Normoxic induction of the hypoxia-inducible factor 1α by insulin and interleukin-1β involves the phosphatidylinositol 3-kinase pathway

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Received 12 October 2001; revised 19 December 2001; accepted 28 December 2001

First published online 16 January 2002

Edited by Veli-Pekka Lehto

Abstract Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric DNA-binding complex of the subunits α and β with relevance in O_2 and energy homeostasis. The labile component, HIF-1α, is not only activated by hypoxia but also by peptides such as insulin and interleukin-1 (IL-1) in normoxia. We investigated whether inhibitors of mitogen-activated protein kinase kinases (MAPKKs: PD 98059, U0126) and phosphatidylinositol 3-kinase (PI3K: LY 294002) do not only lower the hypoxia-induced, but also the insulin- and IL-1-induced HIF-1α accumulation and HIF-1 DNA-binding in human hepatoma cell cultures (line HepG2). The results show that LY 294002 suppressed HIF-1 activation in a dose-dependent manner irrespective of the stimulus. With respect to target proteins controlled by HIF-1, the production of erythropoietin was fully blocked and that of vascular endothelial growth factor reduced following inhibition of the PI3K pathway. The role of MAPKKs in this process remained in question, because PD 98059 and U0126 did not significantly reduce HIF-1α levels at non-toxic doses. We propose that PI3K signaling is not only important in the hypoxic induction of HIF-1 but it is also crucially involved in the response to insulin and IL-1.

Key words: Hypoxia-inducible factor 1; Vascular endothelial growth factor; Phosphatidylinositol 3-kinase; Insulin; Interleukin-1

1. Introduction

Hypoxia-inducible factor 1 (HIF-1) is a dimeric (α/β) transcription factor of major importance for the cellular adaptation to O_2 deficiency [1,2]. While the β-subunit of HIF-1 is always available in the nucleus, the α-subunit is highly susceptible to O_2 and undergoes rapid proteasomal degradation under normoxic conditions. In the presence of O_2 and iron, proline residue 564 in HIF-1α is hydroxylated which enables binding of the von Hippel-Lindau tumor suppressor E3 ligase complex [3,4]. In hypoxia, the α- and β-subunits can dimerize in the nucleus and bind to the consensus sequence (A)CGTG present in the hypoxia response element of O_2-controlled target genes such as those encoding vascular endothelial growth factor (VEGF), erythropoietin (EPO) and distinct glycolytic enzymes. Evidence suggests that the transcriptional activity of HIF-1 is modulated by phosphorylation [5]. The p42/p44 (Erk2/Erk1) mitogen-activated protein kinases (MAPKs) can catalyze HIF-1α phosphorylation [6–11], yielding larger molecular forms (~110 kDa) on electrophoretic separation [7]. The p42/p44 MAPK pathway does not seem to be involved in the stabilization or degradation of HIF-1α but is likely to increase the transactivation potential of the HIF-1 complex. However, MAPKs do not appear to be activated by hypoxia in general [7]. In addition, hypoxia exposure causes activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in some cell lines [12–14]. Pharmacological blockade of PI3K lowers HIF-1-dependent cellular responses to hypoxia [14–16]. Endogenously, the product of the tumor suppressor gene PTEN inhibits effects of PI3K, as it encodes a phosphatase acting on D3 position phosphorylated phosphoinositides. Loss of the tumor suppressor function of PTEN augments HIF-1-mediated gene expression [17].

Recent studies have shown that HIF-1 is not only induced by hypoxia, but is activated also in normoxic cells in response to a variety of peptide mediators, including insulin and insulin-like growth factors [18,19], interleukin-1 (IL-1) and tumor necrosis factor α (TNFα) [20–22], angiotensin II [23] and thrombin [24]. Upon binding of these mediators to their specific membrane receptors various kinases are activated including MAPKs and PI3K. The relevance of these pathways with respect to HIF-1 needs to be further elucidated. In vascular smooth muscle cells, the induction of HIF-1α by angiotensin II is not abolished by inhibitors of p42/p44 MAPK kinases (MAPKKs) or PI3K [23]. In contrast, inhibition of PI3K signaling prevents the induction of HIF-1α in insulin-treated human prostate carcinoma cell cultures [25]. Whether IL-1-induced HIF-1 DNA-binding in normoxic cells involves MAPK or PI3K recruitment is unknown.

Herein, effects were studied of specific inhibitors of MAPKKs (PD 98059, U0126) and of the p110 subunit of PI3K (LY 294002) on hypoxia-, insulin- and IL-1-induced HIF-1α accumulation, HIF-1 DNA-binding, and VEGF as well as EPO synthesis in a human hepatoma cell line (HepG2) that is an established model for O_2-dependent gene expression [26].

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Abbreviations: EMSA, electrophoretic mobility shift assay; EPO, erythropoietin; HIF-1, hypoxia-inducible factor 1; IL, interleukin; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; PI3K, phosphatidylinositol 3-kinase; VEGF, vascular endothelial growth factor

0014-5793/02/$22.00
PII: S0014-5793(02)02247-0

2. Materials and methods

2.1. Cell culture

HepG2 cells from the American Type Culture Collection (ATCC no. HB 8065) were maintained in RPMI 1640 medium (Gibco BRL, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (FBS; Sigma, Deisenhofen, or Life Technologies, Karlsruhe, Germany), penicillin (90 U/ml) and streptomycin (90 μg/ml) in a humidified atmosphere (5% CO_2 in air) at 37°C (Heraeus Incubators, Hanau, Germany). For experiments cells were grown to 25–50% confluence in tissue culture dishes of 145 mm diameter (Greiner, Frickenhausen, Germany). For studies with insulin, FBS-containing medium was replaced by serum-free medium with bovine serum albumin (BSA; 1 g/l) at least 16 h before the experiments were started. For induction of hypoxia dishes were placed in a humidified incubator with 3% O_2, 5% CO_2 and 92% N_2. Cells were treated with human insulin (stock solution 40 U/ml = 1.7 mg/ml; Aventis Pharma, Bad Soden, Germany) or recombinant human IL-1β (Ciba-Geigy, Basel, Switzerland). The MAPKK inhibitors PD 98059 (Calbiochem, Bad Soden, Germany) [27] and U0126 (Promega, Heidelberg, Germany) [28] were dissolved in dimethyl sulfoxide (DMSO) at 10 mM, stored in aliquots at −40°C, and diluted in culture medium to ≤100 μM just prior to use. The specific PI3K inhibitor LY 294002 (Calbiochem) [29] was prepared similarly. Cultures were pre-incubated with PD 98059 60 min and with LY 294002 10 min prior to the experiments. The final DMSO concentration was 5‰ in all cultures, including the controls. The experimental incubation periods were 4 h, 6 h or 24 h, as specified. In order to quantify cytotoxic drug effects, the colorimetric tetrazolium salt/formazan method was applied, which is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) to purple formazan in living cells. Confluent HepG2 cultures in 96-well dishes were used for these studies as described [30].

2.2. Nuclear extract preparation

Cells were washed with ice-cold phosphate-buffered saline (PBS), harvested in 6 ml PBS, and centrifuged at 800×*g* for 6 min at 4°C. The cell pellets were washed with 4 ml ice-cold buffer A (10 mM Tris, pH 7.8, 1.5 mM $MgCl_2$, and 10 mM KCl), resuspended in this buffer and kept on ice for 10 min. Cell lysis was controlled by trypan blue staining. Nuclei were pelleted at 3500×*g* for 6 min at 4°C, and resuspended in 100 μl ice-cold buffer C (420 mM KCl, 20 mM Tris, pH 7.8, 1.5 mM $MgCl_2$, and 20% glycerol) and incubated for 30 min on ice with occasional flicking of the tubes. Just prior to use, both buffers were supplemented with 2 μg/ml aprotinin, 10 μg/ml leupeptin, 20 μg/ml pepstatin, 1 mM Na_3VO_4, 0.5 mM benzamidine, 2 mM levamisole, 10 mM β-glycerophosphate, 0.5 mM dithiothreitol (DTT), and 0.4 mM phenylmethylsulfonyl fluoride. Nuclei were centrifuged at 13 000×*g* for 30 min at 4°C. The supernatants were stored at −80°C. Protein concentrations were determined by the Bradford method using BSA as standard.

2.3. Western blot analysis

For determination of immunoreactive HIF-1α protein, nuclear extracts were subjected to Western blot analysis as described [20]. Samples were run on sodium dodecyl sulfate/7.5% polyacrylamide gels and transferred to PVDF membranes (Roth, Karlsruhe, Germany). Equal loading and transfer efficiency was verified by staining with 2% Ponceau S. Membranes were blocked overnight with PBS/5% fat-free skim milk and then incubated for 1.5 h at room temperature with a 1:1000 diluted monoclonal mouse antibody against human HIF-1α (Transduction Laboratories, Heidelberg, Germany). For detection, a horseradish peroxidase-linked anti-mouse IgG antibody (1:2000, 1 h at room temperature; Santa Cruz, Heidelberg, Germany) and enhanced chemiluminescence substrate (Amersham, Freiburg, Germany) were used.

2.4. Electrophoretic mobility shift assay (EMSA)

Oligonucleotides for gel shift assays were synthesized by MWG (Ebersberg, Germany). The sequences containing the HIF-1 binding sites were derived from the human transferrin gene (*TfHBS*) according to Rolfs et al. [31]. Sequences were: *TfHBS* (sense), 5′-TTCCTGCACGTACACACAAAGCGCACGTATTTC-3′, and *TfHBS* (antisense), 5′-GAAATACGTGCGCTTTGTGTGTACGTG-CAGGAA-3′. After 5′ end labeling of the sense strand unincorporated [γ-^{32}P]-labeled ATP (New England Nuclear, Cologne, Germany) was removed with a Sephadex G50 (Pharmacia, Uppsala, Sweden) column. The annealing reaction was performed in the presence of a two-fold molar excess of unlabeled antisense oligonucleotides and 1 mM $MgCl_2$. Binding reactions were set up in a volume of 20 μl. Nuclear extracts (5 μg of protein) were pre-incubated on ice for 30 min in a buffer containing 50 mM KCl, 10 mM Tris, pH 7.7, 5 mM DTT, 1 mM EDTA, 1 mM $MgCl_2$, 5% glycerol, 0.03% NP40, and 500 ng salmon testes DNA. Following the addition of the ^{32}P-labeled oligonucleotide, the reactions were incubated at 4°C overnight. Samples were resolved by electrophoresis on native 5% polyacrylamide gels at room temperature. Gels were dried and analyzed by phosphoimaging (BAS 1000; Fuji, Düsseldorf, Germany). For competition experiments, a 250-fold molar excess of unlabeled annealed oligonucleotide or an unspecific unlabeled nucleotide (nuclear factor (NF)-κB sequence) was added before the labeled probes. For supershift experiments, 1 μl undiluted anti-HIF-1α antibody (Transduction Laboratories) was added 30 min before the gel was run.

2.5. Luciferase assay

HepG2 cells were stably transfected with a hypoxia-responsive reporter gene construct as described previously [32]. Briefly, the firefly luciferase reporter gene plasmid pH3SVL [32], containing a total of six HIF-1 binding sites derived from the transferrin gene, was linearized with *Xmn*I, mixed with the *Eco*RI-linearized neomycin expression vector pSV2neo at a molar ratio of 10:1, and co-electroporated into HepG2 cells. Following limited dilution and selection in 250–500 μg/ml G418 (PAA Laboratories, Cölbe, Germany), a hypoxia reporter cell line (termed HRG1) was chosen based on the efficiency of hypoxic reporter gene induction. HRG1 (4×10^4 cells per well; 24-well; Nunc, Wiesbaden, Germany) was seeded and grown for 48 h. Following 24 h of hypoxic (3% O_2) incubation in the absence or presence of kinase inhibitors the cells were lysed ('passive lysis'; Promega) and luciferase activity measured in a MicroLumate LB 96P (Berthold EG&G, Bad Wildbach, Germany).

2.6. Assay of VEGF and EPO

$VEGF_{121/165}$ in the culture media was measured by commercial enzyme-linked immunoassay (ELISA; Quantikine; R&D Systems, Minneapolis, MN, USA). EPO was measured using a commercial ELISA test kit calibrated against the World Health Organization Standard Preparation (Medac, Hamburg, Germany). The rates of the production of VEGF and EPO were related to cellular protein as a measure of culture density. To this aim, the cultures were rinsed with PBS, prior to the addition of lysis buffer (25 mM Tris, 1 mM DTT, 1 mM EDTA, 15% glycerol, 1% Triton X-100; pH 7.8). Protein was measured by the Bradford method. The results are given as the mean ± S.D. Dunnett's test was applied to determine the significance of difference ($P < 0.05$) between a control mean and several treatment means and Tukey–Kramer's test for multiple comparisons.

3. Results and discussion

HIF-1α protein was barely detectable on Western blots of nuclear extracts from normoxic HepG2 cultures without specific hormonal stimulation. However, on exposure to hypoxia (4 h at 3% O_2) significant amounts of HIF-1α were demonstrated (Fig. 1A). The presence of DMSO (5‰) in the culture media did not alter the response to hypoxia. The addition of the PI3K inhibitor LY 294002 produced a dose-dependent decrease in the level of HIF-1α. The MAPKK inhibitor PD 98059 moderately lowered the level of HIF-1α, but there was no clear dose-dependency. Likewise, U0126 moderately lowered HIF-1α levels in hypoxic HepG2 cultures at 10 μM, while it was ineffective at 1 μM (Fig. 2A). Note that the anti-HIF-1α antibody reacts with HIF-1α forms of varying molecular mass, which may range from 104 to 116 kDa. There is evidence that the high molecular forms arise as a product of phosphorylation [7]. Accordingly, the high molecular forms of HIF-1α were more strongly reduced by PD 98059 treatment. This observation supported the concept that the p42/p44

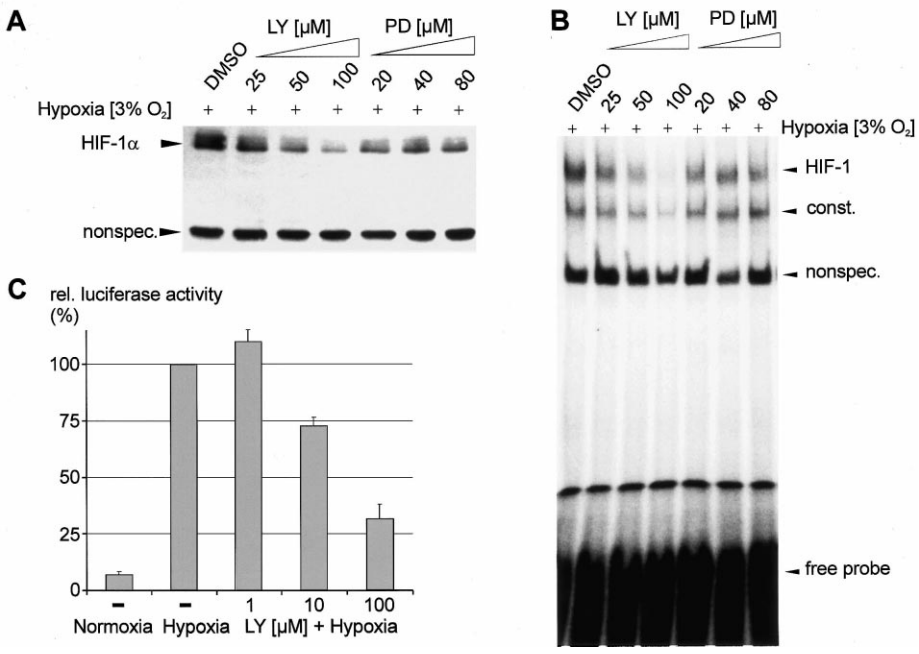

Fig. 1. A: Western blot analysis of HIF-1α in nuclear extracts of hypoxia-exposed (4 h at 3% O_2) HepG2 cells treated with LY 294002 or PD 98059. Hypoxic control cultures were treated with DMSO only. B: Gel shift analysis with nuclear extracts of similarly treated cells. HIF-1, inducible HIF-1 binding; const., constitutive binding; nonspec., non-specific binding. C: Luciferase activities in the stably transfected hypoxia reporter cell line HRG1 maintained in normoxia (20% O_2) or hypoxia (3% O_2) (black bars) for 4 h. Results from normoxic cultures and hypoxic cultures treated with LY 294002 were related to the activity of the hypoxic control cultures (100%). The figure shows the mean ± S.D. of four parallel cultures. Similar results were obtained in two additional independent experiments.

MAPK pathway is involved in the phosphorylation of HIF-1α, while blockade of this pathway results in smaller forms of HIF-1α [7]. Next, corresponding nuclear extracts from hypoxic HepG2 cultures were subjected to EMSA (Fig. 1B). The HIF-1 DNA complex was dose-dependently lowered on LY 294002 treatment, while PD 98059 exerted a smaller effect in hypoxic HepG2 cells. To test the functional relevance of the lowered HIF-1α protein levels and HIF-1 DNA-binding, reporter gene assays were performed using the stably transfected HepG2 subline HRG1. LY 294002 (≥10 μM) produced a significant decrease in relative luciferase activity when compared to hypoxic control cells ($P < 0.05$, Dunnett's test), indicating that the PI3K pathway is involved in hypoxia-induced HIF-1 activation (Fig. 1C). These findings are in line with previous observations made on Ha-*ras*-transformed NIH3T3R cells [12] and on human prostate cancer cell cultures [16].

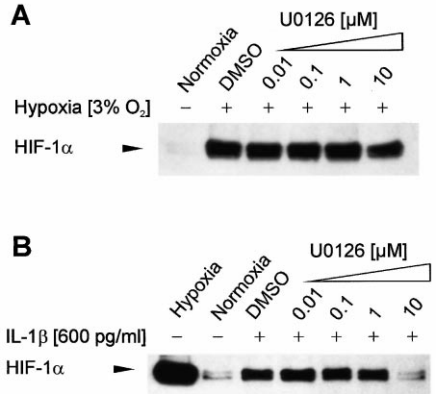

Fig. 2. A: Western blot analysis of HIF-1α in nuclear extracts of normoxic and of hypoxic HepG2 cells treated with U0126 for 4 h. B: Western blot analysis of HIF-1α in nuclear extracts of hypoxic and normoxic HepG2 cells, and of normoxic cells treated with IL-1β and U0126 for 4 h.

To view proteins encoded by HIF-1 target genes, effects of kinase inhibitors were studied on the 24 h rates of the production of EPO and VEGF. In hypoxic control cultures, the production of EPO amounted to 86 mU/mg cell protein (± 26; mean ± S.D., $n = 5$) and of VEGF to 39.0 ng/mg cell protein (± 2.0; mean ± S.D., $n = 4$). These values were significantly reduced to 3 mU EPO/mg (± 4) and 31.6 ng VEGF/mg (± 1.6) in LY 294002 (50 μM)-treated cells. Upon PD 98059 (40 μM) treatment, EPO synthesis was unaltered (102 mU/mg, ± 15), while that of VEGF was lowered to 22.8 ng/mg (± 0.5). To the best of our knowledge, effects of the kinase inhibitors on EPO synthesis have not been reported previously. In contrast, the inhibition of the production of VEGF observed herein is in line with measurements of VEGF expression at the mRNA and/or protein level in NIH3T3R cells [12], human prostate TSU cancer cells [16] and human breast cancer cells [33] following LY 294002 treatment, and of VEGF mRNA in hamster CCL 39 fibroblasts following PD 98059 treatment [6]. In the human glioblastoma cell line U87 MG PI3K inhibition by wortmannin lowers hypoxia-induced VEGF mRNA expression, while PD 98059 does not [34].

Taken together, our findings support the concept that HIF-1α expression, HIF-1 DNA-binding and induction of HIF-1-controlled proteins (EPO and VEGF) in response to hypoxia depend on a functioning PI3K signaling pathway in HepG2 cells. Regarding the effects of the MAPKK inhibitor PD 98059 a clear dose-dependency could not be established. Here, it should be noted that few studies have considered

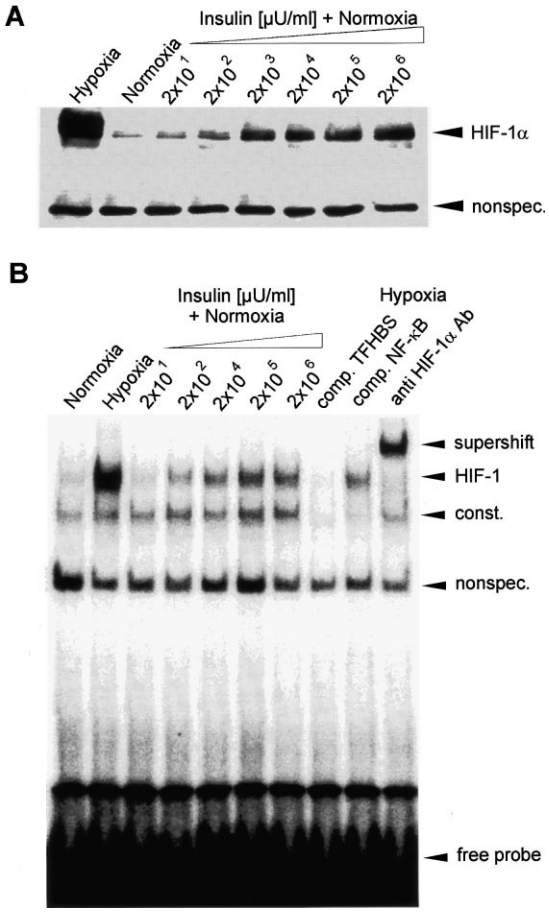

Fig. 3. A: Western blot analysis of HIF-1α in nuclear extracts of hypoxic, normoxic, and normoxic HepG2 cells treated with insulin for 4 h. B: Gel shift analysis with nuclear extracts of similarly treated cells (for abbreviations, see Fig. 1). Specificity was confirmed in competition experiments with a 250-fold molar excess of unlabeled annealed *TfHBS* oligonucleotide, the unspecific NF-κB oligonucleotide and in a supershift experiment with anti-HIF-1α antibodies (Ab) with nuclear extracts from hypoxic HepG2 cells.

the potential negative effects of the kinase inhibitors on cell viability. Based on MTT assay, LY 294002 was not toxic even at the highest concentration tested (100 μM). In contrast, PD 98059 reduced formazan formation significantly at 100 μM (by 11 ± 9%, $n = 8$) during 6 h incubation periods, and at 40 μM (by 14 ± 3%, $n = 8$) and 100 μM (by 16 ± 5%, $n = 8$) during 24 h of incubation ($P < 0.05$, Dunnett's test). U0126 reduced formazan formation significantly at 3 μM (by 10 ± 3%, $n = 8$) during 6 h of incubation and at 1 μM (by 9 ± 4%, $n = 8$) during 24 h of incubation ($P < 0.05$, Dunnett's test). Interestingly, a recent study has shown that the addition of PD 98059 causes marked DNA fragmentation and blocks the anti-apoptotic effects of hypoxia and VEGF in serum-deprived HepG2 cultures [35]. Furthermore, based on studies with the human breast carcinoma cell line MCF-7 a model has been proposed suggesting that dephosphorylated HIF-1α may activate the p53-dependent apoptotic pathway [36].

The addition of insulin (dose range 20 μU/ml to 2 U/ml) to normoxic HepG2 cultures resulted in a dose-dependent increase in nuclear HIF-1α levels as determined by Western blot analysis (Fig. 3A). In addition, insulin produced an increase in HIF-1 DNA-binding (Fig. 3B), which confirms findings by Zelzer et al. [18]. Furthermore, the production of VEGF was measured to increase on insulin treatment (Fig. 4). Others have shown that insulin increases VEGF mRNA levels in chicken embryo fibroblasts and chorioallantoic membrane-derived endothelial cells [15]. EPO production was unaltered during the 6 h period of normoxic incubation. The lack of stimulation of EPO production of normoxic HepG2 cells by insulin is in line with earlier studies [37]. Apparently, an increase in the formation of HIF-1 DNA complex alone does not suffice to stimulate EPO gene expression in normoxic human hepatoma cells.

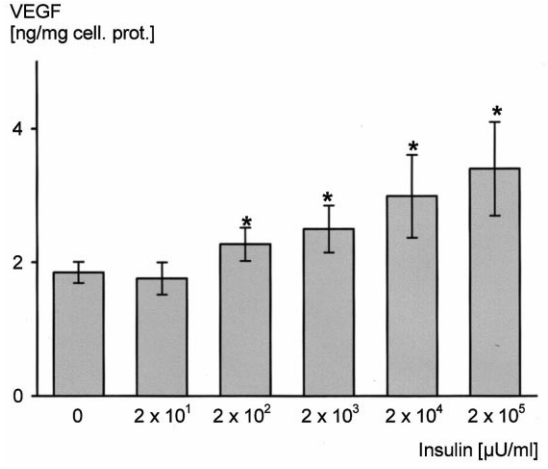

Fig. 4. Effects of insulin treatment on the production of immunoreactive VEGF by normoxic HepG2 cultures during 6 h incubation periods (mean ± S.D. of four parallel cultures).

Activation of PI3K and Akt are early events in insulin signaling. Indeed, LY 294002 strongly suppressed HIF-1α expression in insulin-treated normoxic HepG2 cultures, while PD 98059 was much less effective (Fig. 5). Similar to the action in hypoxic HepG2 cultures, inhibition of PI3K signaling by LY 294002 completely blocked EPO production in normoxic HepG2 cells treated with insulin (Table 1). PD 98059 moderately reduced EPO production. However, like in hypoxic HepG2 cultures, PD 98059 was more potent than LY 294002 in lowering VEGF synthesis in insulin-treated HepG2 cells (Table 1). Previous studies have shown that LY 294002 inhibits insulin-induced VEGF mRNA expression in chicken chorioallantoic endothelial cells but not in chicken embryo fibroblasts [15]. Wortmannin prevents the effect of insulin in NIH3T3 fibroblasts while PD 98059 is not inhibiting in this model [38]. Apparently, there is tissue-specificity in the potential of the kinase inhibitors to modulate

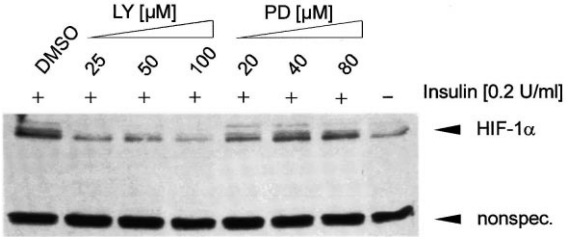

Fig. 5. Western blot analysis of HIF-1α in nuclear extracts of normoxic HepG2 cultures treated with insulin (0.2 U/ml) in the absence (DMSO) or presence of LY 294002 or PD 98059 (4 h incubation). A control without insulin is also shown.

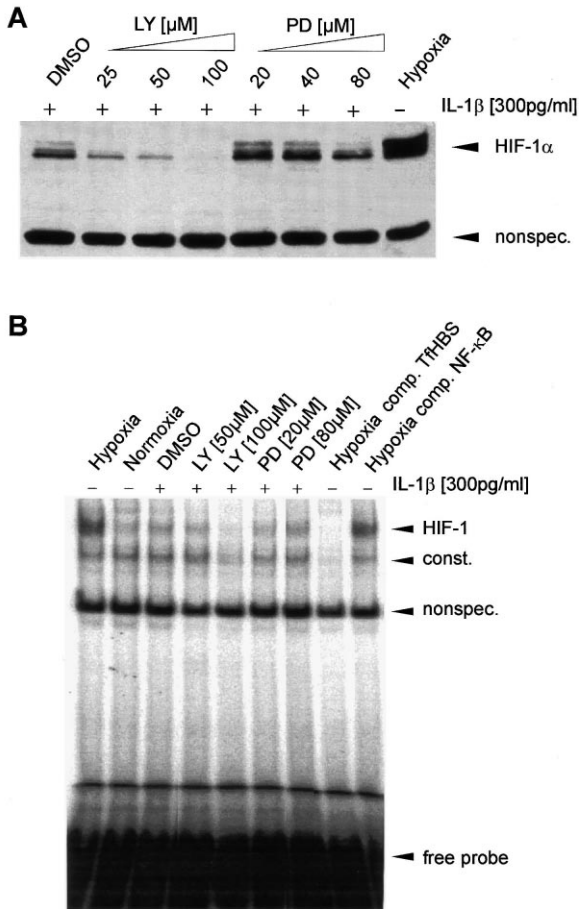

Fig. 6. A: Western blot analysis of HIF-1α in nuclear extracts of normoxic HepG2 cultures treated with IL-1β in the absence (DMSO) or presence of LY 294002 or PD 98059 (4 h incubation). For comparison, the effect of hypoxia exposure (3% O_2) is shown. B: Gel shift analysis with nuclear extracts of similarly treated cells (for abbreviations, see Fig. 1).

insulin-induced VEGF mRNA expression and VEGF synthesis.

In contrast to hypoxia or insulin, IL-1 does not significantly stimulate VEGF production in HepG2 cultures, although this cytokine increases HIF-1 DNA-binding [20]. Moreover, IL-1 lowers EPO gene expression in hypoxic HepG2 cells [39]. The present studies show that IL-1β-induced HIF-1α accumulation in nuclei of normoxic HepG2 cells was dose-dependently blocked by LY 294002, while PD 98059 (Fig. 6A) or U0126 (Fig. 2B) were not effective in non-toxic concentrations. In addition, LY 294002 suppressed the formation of the HIF-1 DNA complex as studied by EMSA (Fig. 6B). Effects of kinase inhibitors on IL-1-induced HIF-1 activation have not been studied previously. The biological meaning of the effect of IL-1 on HIF-1 DNA-binding needs to be further investigated, although it has been implicated in VEGF production in human proximal tubular cells [21] and synovial fibroblasts [22].

Table 1
Effects of LY 294002 and PD 98059 on EPO and VEGF production in normoxic HepG2 cultures

Treatment	EPO (mU/mg cell protein)	VEGF (ng/mg cell protein)
Insulin (0.2 U/ml)	10.8 ± 2.8	2.5 ± 0.1
Insulin+LY (50 μM)	n.d. (< 1.0)*	2.8 ± 0.8
Insulin+PD (40 μM)	5.4 ± 2.0*	1.6 ± 0.3*
None	7.9 ± 3.6	1.5 ± 0.6*

Mean ± S.D. of six parallel cultures incubated for 6 h (n.d.: not detectable).
*$P < 0.05$ vs. cultures treated with insulin alone (Tukey–Kramer multiple comparisons test).

In conclusion, the present findings show that the PI3K/Akt signaling pathway is essential in the accumulation of HIF-1α and HIF-1 DNA complex in response to hypoxia, insulin and IL-1 in HepG2 cells. The role of MAPKs in this process is still in question. Regarding the normoxic activation of HIF-1 by insulin and IL-1 it seems worthy to follow the recent concept that the PI3K/Akt pathway is important for HIF-1α stabilization, while MAPKs increase the transactivation activity of HIF-1α [5,40]. Also, our studies do not preclude the existence of other pathways of activation of HIF-1, as has been shown for angiotensin II, which is thought to increase HIF-1α levels via the formation of reactive O_2 species, ROS [23]. Studies utilizing rat lung epithelial cells indicate that the activation of HIF-1 by TNFα is also mediated by ROS [41]. However, the rates of transcription of the VEGF and EPO genes are controlled by transacting factors other than HIF-1. Like many other cell lines [42], normoxic HepG2 cells exhibit a high level of VEGF mRNA [20] that is only little increased by hypoxia. Accordingly, LY 294002 did not inhibit VEGF synthesis in normoxic HepG2 cells and exerted only a moderate inhibition in hypoxic cultures, despite the decrease in HIF-1 DNA-binding. In contrast, EPO synthesis seems to be more strictly HIF-1-dependent. EPO production was almost completely suppressed on LY 294002 treatment along with the loss of nuclear HIF-1α accumulation, HIF-1 DNA-binding and reporter gene activity.

Acknowledgements: These studies were supported by the Deutsche Forschungsgemeinschaft (DFG, SFB 367). Thanks are due to Tanja Svensson for excellent technical assistance.

References

[1] Semenza, G.L. (2001) Cell 107, 1–3.
[2] Wenger, R.H. and Gassmann, M. (1999) in: Environmental Stress and Gene Regulation (Storey, K.B., Ed), pp. 25–45, BIOS Scientific Publishers, Oxford.
[3] Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, A., Hebestreit, H.F., Mukherji, M., Schofield, C.J., Maxwell, P.H., Pugh, C.W. and Ratcliffe, P.J. (2001) Science 292, 468–472.
[4] Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J.M., Lane, W.S. and Kaelin Jr., W.G. (2001) Science 292, 464–468.
[5] Sodhi, A., Montaner, S., Miyazaki, H. and Gutkind, J.S. (2001) Biochem. Biophys. Res. Commun. 287, 292–300.
[6] Milanini, J., Vinals, F., Pouyssegur, J. and Pages, G. (1998) J. Biol. Chem. 273, 18165–18172.
[7] Richard, D.E., Berra, E., Gothie, E., Roux, D. and Pouyssegur, J. (1999) J. Biol. Chem. 274, 32631–32637.
[8] Sodhi, A., Montaner, S., Patel, V., Zohar, M., Bais, C., Mesri, E.A. and Gutkind, J.S. (2000) Cancer Res. 60, 4873–4880.
[9] Berra, E., Milanini, J., Richard, D.E., Le Gall, M., Vinals, F., Gothie, E., Roux, D., Pages, G. and Pouyssegur, J. (2000) Biochem. Pharmacol. 60, 1171–1178.
[10] Minet, E., Arnould, T., Michel, G., Roland, I., Mottet, D., Raes, M., Remacle, J. and Michiels, C. (2000) FEBS Lett. 468, 53–58.
[11] Hur, E., Chang, K.Y., Lee, E., Lee, S.-K. and Park, H. (2001) Mol. Pharmacol. 59, 1216–1224.

[12] Mazure, N.M., Chen, E.Y., Laderoute, K.R. and Giaccia, A.J. (1997) Blood 90, 3322–3331.
[13] Alvarez-Tejado, M., Naranjo-Suarez, S., Jimenez, C., Carrera, A.C., Landazuri, M.O. and del Peso, L. (2001) J. Biol. Chem. 276, 22368–22374.
[14] Chen, E.Y., Mazure, N.M., Cooper, J.A. and Giaccia, A.J. (2001) Cancer Res. 61, 2429–2433.
[15] Jiang, B.H., Zheng, J.Z., Aoki, M. and Vogt, P.K. (2000) Proc. Natl. Acad. Sci. USA 97, 1749–1753.
[16] Zhong, H., Chiles, K., Feldser, D., Laughner, E., Hanrahan, C., Georgescu, M.M., Simons, J.W. and Semenza, G.L. (2000) Cancer Res. 60, 1541–1545.
[17] Zundel, W., Schindler, C., Haas, K.D., Koong, A., Kaper, F., Chen, E., Gottschalk, A.R., Ryan, H.E., Johnson, R.S., Jefferson, A.B., Stokoe, D. and Giaccia, A.J. (2000) Genes Dev. 14, 391–396.
[18] Zelzer, E., Levy, Y., Kahana, C., Shilo, B.Z., Rubinstein, M. and Cohen, B. (1998) EMBO J. 17, 5085–5094.
[19] Feldser, D., Agani, F., Iyer, N.V., Pak, B., Ferreira, G. and Semenza, G.L. (1999) Cancer Res. 59, 3912–3918.
[20] Hellwig-Bürgel, T., Rutkowski, K., Metzen, E., Fandrey, J. and Jelkmann, W. (1999) Blood 94, 1561–1567.
[21] El Awad, B., Kreft, B., Wolber, E.-M., Hellwig-Bürgel, T., Metzen, E., Fandrey, J. and Jelkmann, W. (2000) Kidney Int. 58, 43–50.
[22] Thornthon, R.D., Lane, P., Borghaei, R.C., Pease, E.A., Caro, J. and Mochan, E. (2000) Biochem. J. 350, 307–312.
[23] Richard, D.E., Berra, E. and Pouyssegur, J. (2000) J. Biol. Chem. 275, 26765–26771.
[24] Görlach, A., Diebold, I., Schini-Kerth, V.B., Berchner-Pfannschmidt, U., Roth, U., Brandes, R.P., Kietzmann, T. and Busse, R. (2001) Circ. Res. 89, 47–54.
[25] Jiang, B.H., Jiang, G., Zheng, J.Z., Lu, Z., Hunter, T. and Vogt, P.K. (2001) Cell Growth Differ. 12, 363–369.
[26] Goldberg, M.A., Glass, G.A., Cunningham, J.M. and Bunn, H.F. (1987) Proc. Natl. Acad. Sci. USA 84, 7972–7976.
[27] Alessi, D.R., Cuenda, A., Cohen, P., Dudley, D.T. and Saltiel, A.R. (1995) J. Biol. Chem. 270, 27489–27494.
[28] Favata, M.F., Horiuchi, K.Y., Manos, E.J., Daulerio, A.J., Stradley, D.A., Fesser, W.S., Van Dyk, D.E., Pitts, W.J., Earl, R.A., Hobbs, F., Copeland, R.A., Magolda, R.L., Scherle, P.A. and Trzaskos, J.M. (2001) J. Biol. Chem. 273, 18623–18632.
[29] Vlahos, C.J., Matter, W.F., Hui, K.Y. and Brown, R.F. (1994) J. Biol. Chem. 269, 5241–5248.
[30] Wolff, M. and Jelkmann, W. (1993) Ann. Hematol. 66, 27–31.
[31] Rolfs, A., Kvietikova, I., Gassmann, M. and Wenger, R.H. (1997) J. Biol. Chem. 272, 20055–20062.
[32] Wanner, R.M., Spielmann, P., Stroka, D.M., Camenisch, G., Camenisch, I., Scheid, A., Houck, D.R., Bauer, C., Gassmann, M. and Wenger, R.H. (2000) Blood 96, 1558–1565.
[33] Blancher, C., Moore, J.W., Robertson, N. and Harris, A.L. (2001) Cancer Res. 61, 7349–7355.
[34] Maity, A., Pore, N., Lee, J., Solomon, D. and O'Rourke, D.M. (2000) Cancer Res. 60, 5879–5886.
[35] Baek, J.H., Jang, J.E., Kang, C.M., Chung, H.Y., Kim, N.D. and Kim, K.W. (2001) Oncogene 19, 4621–4631.
[36] Suzuki, H., Tomida, A. and Tsuruo, T. (2001) Oncogene 20, 5779–5788.
[37] Scholz, H., Baier, W., Ratcliffe, P., Eckardt, K., Zapf, J., Kurtz, A. and Bauer, C. (1992) Am. J. Physiol. 263, C474–C479.
[38] Miele, C., Rochford, J.J., Filippa, N., Giorgetti-Peraldi, S. and Van Obberghen, E. (2000) J. Biol. Chem. 275, 21695–21702.
[39] Fandrey, J., Huwiler, A., Frede, S., Pfeilschifter, J. and Jelkmann, W. (1994) Eur. J. Biochem. 226, 335–340.
[40] Minet, E., Michel, G., Mottet, D., Raes, M. and Michiels, C. (2001) Free Radic. Biol. Med. 31, 847–855.
[41] Haddad, J.J. and Land, S.C. (2001) FEBS Lett. 505, 269–274.
[42] White, F.C., Carroll, S.M. and Kamps, M.P. (1995) Growth Factors 12, 289–301.