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flasks. The cells were incubated in normoxia or in hypoxia. Then the cells were washed with ice-cold PBS and lysed using the extraction buffer (Tris 40 mM pH 7.5, KCl 300 mM, EDTA 2 mM, Triton X-100 1%, protease and phosphatase inhibitors). The lysate was incubated on ice for 30 min, centrifuged 15 min at 15 000 rpm, 4°C, and the clear lysate was boiled and kept frozen until use. The different extracts were resolved on SDS–PAGE gels and then transferred to PVDF membrane. After blocking in phosphate saline buffer containing 0.2% Tween and 5% dried milk, the blot was probed with the suitable antibody. The phospho-specific p44/p42 MAPK antibody (Westburg, Biolabs, UK) [16] was used (1/5000) to detect phospho-ERKs, monoclonal anti-ERK2 and monoclonal anti-ERK1 antibodies (PharMingen, Becton Dickinson, USA) were used (1/2000) to probe total ERK1 and ERK2. Anti-HIF-1α (Transduction Laboratories, Becton Dickinson, UK) was used to probe HIF-1α (1/1000) and anti-α-tubulin (1/2000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to probe tubulin as control of loaded protein amount. Horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia, Sweden) were used (1/2000) for detection by chemiluminescence.

2.3. Immunofluorescence

HMEC-1 were grown on glass coverslip. The cells were incubated during 2 h in normoxia or in hypoxia. Then the cells were washed with PBS and fixed 10 min with PBS containing 4% paraformaldehyde. The fixed cells were washed three times with PBS+BSA 3%, the cells were incubated 2 h with either an anti-ERK1 or an anti-ERK2 antibody (1/2000) (Transduction Laboratories, Becton Dickinson, USA) in PBS+BSA 3%. Then the cells were washed three times with PBS+BSA and secondary anti-mouse TRITC-conjugated antibodies were added to the cells (1/2000). After 1 h of incubation, the cells were washed three times with PBS+BSA before fixation in mowiol. Observation were performed using a confocal microscope (Leica, Germany).

2.4. Immunoprecipitations and kinase assay

Total cell extracts from normoxic and hypoxic cells incubated in the presence or in the absence of PD98059 20 μM (Calbiochem) were prepared as described above. After having collected the supernatant, ERK1 (Santa Cruz Biotechnology) or ERK2 (PharMingen International, Becton Dickinson, USA) antibodies (1/1000) were added to the samples followed by an incubation of 2 h at 4°C. The immune complexes were immobilized with 40 μl of Sepharose-protA beads and incubated for 2 h, followed by three washes with 800 μl of cold lysis buffer. An additional washing step was performed with 800 μl of kinase assay buffer (25 mM HEPES pH 7.6, 20 mM $MgCl_2$, Na_3VO_4 0.1 mM, DTT 2 mM). 100 μl of each sample were kept for Western blot analysis. The beads with ERK kinase were collected by centrifugation and resuspended in 40 μl of kinase assay buffer. 2 μl of ATP 1 mM, 1 μl of [γ-^{32}P]dATP (10 μCi) and 2 μg of ERKs-peptide substrate (Santa Cruz Biotechnology) (RRRELVEPLTPSGE) were added to the assay mix. The mix was incubated 30 min at 37°C and the reaction was stopped by boiling. Then the peptide was adsorbed on phosphocellulose SpinZyme membranes (Pierce, USA). The membranes were placed into a scintillation vial and the radiolabelled peptide of each sample was quantitated using a scintillation counter.

2.5. Transient transfection and reporter gene assays

To assay the transcriptional activity of HIF-1, we used the pGL3-SV40HRE vector which contains three copies of the EPO HRE downstream from the luciferase gene of which the expression is driven by a heterologous SV40 promoter [17]. The pGL3-SV40 was used as negative control and does not respond to hypoxia (not shown in figures) [9,17]. HMEC-1 transfection was performed in a 96-well plate at 80% confluency. These vectors were co-transfected with the control vector pRL-SV40 (Promega, USA) as transfection efficiency control. Regarding the dominant negative vectors, we used ERK1 (pcDNA-ERK1-) and ERK2 (pcDNA-ERK2-) dominant negative expression vectors which encode ERKs mutated in their ATP binding sites [18] and as null vector, we used the pBSKII+ (Stratagene, USA). 3 μg of DOTAP transfection reagent (Boehringer, Germany) were mixed with a total of 500 ng of the described plasmids. The transfection solution was added to the cells for 4 h. The cell medium was replaced with CO_2-independent medium. Thereafter, the transfected cells were placed in either normoxia or hypoxia for 24 h. After the incubation, the luciferase activity was quantitated in a luminometer using the Dual-Luciferase-Reporter system (Promega, USA). pc-fos promoter reporter was transfected following the same procedure.

2.6. EGFP-CHIF-1α kinase assay

COS-7 cells grown in 25 cm^2 flasks were transfected with the pEGFP-CHIF-1α or EGFP expression vectors using DOTAP transfection reagent (Boehringer, Germany). HMEC-1 were also grown in 25 cm^2 flasks and incubated in normoxia or in hypoxia in the presence of 20 μM PD98059. COS-7 cells were lysed with the lysis buffer (Tris 40 mM pH 7.5, KCl 300 mM, EDTA 2 mM, Triton X-100 1%) containing protease inhibitors but not phosphatase inhibitors. HMEC-1 were also lysed with the same buffer but containing phosphatase inhibitors. ERK1, EGFP and EGFP-CHIF-1α were immunoprecipitated using the procedure described above with respectively ERK1 (1/1000) (Santa Cruz Biotechnology) and EGFP antibodies (1/1000) (Clontech, USA). Then EGFP-CHIF-1α and EGFP were incubated together with ERK1 samples during 1 h in kinase assay buffer (described above), with 2 μl of ATP 1 mM, 1 μl of [γ-^{32}P]dATP (10 μCi) and under agitation. At last, the samples were loaded on a 12% polyacrylamide gel. The gel was dried and phospho-proteins were revealed by autoradiography and quantitated by phospho-imager (Packard, USA).

3. Results

3.1. ERK1 and ERK2 MAPKs are phosphorylated during hypoxia

Phosphorylation of the HIF-1α sub-unit seems to be important for HIF-1α stabilization and hence HIF-1 activation upon hypoxia [10,13]. The identity of the kinase(s) involved in HIF-1α phosphorylation in hypoxia is not known. Since HIF-1α is stabilized by FGF-2 and EGF [19] hormones known to activate MAPKs [20], we sought whether ERK1 and ERK2 MAPKs are activated during hypoxia. Active ERKs are known to be phosphorylated among others on tyrosine (204), this phosphorylation can be quantitated by Western blot analysis. HMEC-1 were incubated during increasing time (0.5, 1 and 2 h) in either hypoxia (1% O_2) or normoxia (21% O_2). Then the cells were lysed for total protein extract

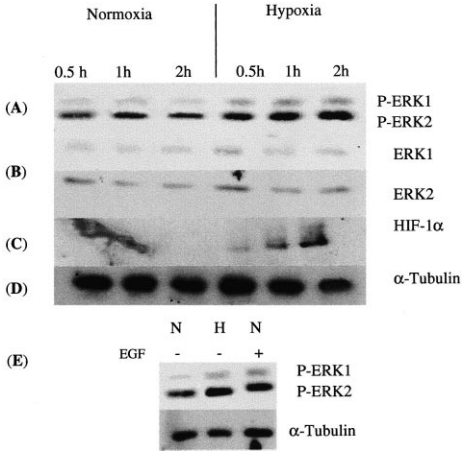

Fig. 1. A: Western blot analysis of ERK1 and ERK2 phosphorylation. HMEC-1 were incubated during 0.5, 1 and 2 h in either normoxia or hypoxia. Total proteins were extracted and blotted on PVDF membrane. Phospho-ERKs were probed with an anti-phospho p42/p44 antibody. B: A Western blot was performed on the same extracts using monoclonal anti-ERK1 and anti-ERK2 in order to quantitate the total amount of ERKs loaded. C: Western blot analysis of HIF-1α protein amount during hypoxia and normoxia. D: α-Tubulin control for the amount of proteins loaded on the gel. E: Comparison of ERK phosphorylation in either hypoxic conditions (0.5 h, 1% O_2) or with EGF stimulation (0.5 h, 10 ng/ml).

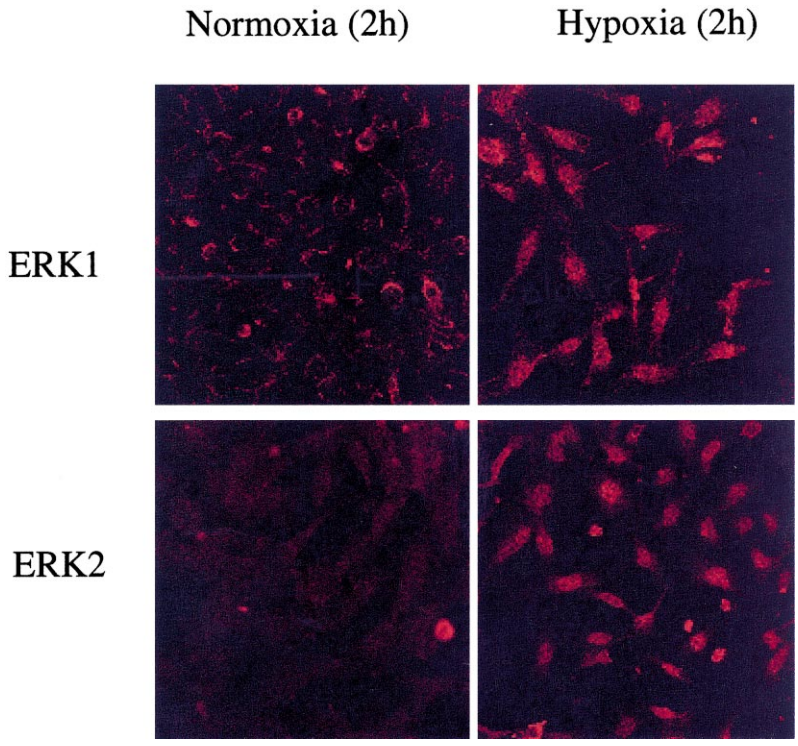

Fig. 2. Immunofluorescence detection of ERK1 and ERK2 in both normoxia and hypoxia. HMEC-1 were incubated during 2 h in either normoxia or hypoxia. Then the cells were labelled with ERK1 or ERK2 antibodies and TRITC-conjugated secondary antibody. Sub-cellular localization of ERK1 and ERK2 was observed with a confocal microscope (magnification 400×).

preparation. 5 μg of total protein extracts were loaded on polyacrylamide gel for Western blot analysis. Using an anti-phospho-ERK antibody which recognizes the active forms of ERK1 and ERK2 [16], an increase in both ERK1 and ERK2 phosphorylation was observed during hypoxia (Fig. 1A). Using monoclonal antibodies raised against total ERK1 and ERK2, no modification in the level of ERK kinases expression was detected (Fig. 1B). This indicates that the increase in ERKs phosphorylation was not due to an increase in total ERK proteins. Hypoxia also leads to the rapid stabilization of HIF-1α detected by Western blot analysis, with similar kinetics (Fig. 1C). α-Tubulin was used as control for the total amount of proteins loaded on the gel (Fig. 1D). We also compared the ERKs phosphorylation level following hypoxia or EGF stimulation of a known ERK activator. Both EGF and hypoxia induced ERKs phosphorylation to similar levels (Fig. 1E).

3.2. *ERK1 and ERK2 are translocated into the nucleus during hypoxia*

Phosphorylation of cytosolic ERKs by the cytosolic MAPK kinase MEK1 leads to their activation [21,22]. They are then translocated within the nucleus where they will phosphorylate various substrates such as transcription factors (c-jun, c-myc, Elk-1) [21,22]. Since hypoxia induces the phosphorylation of ERKs, we looked for their translocation into the nucleus. Immunofluorescence experiments were performed on HMEC-1 incubated during 2 h in either normoxia or hypoxia. Monoclonal anti-ERK1 and anti-ERK2 were used as primary antibodies and anti-mouse TRITC-conjugated antibodies were used as secondary antibodies. In normoxia, ERK1 is perinuclear while ERK2 is located throughout the cytosol (Fig. 2). A clear translocation of both ERK1 and ERK2 was observed after 2 h of hypoxia (Fig. 2).

3.3. *ERK1 and ERK2 activity increase during hypoxia*

In order to confirm ERK activation during hypoxia, we performed a kinase assay using a EGF-R peptide which is a substrate for ERK kinases. HMEC-1 were incubated during 2 h in either normoxia or hypoxia and with or without PD98059 (20 μM), an inhibitor of the ERK kinase pathway [14]. Then the cells were lysed and ERK1 or ERK2 were immunoprecipitated using a rabbit anti-ERK2 antibody or a goat anti-ERK1 antibody. None of these antibodies cross-reacts with the other ERK. Immunoprecipitated proteins were then incubated 30 min with [γ-^{32}P]ATP and the peptide substrate. Incorporated radiolabelled ATP was quantitated in a scintillation counter. As shown in Fig. 3A activation of ERK1 and of ERK2 was observed in hypoxia compared to normoxia. This activation was completely inhibited when cells were incubated in the presence of PD98059 (20 μM). The total amount of immunoprecipitated ERK1 and ERK2 was analyzed by Western blotting. As shown in Fig. 3B the same amount of ERK kinase was immunoprecipitated for each condition.

3.4. *ERK kinases are not involved in HIF-1α stabilization during hypoxia*

Results shown here indicate that hypoxia induces phosphorylation, nuclear translocation and activation of ERK1 and ERK2. We next investigated whether these kinases are involved in the hypoxia-induced stabilization of HIF-1α and activation of HIF-1. The effect of PD98059 on HIF-1α stabilization upon hypoxia was first studied. Western blot analysis using anti-HIF-1α antibody was performed on protein ex-

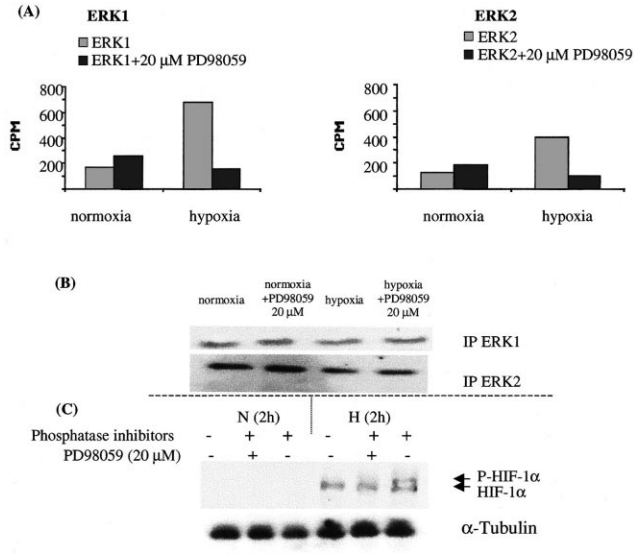

Fig. 3. Quantification of ERK1 and ERK2 activity using a kinase assay experiment. HMEC-1 were incubated 2 h in normoxia or in hypoxia in the presence or in the absence of 20 μM of PD98059. ERK1 and ERK2 immunoprecipitated from the cell extracts were incubated with a peptide-substrate and [γ-^{32}P]dATP. A: Quantification of radiolabelled phosphate incorporated in the peptide substrate by ERK1 and ERK2. B: Western blot analysis of the amount of ERK1 and of ERK2 immunoprecipitated for each experimental condition. C: PD98059 does not inhibit HIF-1α stabilization during hypoxia. HMEC-1 were incubated during 2 h in either normoxia or in hypoxia in the presence or in the absence of 20 μM PD98059. HIF-1α was detected by Western blot analysis. HIF-1α is phosphorylated in hypoxia and PD98059 inhibits the HIF-1α phosphorylation. Extracts that were not treated with phosphatase inhibitors were used as phosphorylation negative control. α-Tubulin was used as control for total protein amount loaded on the gel.

tracts from normoxic and hypoxic HMEC-1 incubated with or without 20 μM PD98059. In normoxia, HIF-1α is rapidly degraded and no protein is detectable (Fig. 3C). In hypoxia the protein is stabilized and is detected by Western blotting (Fig. 3C). PD98059 did not inhibit the hypoxia-induced stabilization of HIF-1α (Fig. 3C). This result is in accordance with the results from Semenza [23] and Salceda [13] who showed that PD98059 did not inhibit the hypoxia-induced DNA binding activity of HIF-1 upon hypoxia. Moreover, using the HIF-1α antibody to a dilution 1/1000 and extensive exposition time, we were able to detect HIF-1α phosphorylation (Fig. 3C). This phosphorylation is present in hypoxia and is inhibited in the presence of PD98059 (Fig. 3C). A control of phosphorylation was performed using extracts which were not treated with phosphatase inhibitors (Fig. 3C). All together, these results indicate that ERKs are not involved in HIF-1α stabilization nor in the regulation of the DNA binding activity of HIF-1. However, HIF-1α phosphorylation is dependent on the ERK pathway.

3.5. PD98059 inhibits the activity of HIF-1

We then investigated the role of ERK kinases in the HIF-1 transcriptional activity. HMEC-1 were transfected with a luciferase reporter gene [17] (pGL3-SV40HRE) which expression is under the control of three copies of the HRE *cis*-elements. The pRL-SV40 vector was used as transfection efficiency control. Transfected cells were incubated in either normoxia or hypoxia without or in the presence of 20 μM PD98059. 24 h later the luciferase activity was quantitated. As shown in Fig. 4, hypoxia increased by 3.5-fold the luciferase activity indicating that HIF-1 was indeed activated in these conditions. PD98059 strongly inhibited the HIF-1 induced response to hypoxia. This result indicates that ERK kinases could be involved in the regulation of hypoxia-induced activity of HIF-1.

3.6. ERK1 but not ERK2 is involved in HIF-1 activity

In order to confirm that ERK kinases are indeed involved in the regulation of HIF-1 activity, we used ERK1 and ERK2 dominant negative mutants [18]. HMEC-1 were co-transfected with the pGL3-SV40HRE, the pRL-SV40 and either with a null-vector or with a ERK1 or a ERK2 dominant negative expressing vector. Thereafter, the cells were placed in normoxia or in hypoxia and 20 h later, the luciferase activity was quantitated. A complete inhibition of HIF-1 activity was obtained in cells transfected with the ERK1 dominant negative (Fig. 5A). No such effect was observed in cells expressing a ERK2 dominant negative protein (Fig. 5A). This result confirms that MAPKs are actually involved in the regulation of HIF-1 transcriptional activity upon hypoxia and that HIF-1 activation requires active ERK1. ERK1 and ERK2 kinase dead alleles are also able to inhibit the c-fos promoter reporter activity (Fig. 5B), this activation is induced by Elk-1 [24] which has been identified as a ERKs target in hypoxia [24,25]. This demonstrates that the dominant negative mutants are expressed in HMEC-1 and that they are active. This also confirmed ERKs activation upon hypoxia.

3.7. The HIF-1α carboxy-terminal domain is phosphorylated by ERK1

Inhibition of HIF-1 activity by ERK1 dominant negative does not indicate that ERK1, while activated by hypoxia is able to directly phosphorylate the HIF-1 sub-units. Salceda and Semenza proposed that the carboxy-terminal domain of HIF-1α is also regulated independently of the HIF-1 DNA binding activity [11,13] and that this regulation involves phosphorylation(s). That is why we tested the capability of hypoxically activated ERK1 to directly phosphorylate the carboxy-terminal domain of HIF-1α. COS-7 cells were transfected with the pEGFP-CHIF-1α (carboxy terminal domain of

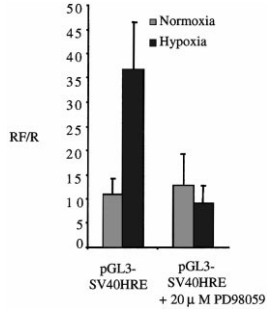

Fig. 4. PD98059 inhibits the hypoxia-induced transcriptional activity of HIF-1. HMEC-1 were transfected with the pGL3-SV40HRE together with the pRL-SV40 control vector. Then the cells were incubated 24 h in either normoxia or hypoxia in the absence or in the presence of 20 μM PD98059. Thereafter, the cells were lysed for luciferase assays. Data represent the ratio between test firefly luciferase activity and renilla luciferase activity (RF/R). Results are presented as means ± S.D. for triplicates.

HIF-1α) vector which expresses a EGFP-CHIF-1α chimeric protein or with the pEGFP as a negative control. Then the cells were lysed and EGFP-CHIF-1α or EGFP proteins were immunoprecipitated using anti-EGFP antibodies. In parallel, ERK1 proteins were immunoprecipitated from HMEC-1 incubated 2 h in normoxia or in hypoxia in the presence or in the absence of 20 μM PD98059. Then EGFP-CHIF-1α was incubated with [γ-P^{32}]dATP in the presence of the different ERK1 samples. EGFP was incubated in the presence of ERK1 immunoprecipitated from hypoxic HMEC-1. The samples were then loaded on a polyacrylamide gel. As shown in Fig. 6A,B, the HIF-1α carboxy-terminal domain is phosphorylated by ERK1, activated by hypoxia and PD98059 indeed inhibited the HIF-1α phosphorylation. EGFP is not phosphorylated by ERK1. The amount of immunoprecipitated ERK1 is similar in all samples as shown by Western blot (Fig. 6C). These results indicate that the carboxy-terminal domain of HIF-1α can be directly phosphorylated by ERK1 kinase only when activated by hypoxia.

4. Discussion

Phosphorylation plays a key role in transcription factor regulation. HIF-1 is a transcription factor specifically activated under hypoxic conditions [1]. Its phosphorylation is necessary to obtain its activation [10,13]. Semenza et al. demonstrated that the carboxy-terminal domain of HIF-1α is involved in the transactivation of hypoxia-responsive reporter genes [11]. Hence, it appears that HIF-1 regulation involves at least two mechanisms: regulation of the HIF-1α degradation and regulations occurring at the transactivation domain. Phosphorylation seems to be an important way of regulating HIF-1 activity. These data infers a transduction pathway from a putative oxygen sensor to unidentified kinase(s).

HIF-1α stabilization was observed in the presence of EGF [19]. EGF is known to activate the ERKs MAPK pathway [19,20]. Interestingly, Mukhopadhyay et al. demonstrated that 6-thioguanine an ERK inhibitor, impairs the hypoxic activation of the expression of the VEGF gene [26]. Salceda et al.

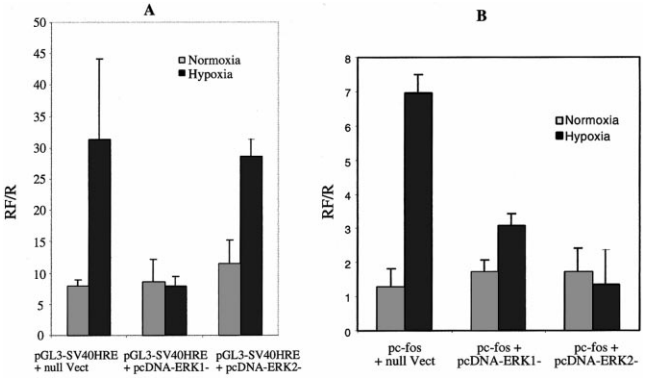

Fig. 5. ERK1 but not ERK2 dominant negative mutants inhibit HIF-1 transcriptional activity. HMEC-1 were cotransfected with the pGL3-SV40HRE (A) or pc-fos (B) together with the pRL-SV40 control vector and with either a null vector (pBSKII) or a dominant negative ERK1 vector (pcDNA-ERK1-) or a dominant negative ERK2 vector (pcDNA-ERK2-). The cells were incubated 20 h in either normoxia or hypoxia. The cells were lysed for luciferase assays. Data represent the ratio between test firefly luciferase activity and renilla luciferase activity (RF/R). Results are presented as means ± S.D. for triplicates.

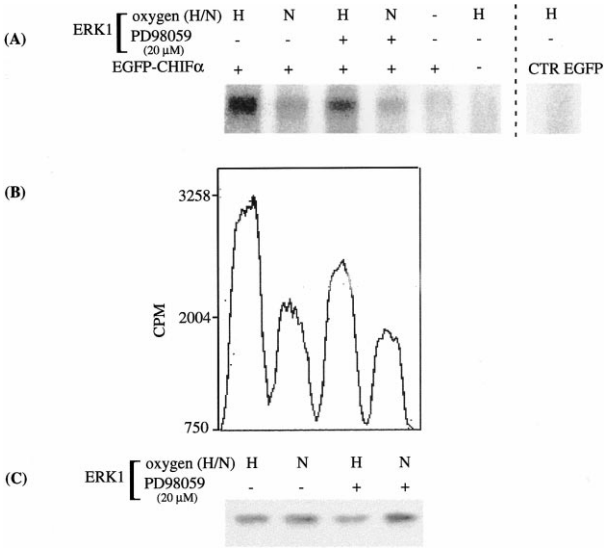

Fig. 6. Phosphorylation of the carboxy-terminal domain of HIF-1α by ERK1. Immunoprecipitated EGFP-CHIF-1α was incubated with immunoprecipitated ERK1 from HMEC-1 incubated in either normoxia (N) or hypoxia (H) in the presence (+) or in the absence (−) of 20 μM PD98059 (A). EGFP was used as negative control and incubated with ERK1 from hypoxic HMEC-1 (A). The amount of phospho-EGFP-CHIF-1α was quantitated in a phospho-imager (B). The amount of immunoprecipitated ERK1 was quantitated by Western blot (C).

also showed that PD98059, an inhibitor of the ERKs upstream kinase MEK1, was able to impair the HIF-1 induced transactivation activity but did not inhibit its DNA binding activity [13]. In order to search for kinases activated in hypoxic conditions, we investigated the putative role of MAPK(s) in HIF-1 activation. In this report, we indeed showed that in HMEC-1, hypoxia is able to induce the phosphorylation of both ERK1 and ERK2 MAPKs. This phosphorylation led to their nuclear translocation and to their activation as assessed by a kinase assay and c-fos promoter reporter assay.

We then sought whether the activation of ERK kinases by hypoxia is involved in the HIF-1-dependent response. As HIF-1α is a cytosolic protein rapidly degraded by the proteasome [5,6] and ERK are nuclear active kinases [21,22], we presumed that MAPKs were not involved in the hypoxic stabilization mechanism of HIF-1α. Indeed, treatment with an ERK inhibitor (PD98059) did not impair the HIF-1α stabilization in hypoxia nor did it impair the DNA binding activity of HIF-1 as shown by Salceda and Semenza [13,23], but it is involved in HIF-1α phosphorylation.

On the other hand, in HMEC-1, PD98059 was able to inhibit the hypoxia-induced transcription of a reporter gene which is regulated by HRE *cis*-elements. Inhibition observed with dominant negative mutants of ERK1 and ERK2 confirmed in vivo that ERKs MAPKs are activated by hypoxia as assayed with the c-fos promoter reporter and that ERK1 is involved in the activation of HIF-1 transcriptional activity by hypoxia whereas ERK2 did not seem to be involved. Moreover, we showed that ERK1 activated by hypoxia is able to directly phosphorylate the HIF-1α carboxy-terminal domain. Interestingly, the HIF-1α ID domain contains several X-X-S-P [27] motives which are putative phosphorylation sites for ERKs. However, the regulation of HIF-1 activity by ERK1

could also involve phosphorylation of co-activator(s) [12]. This has to be tested.

The nature of the transduction pathway from the putative oxygen sensor to the activation of kinases is still unknown. Several models were hypothesized [1,28]. Interestingly in cells unable of mitochondrial respiration following mitochondrial DNA deletion, HIF-1 activation is inhibited in hypoxia [28]. When hypoxic cells are treated with the reducing agents PDTC, PDI or ebselen, HIF-1 remains inactive [1,28]. These results suggested that mitochondrial ROS production during hypoxia could be one event involved in the hypoxia signalling pathway [28]. However, the increase of ROS production during hypoxia is still much debated. Interestingly, there are growing evidences that ERKs MAPK pathway is activated by reactive oxygen species [29,30].

Richard et al. [31], also recently demonstrated in vitro that ERKs are indeed able to phosphorylate HIF-1α. However they did not detect ERKs activation upon hypoxia in their cell line. We show here that in microvascular endothelial cells hypoxia is able to activate ERKs MAPKs and that in vivo ERK1 directly participates in HIF-1 activation and is able to phosphorylate its transactivation domain. This evidences that MAPK are involved in the transcriptional response to hypoxia.

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