Regulation of Hypoxia-inducible Factor-1α Protein Level during Hypoxic Conditions by the Phosphatidylinositol 3-Kinase/Akt/Glycogen Synthase Kinase 3β Pathway in HepG2 Cells*

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Hypoxia initiates an intracellular signaling pathway leading to the activation of the transcription factor hypoxia-inducible factor-1 (HIF-1). HIF-1 activity is regulated through different mechanisms involving stabilization of HIF-1α, phosphorylations, modifications of redox conditions, and interactions with coactivators. However, it appears that some of these steps can be cell type-specific. Among them, the involvement of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in the regulation of HIF-1 by hypoxia remains controversial. Here, we investigated the activation state of PI3K/Akt/glycogen synthase kinase 3β (GSK3β) in HepG2 cells. Increasing incubation times in hypoxia dramatically decreased both the phosphorylation of Akt and the inhibiting phosphorylation of GSK3β. The PI3K/Akt pathway was necessary for HIF-1α stabilization early during hypoxia. Indeed, its inhibition was sufficient to decrease HIF-1α protein level after 5-h incubation in hypoxia. However, longer exposure (16 h) in hypoxia resulted in a decreased HIF-1α protein level compared with early exposure (5 h). At that time, Akt was no longer present or active, which resulted in a decrease in the inhibiting phosphorylation of GSK3β on Ser-9 and hence in an increased GSK3β activity. GSK3 inhibition reverted the effect of prolonged hypoxia on HIF-1α protein level; more stabilized HIF-1α was observed as well as increased HIF-1 transcriptional activity. Thus, a prolonged hypoxia activates GSK3β, which results in decreased HIF-1α accumulation. In conclusion, hypoxia induced a biphasic effect on HIF-1α stabilization with accumulation in early hypoxia, which depends on an active PI3K/Akt pathway and an inactive GSK3β, whereas prolonged hypoxia results in the inactivation of Akt and activation of GSK3β, which then down-regulates the HIF-1 activity through down-regulation of HIF-1α accumulation.

Mammalian cells require a constant supply of oxygen to maintain adequate energy production, which is essential for maintaining normal function and for ensuring cell survival. The cellular response to decreased oxygen levels is regulated by a hypoxia-inducible factor-1 (HIF-1)[1] heterodimeric complex composed of two subunits, HIF-1α and arylhydrocarbon receptor nuclear translocator (1).

This transcription factor binds to conserved regulatory sequences known as hypoxia-responsive element (HRE) found in the promoter of several target genes such as vascular endothelial growth factor (VEGF), erythropoietin, or glycolytic enzymes (aldolase A, enolase-α, etc.) and controls their expression in response to hypoxia, leading to the adaptation of cells to decreased oxygen level (2).

Only the HIF-1α subunit is regulated by a reduced oxygen level, and the regulation occurs in large part at post-translational modifications, resulting in its stabilization, nuclear translocation, DNA binding activity, and proper transcriptional activity. Under normal conditions, HIF-1α is hydroxylated at the prolines 564 and 462 residues in the oxygen-dependent degradation domain and, hence, interacts with an E3 ubiquitin-ligase complex containing the pVHL protein (von Hippel Lindau protein) (3–5) and other components, leading to the ubiquitinylation and degradation of HIF-1α protein by proteasome 26 S (6). This process is regulated by an O_2-sensitive enzyme called HIF-α prolyl-hydroxylase (7, 8).

Upon hypoxia, stabilized HIF-1α subunit translocates into the nucleus, heterodimerizes with arylhydrocarbon receptor nuclear translocator, and is phosphorylated and reduced, respectively, by mitogen-activated protein kinase kinase (9) and by the redox factors thioredoxin and Ref-1 (10).

The mechanism of HIF-1α degradation is becoming more and more clear, but, in addition to the proline hydroxylation of HIF-1α, other regulatory pathways have been reported to be important for the control of HIF-1α protein level by hypoxia such as the PI3K/Akt pathway (11–15). However, the involvement of these kinases in the regulation of HIF-1 activity remains controversial and seems to be cell type-specific, whereas their activation is not always observed in hypoxia.

Many stimuli are able to activate Akt, and the common mechanism by which Akt is regulated is known. In general, phosphatidylinositol 3,4,5-P_3 targets Akt in the inner leaflet of the plasma membrane. Akt is phosphorylated on Ser-473 (in the regulatory domain) and Thr-308 (in the catalytic domain) by phosphoinositide-dependent protein kinase, an upstream kinase, resulting in enzymatic activation of Akt. Activated Akt

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[1] The abbreviations used are: HIF-1, hypoxia-inducible factor-1; HRE, hypoxia-responsive element; VEGF, vascular endothelial growth factor; E3, ubiquitin-protein isopeptide ligase; PI3K, phosphatidylinositol 3-kinase; GSK3β, glycogen synthase kinase 3β; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

Regulation of HIF-1α Protein Level

can phosphorylate various substrates, and glycogen synthase kinase 3 (GSK3), a ubiquitous Ser/Thr kinase, is one of the well characterized downstream targets of this kinase (16–18).

Akt phosphorylates the two isoforms of GSK3 (α and β), respectively on Ser-21 and Ser-9; this results in an inhibition of its activity. On the other hand, GSK3 activity is up-regulated by Tyr-279/216 phosphorylation, but it is not clear whether a separate kinase regulates the phosphorylation state on this residue or whether this residue is an autophosphorylation site (19). GSK3 does not seem to be phosphorylated on both sites together.

GSK3 has been demonstrated to regulate glycogen synthase activity and was first identified as a negative regulator of glycogen synthesis (20). Now GSK3 is known to phosphorylate and regulate the activation of numerous transcription factors such as c-Myc (21), AP1 (22, 23), and NF-κB (24, 25). A recent study performed by Sodhi *et al.* (26) reported that there was a potential consensus site in the oxygen-dependent degradation domain of HIF-1α for GSK3β and that this site could play a role in the regulation of HIF-1α protein stability, but no more precise data are available.

In this context, the aim of this work was to determine the activation state of the PI3K/Akt/GSK3β pathway in hypoxia in human hepatoma cells (HepG2). The second goal of the study was to elucidate the role of the PI3K/Akt pathway and of the GSK3β kinase in the regulation of HIF-1α stability and/or HIF-1 activity.

The results presented here demonstrated that in HepG2 cells, the PI3K/Akt pathway is constitutively active in normoxia. This activation decreased during "early" hypoxia (5 h), but GSK3β is maintained inactive through phosphorylation of Ser-9. On the other hand, "prolonged" hypoxia (16 h) totally inactivated the PI3K/Akt pathway and hence promoted GSK3β activity. Activated GSK3β plays a role in the regulation of HIF-1α protein accumulation in hypoxia and hence seems to control the turnover of the HIF-1α protein in these conditions.

MATERIALS AND METHODS

HepG2 Cell Culture—Human Hepatoma cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal calf serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 50 ng/ml amphotericin B. Normoxia (21% O_2) and hypoxia (1% O_2) incubations were performed in serum-free CO_2-independent medium (Invitrogen) supplemented with 100 units/ml penicillin G, 100 μg/ml streptomycin, 50 ng/ml amphotericin B, and 10 mM glutamine.

Western Blot Analysis—Total cell extracts were prepared from HepG2 grown in T25 flasks at 90% confluence. Cell medium was replaced by CO_2-independent medium, and cells were exposed to 20% O_2 or 1% O_2 for 5 or 16 h with or without inhibitors, LY294002 (Calbiochem) at 50 μM or LiCl (Sigma) at 20 or 50 mM. After the incubation, the cells were lysed using lysis buffer (20 mM Tris, pH 7.5 (Merck), 150 mM KCl (Merck), 1 mM EDTA, 1% Triton X-100 (Janssen Chemica), protease inhibitor (Complete®; Roche Applied Science), and phosphatase inhibitor mixture). The lysate was centrifuged for 5 min at 15,000 rpm at 4 °C, and the supernatant was kept frozen. Extracts were separated on a 10% SDS-PAGE and then transferred to a polyvinylidene difluoride membrane (Amersham Biosciences). After adequate blocking, the blot was probed with anti-HIF-1α antibodies (Transduction Laboratories; diluted 1:1000; secondary antibody was anti-mouse (Amersham Biosciences) diluted 1:2000), anti-Akt antibodies (Cell Signaling; diluted 1:1000; secondary antibody was anti-rabbit (Amersham Biosciences), diluted 1:2000)), anti-GSK3β antibodies (Transduction Laboratories; diluted 1:1000; secondary antibody was anti-mouse (Amersham Biosciences), diluted 1:2000)), anti-phospho-Ser-21/9 GSK3α/β (Transduction Laboratories; diluted 1:1000; secondary antibody was anti-rabbit (Amersham Biosciences), diluted 1:2000)), and anti-phospho-Tyr-279/216 GSK3α/β (BIOSOURCE; diluted 1:1000; secondary antibody was anti-rabbit (Amersham Biosciences), diluted 1:2000)). Anti-α-tubulin antibodies (Innogenex; diluted 1:2000; secondary antibody was anti-mouse (Amersham Biosciences), diluted 1:2000) were used to probe tubulin as a control for the total amount of proteins loaded on the gel. Chemiluminescent detection was performed using

horseradish peroxidase conjugated to the secondary antibody.

Transfection Experiments and Reporter Gene Assay—To assay the transcriptional activity of HIF-1, the pGL3-SV40/6HRE reporter vector containing an artificial promoter with TATA box and six copies of the erythropoietin HRE cis-element upstream of the firefly luciferase gene was used. HepG2 transfection was performed in a 24-well plate with DOTAP transfection reagent (Roche Molecular Biochemicals) and 200 ng of PGL3-SV40/6HRE. This vector was co-transfected with 100 ng of the control vector pRL-SV40 (Promega, Madison, WI) and with or without 1 μg of expression vector. Regarding the dominant negative mutant, a vector expressing a kinase-inactive mutant of GSK3 was used (27). pCMV-Myc was used as the empty vector. 24 h post-transfection, the medium was replaced by CO_2-independent medium (Invitrogen) without serum and with or without inhibitor, and cells were incubated for 16 h in normoxia or hypoxia. After the incubation, the luciferase activity was measured. Luciferase activity was quantitated in a luminometer using the Dual Luciferase Reporter System (Promega). Experiments were performed in triplicate. Results are expressed as means of the ratio between firefly luciferase activity and *Renilla* luciferase activity.

Immunofluorescence—HepG2 cells grown on a glass coverslip were incubated in normoxia or hypoxia with or without inhibitor. After the incubation, the medium was removed, and cells were fixed 10 min with PBS containing 4% paraformaldehyde (Merck). The fixed cells were washed three times with PBS and permeabilized or not with a solution of PBS-Triton X-100 1% (Sigma). After three washing steps with PBS plus 3% bovine serum albumin (Sigma), cells were incubated at 4 °C overnight with anti-HIF-1α antibodies (diluted 1:100; Transduction Laboratories), with anti-Akt antibodies (diluted 1:100; Transduction Laboratories), with anti-phospho-Ser 473-Akt antibodies (diluted 1:50; Cell Signaling), with anti-phospho-Tyr-279/216 GSK3α/β (diluted 1:100; BIOSOURCE), or with anti-Glut-1 antibodies (diluted 1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Then cells were washed three times with PBS plus 3% bovine serum albumin, and the secondary antibodies conjugated to Alexa fluorochrome (488) (diluted 1:500; Molecular Probes, Inc., Eugene, OR) were added for 1 h. The cells were then washed three times with PBS plus bovine serum albumin 3%. For nucleus labeling, the cells were incubated with Topro-3 (diluted 1:80; Molecular Probes). Finally, the cells were mounted in mowiol. Semiquantitative observations were performed with a constant photomultiplier value using a confocal microscope (Leica).

VEGF Assay—VEGF secreted in the incubation medium was assayed by an ELISA (Quantikine from R & D Systems) according to the procedure provided by the supplier. Results are expressed in ng of VEGF reported to μg of proteins assayed by the Folin method.

Akt and Phospho-Ser 473 Akt Assay—Akt and phospho-Ser 473 Akt present in the cell extract were assayed by an ELISA (BIOSOURCE) according to the procedure provided by the supplier. Results are expressed in ng of Akt reported to mg of proteins assayed by the Folin method for the total of Akt and as the ratio of phospho-Akt to Akt to estimate the proportion of the phosphorylated form.

GSK3 in Vitro Kinase Assay—Cells were collected by cell scraping into a lysis buffer (10 mM Tris, pH 7.5 (Merck), 150 mM NaCl (Merck), 1 mM EDTA, 1 mM EGTA, 1% Triton X-100 (Janssen Chemica), protease inhibitor (Complete®; Roche Applied Science), and phosphatase inhibitor mixture). Whole cell homogenates were centrifuged at 13,000 rpm for 15 min at 4 °C, and the supernatants were transferred to fresh microcentrifuge tubes. 2.5 μg of anti-GSK3β antibodies (Transduction Laboratories) were then added and incubated for 1 h at 4 °C, and GSK3 was immunoprecipitated by adding 60 μl of protein A/protein G-coated Sepharose beads (Oncogene). The immunoprecipitated samples were then incubated with 20 μM cAMP-response element-binding protein synthetic phosphopeptide (Sigma) in 70 μl of reaction mixture (20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 20 μM ATP, 20 μCi of [γ-^{32}P]ATP (PerkinElmer Life Sciences)) for 15 min at 30 °C. The reaction mixture was then loaded on phosphocellulose membrane Spinzyme columns (Pierce). The columns were rinsed twice with 75 mM phosphoric acid. The radioactivity associated with the columns was then measured. Results are presented in counts/min.

RESULTS

Hypoxia Incubation of HepG2 Cells Resulted in the Inactivation of Akt—Several reports showed that hypoxia incubation induces the activation of the PI3K/Akt pathway in some cell lines. To investigate whether hypoxia-induced PI3K activation would result in the stimulation of the downstream Akt, HepG2 cells were exposed to hypoxia for 5 or 16 h. Active phosphoryl-
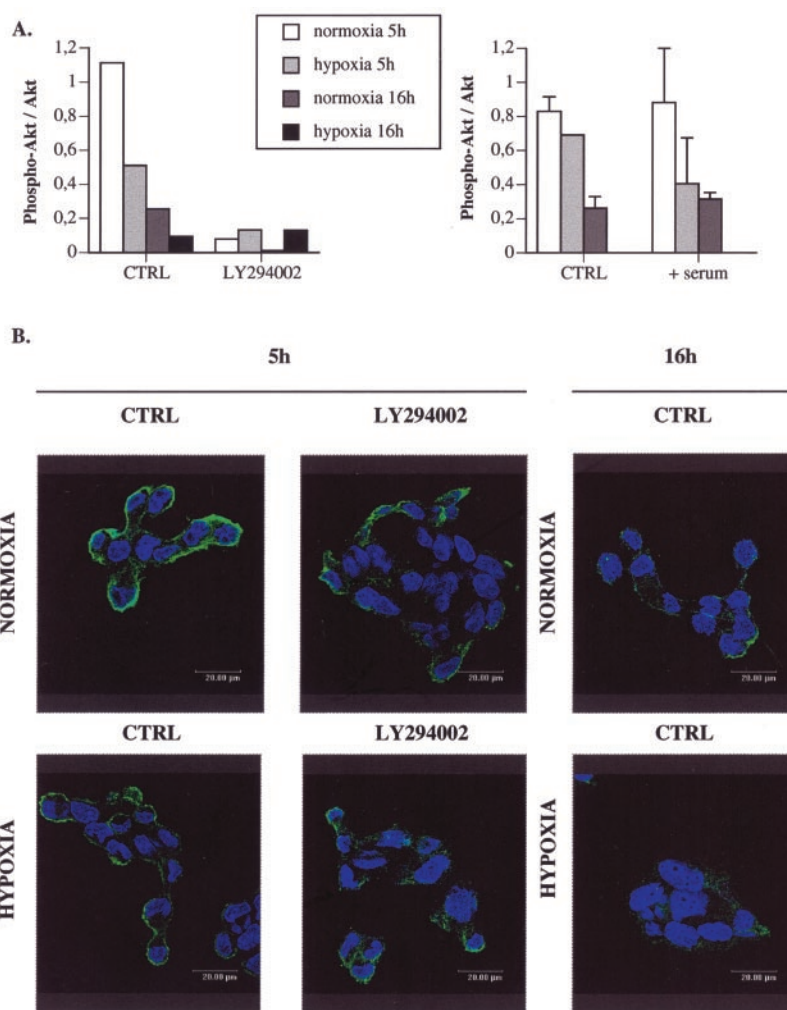
FIG. 1. **Effect of hypoxia incubation and PI3K inhibition on Akt phosphorylation.** HepG2 cells were incubated for 5 or 16 h in either normoxia or hypoxia in the presence or in the absence of 10% serum or LY294002 at 50 μM. *A*, after the incubation, the cells were lysed to determine the level of Akt phosphorylation by ELISA. Results are expressed in the ratio of phospho-Akt to Akt to estimate the proportion of the phosphorylated form as means for duplicates (*left*) or means ± S.D. for triplicates (*right*). *B*, cells were fixed and immunolabeled for phospho-Ser-473 Akt. The nuclei were stained with Topro-3, and cells were observed in semiquantitative confocal microscopy.

ated Akt was quantitated by a specific ELISA as well as visualized by immunofluorescence studies in confocal microscopy. An incubation of 5 h in hypoxia induced a 55% decrease in the amount of phospho-Ser-473 Akt as assayed by ELISA. This effect was even stronger (90% inhibition) after 16-h exposure in hypoxia (Fig. 1*A*). It has been noted that the level of phospho-Akt also decreased in normoxia but to a much lower extent. These effects were independent of the presence or absence of serum (Fig. 1*A*, *right panel*). This effect was probably due to the shift from Dulbecco's modified Eagle's medium to CO_2-independent medium. Results obtained in immunofluorescence studies also showed a decreased in phospho-Akt under hypoxic conditions, which was more marked at 16 h than at 5 h (Fig. 1*B*). These results suggest that increasing incubation times in hypoxia induced a decrease in the activity of Akt.

In order to confirm the role of PI3K in the activation of Akt, we used LY294002, a specific PI3K inhibitor, in normoxia and in hypoxia. The diminished phosphorylation of Akt in both conditions suggested that PI3K was an important component of Akt activation (Fig. 1, *A* and *B*).

To verify that the effect of hypoxia incubation and of LY294002 treatment on Akt phosphorylation was not due to a change in the total amount of Akt protein, an ELISA for total Akt, Western blot for anti-Akt, and immunofluorescence stainings were performed. A 5-h incubation time in the presence or the absence of LY294002 in normoxia or in hypoxia did not alter the level of Akt protein (Fig. 2, *A* and *D*). However, 16-h hypoxia incubation caused an important decrease in Akt expression (Fig. 2, *A–C*), and PI3K inhibition did not influence

this decrease (Fig. 2*D*). This decrease was independent of the absence or presence of serum (Fig. 2, *A*, *right panel*, and *B*). Altogether, these results indicate that early hypoxia decreases the activity of Akt, whereas longer incubation times also diminish the total amount of the protein.

Hypoxia Decreased an Inactivating Phosphorylation of GSK3β—We then determined whether the phosphorylation state of GSK3β, a downstream target of Akt, was following the changes in Akt activity induced by hypoxia. With a kinetics similar to that of Akt activity, 16-h hypoxia incubation caused a marked decrease in the phosphorylation of GSK3β on Ser-9, whereas 5 h had a smaller effect (Fig. 3*A*). PI3K inhibition by LY294002 also induced a decrease in the phosphorylation of GSK3β on Ser-9. The effect of this inhibitor occurred only when cells were incubated for 5 h, probably because there remained very few active Akt at 16 h (Fig. 3, *B* and *C*). Moreover, by using an anti-phospho-Tyr[216] GSK3β antibody, Western blot analysis revealed that the activated form of GSK3β was present in all conditions in the same amount. These results indicate that long duration of hypoxia incubation abolished Akt activity and thus phosphorylation of GSK3β on Ser-9. The fact that both phospho-Ser-9-GSK3β and phospho-Tyr-216-GSK3β can be detected together in cell extracts indicates that there were at least two populations of different phosphorylated forms of GSK3β. A kinase assay for GSK3β activity was performed after immunoprecipitation; an increase of 90% in GSK3 activity was observed after 16-h incubation in hypoxia in comparison with normoxia (Fig. 3*C*). The fact that the Tyr-216-phosphorylated form of GSK3β was present alone only after 16-h incubation in
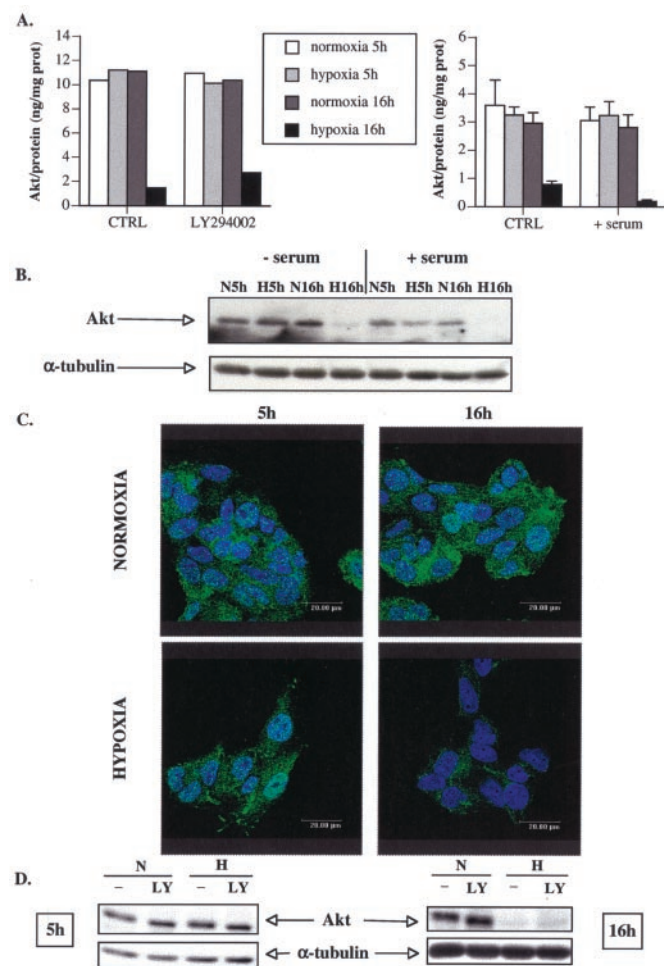
FIG. 2. **Effect of hypoxia incubation and PI3K inhibition on total Akt expression.** HepG2 cells were incubated for 5 or 16 h in either normoxia or hypoxia, in the presence or in the absence of 10% serum or LY294002 at 50 μM. *A*, after the incubation, the cells were lysed to determine the level of total Akt by ELISA. Results are expressed in ng of Akt reported to mg of proteins assayed by the Folin method as means for duplicates (*left*) or means ± S.D. for triplicates (*right*). *B* and *D*, cytosolic extracts were also prepared and resolved by SDS-PAGE. After blotting, Akt was revealed by Western analysis. *C*, cells were fixed and immunolabeled for total Akt. The nuclei were stained with Topro-3, and cells were observed in semiquantitative confocal microscopy.

hypoxia probably explains the increase in GSK3 activity observed at that time. In addition, immunofluorescence studies revealed that the phospho-Tyr-216-GSK3β translocated from the cytosol to the nucleus after a 16-h incubation in hypoxia, hence co-localizing with HIF-1α (Fig. 3*D*).

Effect of LiCl, a GSK3 Inhibitor, on Akt Activity and on the Expression of the Different GSK3β-phosphorylated Forms—The most common inhibitor used to inhibit GSK3β is the Li+ ion, but Chalecka-Franaszek and Chuang (28) described in their reports that lithium treatment can also activate Akt. Mora *et al.* (29) have explained this effect by the fact that lithium inhibits the activity of protein phosphatase-2A, a phosphatase that dephosphorylates Akt. The total level of the Akt protein (Fig. 4*B*) as well as the level its phosphorylated form (Fig. 4*A*) were not affected by lithium as measured by ELISA. Western analysis showed that lithium has no effect on Akt level in hypoxia (Fig. 4*C*).

To determine whether the effects of lithium on Akt activity can be correlated with modulation of the phosphorylation state of its target GSK3β, Western blot analysis was performed to reveal the different phosphorylation forms of GSK3β (Ser-9
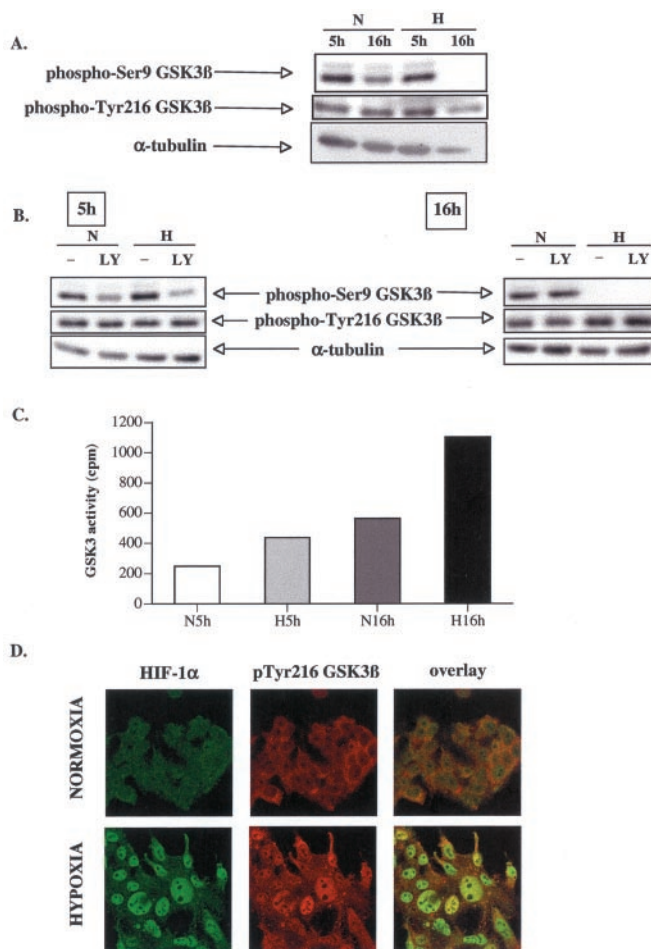

FIG. 3. **Effect of hypoxia incubation and PI3K inhibition on phospho-Ser-9 GSK3β and phospho-Tyr-216 GSK3β expression as well as on GSK3 activity.** HepG2 cells were incubated for 5 or 16 h in either normoxia or hypoxia, in the presence or in the absence of LY294002 at 50 μM. *A* and *B*, after the incubation, cytosolic extracts were prepared and resolved by SDS-PAGE. After corresponding blotting, phospho-Ser-9 GSK3β and phospho-Tyr-216 GSK3β forms were revealed by Western analysis. A Western blot for α-tubulin was performed to check for the total amount of proteins loaded on the gel. *C*, after the incubation, GSK3 was immunoprecipitated, and *in vitro* GSK3 activity was determined. Results are expressed in counts/min. *D*, after the incubation, the cells were fixed, immunolabeled for HIF-1α (*green*) and phospho-Tyr-216 GSK3β (*red*) and were observed in semiquantitative confocal microscopy.

and Tyr-216). Fig. 4, *C* and *D*, shows that lithium did not significantly increase GSK3β phosphorylation on Ser-9. The level of the phospho-Tyr-216 form of GSK3β was also not affected by this incubation.

Effect of PI3K/Akt and GSK3 Inhibition on HIF-1α Stability and HIF-1 Activity—The previous results show that increasing incubation times in hypoxia influenced the activity of Akt, reduced the expression level of the Ser-9-phosphorylated form of GSK3β, and increased GSK3 activity. In order to study whether these modifications affect HIF-1, cells were incubated for 5 or 16 h either in normoxia or in hypoxia, and the protein level of HIF-1α was examined, as the first step in the regulation of HIF-1 activity. Fig. 5 shows whereas both short and long hypoxic incubations led to increased HIF-1α level, there was less HIF-1α at 16-h than at 5-h hypoxia. These results were independent of the absence or presence of serum. The same results were obtained when human umbilical vein endothelial cells (HUVEC-C) were incubated for 5 and 16 h in hypoxia (data not shown). This was observed both in immunofluorescence and Western blot studies. Since after prolonged hypoxia
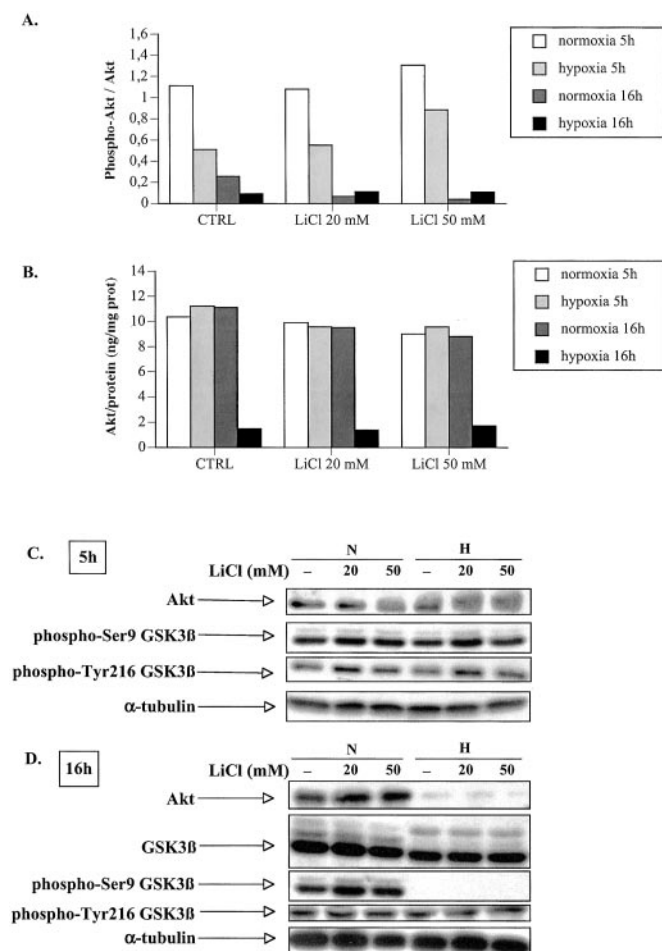
A.

B.

C. 5h

D. 16h

FIG. 4. **Effect of hypoxia incubation and GSK3 inhibition on total Akt expression, Akt phosphorylation, and phospho-Ser-9 GSK3β and phospho-Tyr-216 GSK3β expression.** HepG2 cells were incubated for 5 or 16 h in either normoxia or hypoxia, in the presence or in the absence of LiCl at 20 or 50 mM. *A* and *B*, after the incubation, the cells were lysed to determine the Akt expression and the level of Akt phosphorylation by ELISA. Results are expressed in ng of Akt reported to mg of proteins assayed by the Folin method for total Akt and as the ratio of phospho-Akt to Akt to estimate the proportion of the phosphorylated form as means for duplicates. *C* and *D*, cytosolic extracts were prepared and resolved by SDS-PAGE. After corresponding blotting, Akt, phospho-Ser-9 GSK3β, and phospho-Tyr-216 GSK3β forms were revealed by Western analysis. A Western blot for α-tubulin was performed to check for the total amount of proteins loaded on the gel.

A.

B.

FIG. 5. **Effect of hypoxia incubation on HIF-1α expression.** HepG2 cells were incubated for 5 or 16 h in either normoxia or hypoxia with or without 10% serum. *A*, after the incubation, the cells were fixed, immunolabeled for HIF-1α, and observed in semiquantitative confocal microscopy. *B*, cytosolic extracts were also prepared and resolved by SDS-PAGE. After blotting, HIF-1α was revealed by Western analysis. A Western blot for α-tubulin was performed to check for the total amount of proteins loaded on the gel.

(16 h), activated GSK3β was present in HepG2 cells, we investigated whether GSK3β would play a role in the regulation of HIF-1α protein level. To test this hypothesis, LiCl, the GSK3 inhibitor, was used, and its effects on HIF-1α level were determined. After 16-h incubation in hypoxia, Li⁺ ion induced an increase in HIF-1α protein level in a concentration-dependent manner (Fig. 6*B*). It has to be noted that HIF-1α stabilization in hypoxia was not affected when cells were incubated in the presence of LiCl for 5 h (Fig. 6*A*), which was consistent with the fact that GSK3 activity was lower, probably because the Ser-9 phosphorylated form was detected at this time. On the other hand, a 5-h incubation in the presence of LY294002 was able to inhibit HIF-1α protein accumulation in hypoxia when LY294002 relieved the Akt inhibition on GSK3β (Fig. 7*A*), but this effect was not observed after 16 h, when GSK3β was more active (Fig. 7*B*).

The results described above suggest that GSK3β activity seems to be an important way of regulating HIF-1α subunit accumulation during prolonged hypoxia. However, HIF-1 activation involved at least two mechanisms: regulation of HIF-1α
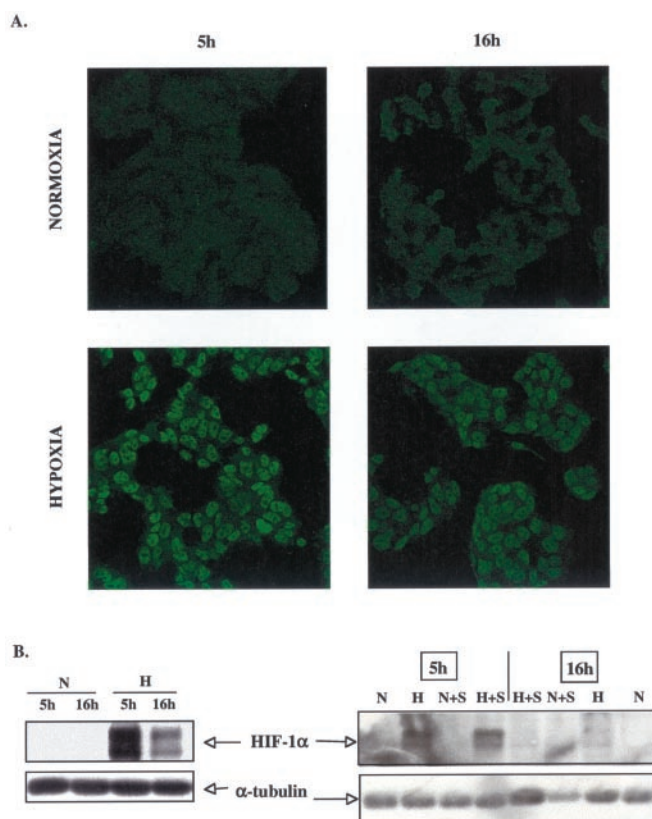
accumulation/degradation and regulation of the actual transcriptional activity. If the HIF-1α stabilization is very important to activate HIF-1, the accumulation of HIF-1α *per se* (*e.g.* through proteasome inhibitor in normoxia) is not always sufficient for inducing HIF-1 transcriptional activity.

In order to test whether GSK3β-induced regulation of HIF-1α stabilization would also regulate HIF-1 activity, we used a GSK3 dominant negative mutant (GSK3 −/−) in a reporter assay. Fig. 8*A* shows that the GSK3 dominant negative mutant increased luciferase activity in hypoxia. The effect of lithium was also tested, but it interfered with the expression of luciferase when using pGL3-SV40 so that the results could not properly be interpreted (data not shown). These results suggest that inhibition of GSK3 would result in an increased HIF-1 activity. HIF-1 transcriptional activity was also measured when the PI3K/Akt pathway was inhibited by LY294002. No modification of luciferase expression was detected after a 16-h incubation in normoxic or hypoxic cells treated with LY294002 (Fig. 8*B*).

Altogether, these results indicate that GSK3 inhibition increase HIF-1α accumulation and hence HIF-1 activation after 16-h hypoxia incubation.

Effect of PI3K/Akt/GSK3β Pathway Inhibitors on HIF-1 Target Gene Levels—HIF-1α subunit stabilization and activation of the HIF-1 transcription factor are two important steps in the cell response to hypoxia, but ultimately, the effectiveness of the hypoxia response depends on the transactivation of HIF-1 target genes. VEGF is one of the most responsive genes whose expression is dependent on HIF-1 (11, 30). In order to study the
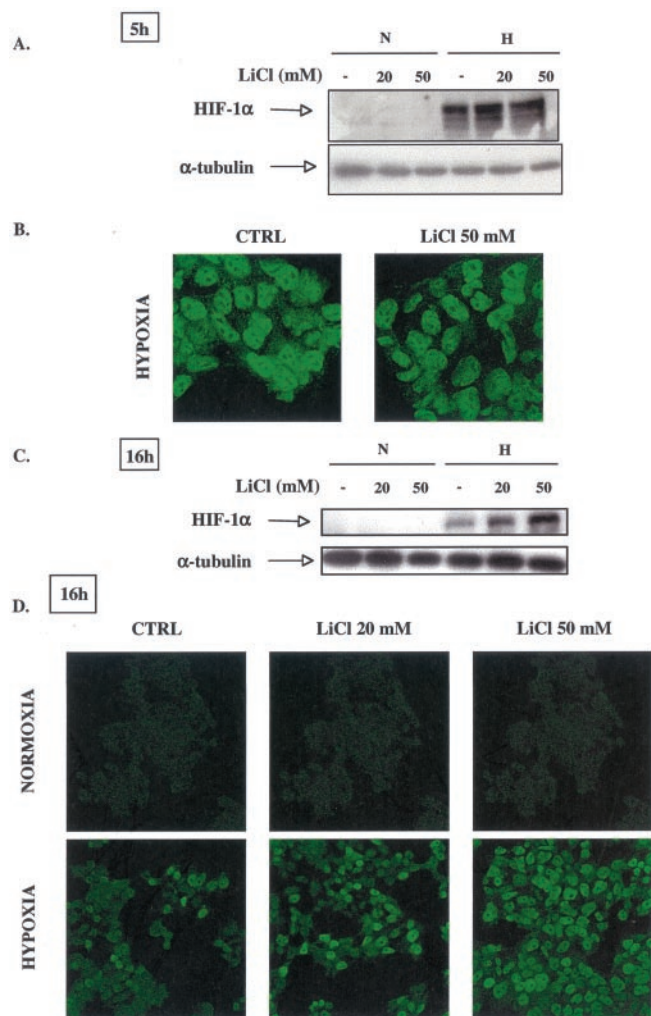
FIG. 6. **Effect of GSK3 inhibition on HIF-1α expression.** HepG2 cells were incubated for 5 h (*A* and *B*) or 16 h (*C* and *D*) in either normoxia or hypoxia in the presence or in the absence of LiCl at 20 or 50 mM. Cytosolic extracts were prepared and resolved by SDS-PAGE. *A* and *C*, after blotting, HIF-1α was revealed by Western analysis. A Western blot for α-tubulin was performed to check for the total amount of proteins loaded on the gel. *B* and *D*, after the incubation, the cells were also fixed and immunolabeled for HIF-1α and were observed in semiquantitative confocal microscopy.

physiological relevance of role of PI3K/Akt/GSK3β pathway in HIF-1 activation, we tested the effect of LY294002 and LiCl on the basal VEGF synthesis and hypoxia-induced VEGF release, after 5- and 16-h incubation. It has been noted that other processes in addition to transcription are involved in the regulation of VEGF expression, for example through mRNA stabilization (31). 5-h incubation in hypoxia in the presence of LY294002 resulted in a decreased synthesis of VEGF (Fig. 9*A*), whereas the effect of LY294002 was less pronounced after a 16-h incubation. In contrast, lithium increased the 16-h hypoxia-induced VEGF release (Fig. 9*B*) but had no effect after 5 h of incubation.

We have also tested the effect of PI3K and GSK3 inhibition on Glut-1 expression, a second HIF-1 target gene. Glut-1 is a transmembrane glucose transporter that regulates glucose uptake. The immunofluorescence experiment presented in Fig. 10 shows that Glut-1 protein is constitutively expressed and targeted to the plasma membrane in normoxia. The expression of Glut-1 was modestly increased after 5 h of hypoxia incubation, but the expression level was much higher after 16 h. Treatment with LY294002 resulted in a dramatically decreased level of Glut-1 after 5-h hypoxia incubation. In addition, in parallel
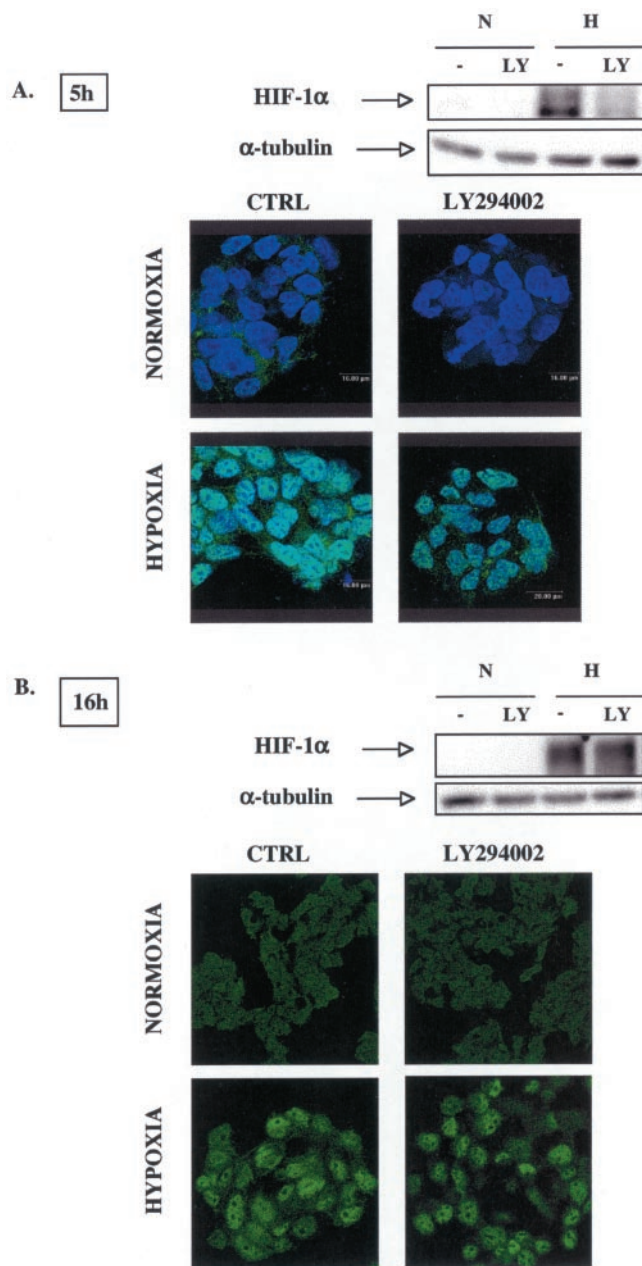

FIG. 7. **Effect of PI3K inhibition on HIF-1α expression.** HepG2 cells were incubated for 5 h (*A*) or 16 h (*B*) in either normoxia or hypoxia in the presence or in the absence of LY294002, a specific PI3K inhibitor, at 50 μM. *A*, after the incubation, the cells were fixed and immunolabeled for HIF-1α and were observed in semiquantitative confocal microscopy. *B*, cytosolic extracts were also prepared and resolved by SDS-PAGE. After blotting, HIF-1α was revealed by Western analysis. A Western blot for α-tubulin was performed to check for the total amount of proteins loaded on the gel.

with the effect of lithium on VEGF release, incubation in the presence of LiCl increased the 16-h hypoxic Glut-1 expression. These results suggest that GSK3 inhibition, which leads to increased HIF-1α stabilization and HIF-1 transactivation, also induced an marked increase in HIF-1-dependent gene expression such as VEGF and Glut-1.

DISCUSSION

The activation of the HIF-1 transcription factor by hypoxia is a multistep process. The regulation of HIF-1 appears to be due to posttranslational modifications such as stabilization of HIF-1α subunit, phosphorylations, and nuclear translocation to lead to proper transactivation activity. Stabilization of
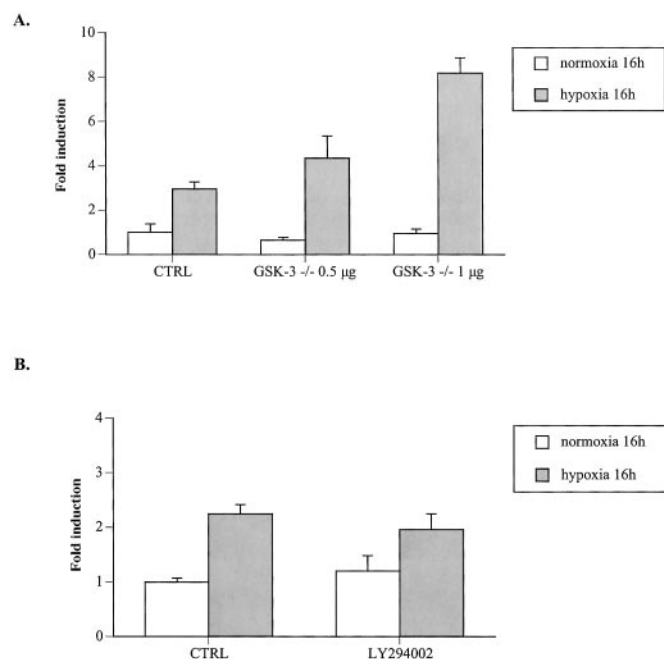
FIG. 8. **Effect of a GSK3 dominant negative mutant and PI3K inhibition on the hypoxia-induced HIF-1 transcriptional activity.** *A*, HepG2 cells were co-transfected with pGL3-SV40/6HRE together with the pRL-SV40 and with the GSK3 dominant negative expressing plasmid (GSK3 −/−) or the null pMyc vector (*CTRL*). The cells were incubated for 16 h in either normoxia or hypoxia. After the incubation, the cells were lysed for a luciferase assay. *B*, HepG2 cells were transfected with pGL3-SV40/6HRE together with the pRL-SV40. The cells were incubated for 16 h in either normoxia or hypoxia in the presence or in the absence of LY294002, a specific PI3K inhibitor, at 50 μM. After the incubation, the cells were lysed for the luciferase assay. Data represent the ratio of firefly luciferase activity to *Renilla* luciferase activity (*L/R*). Results are presented as means ± S.D. for triplicates.

FIG. 9. **Effect of PI3K inhibition and GSK3 inhibition on VEGF secretion.** HepG2 cells were incubated for 5 or 16 h in either normoxia or hypoxia in the presence or in the absence of LY294002 at 50 μM (*A*) or LiCl at 20 mM (*B*). After the incubation, the medium was recovered for assaying VEGF by ELISA, and cells were lysed for a protein assay. Results are expressed in ng of VEGF/μg of proteins and are presented as means ± S.D. for triplicates.

HIF-1α occurs through the inhibition of HIF-α prolyl hydroxylase (7, 8), an enzyme whose activity is diminished under hypoxic conditions.

Several studies have shown that HIF-1 is not only induced by hypoxia but is activated also in normoxia in response to insulin, insulin-like growth factors (32–34), interleukin-1, tumor necrosis factor-α (35, 36), angiotensin II (37), and thrombin (38). It should be noted that, in contrast to hypoxia, insulin regulates HIF-1α protein level and hence HIF-1 DNA binding activity and HIF-1 transcriptional activity through the stimulation of HIF-1α synthesis and not through the inhibition of its degradation. Furthermore, insulin does not affect the transcription of HIF-1α mRNA but regulates HIF-1α through a translation-dependent mechanism. In fact, it seems that HIF-1α synthesis is stimulated by the activation of eukaryotic translation initiation factor 2B through a PI3K/Akt/GSK3 pathway (39, 40). PI3K/Akt/TOR seems to be also involved in the insulin action on HIF-1 activity by a positive regulation of the HIF-1α translation (41–43). The insulin-activated TOR by phosphorylation of 4E-BP1, dissociates eukaryotic translation initiation factor 4E from 4E-BP1 and stimulates the translation initiation of the HIF-1α mRNA (44). Similarly, Her2(neu) signaling (45) or activation of the Src kinase (46) also results in an increased translation of HIF-1α through a PI3K/Akt- or PI3K/mTOR-dependent pathway.

However, many published reports suggest that PI3K and Akt could also play a major role in regulating the stabilization of HIF-1α in hypoxia (11–15), although the PI3K/Akt signaling pathway by hypoxia seems to be cell type-specific. Indeed, some authors have found that PI3K/Akt is activated by hypoxia in
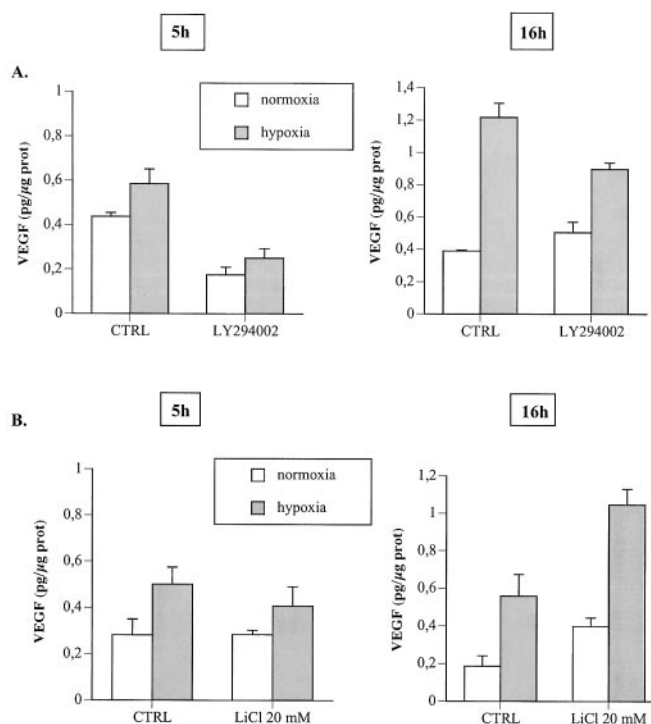
cells such as PC12 cells (47–49), HeLa (47), and HT1080 fibrosarcoma cells (50). In contrast, this effect was not observed in other cell types including some different breast cancer cell lines (51), PC-3 prostate cancer cells (13), COS-7 cells (52), and 3T3 cells (45). In this work, the level of activity of the PI3K/Akt pathway during increasing hypoxia incubations in human hepatoma cells (HepG2) has been examined. Several studies have already shown that HIF-1α is not a direct substrate for Akt (53), but the HIF-1α subunit stabilization is signaled through Akt; this could occur through a downstream target of Akt such as the GSK3 kinase. This led us to study, in the second part of the work, the role of PI3K/Akt and GSK3 on the regulation of the HIF-1α protein level and on the regulation of HIF-1 transcriptional activity in hypoxia.

The results presented here suggest that the PI3K/Akt pathway is constitutively active in HepG2 cells in normoxia. Akt is probably activated by insulin and/or growth factors present in the serum of the culture medium. Active PI3K/Akt signaling pathway maintains GSK3β phosphorylated on Ser-9, keeping it inactive. It should be noted that most of the incubations in normoxia and in hypoxia were performed in serum-free conditions. These experimental conditions induced Akt inactivation and reduced GSK3β phosphorylation on Ser-9. In hypoxia, Akt inactivation is much more marked, independently of the absence or presence of serum. However, the mechanism of the inhibition of the PI3K/Akt pathway by hypoxia is not known. Although the PI3K/Akt pathway activity is diminished after 5-h incubation in hypoxia, the residual activity of this pathway is sufficient and necessary to sustain HIF-1α accumulation. Indeed, both HIF-1α protein accumulation and HIF-1 transcriptional activity were inhibited by chemical inhibition of this pathway. The PI3K inhibition also reduced the GSK3β phosphorylation on Ser-9, and we postulated that the reduction in GSK3β phosphorylation on Ser-9 allows the Tyr-216 phospho-
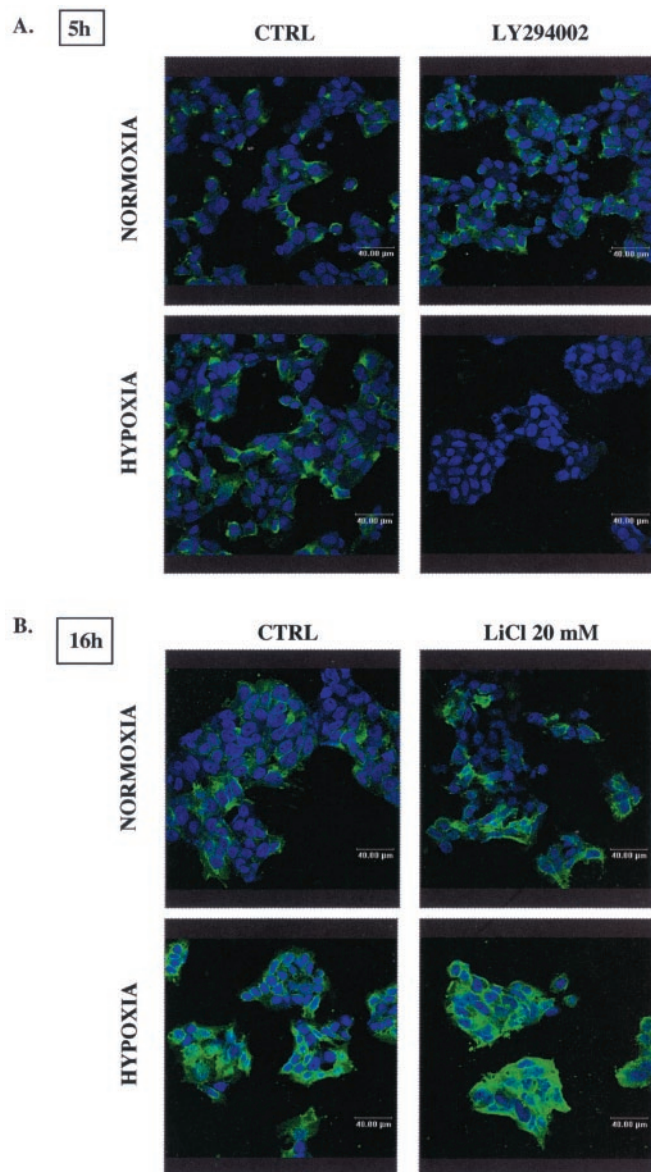
Regulation of HIF-1α Protein Level

FIG. 10. **Effect of PI3K inhibition and GSK3 inhibition on Glut-1 expression.** HepG2 cells were incubated for 5 h (*A*) or 16 h (*B*) in either normoxia or hypoxia, respectively, in the presence or in the absence of LY294002 at 50 μM (*A*) or LiCl at 20 mM (*B*). After the incubation, the cells were fixed and immunolabeled for Glut-1 without being permeabilized. The nuclei were stained with Topro-3, and the cells were observed in semiquantitative confocal microscopy.

FIG. 11. **Schematic representation of the putative role of the PI3K/Akt/GSK3β pathway in the regulation of HIF-1α accumulation in hypoxia.**

rylated form of GSK3β present in the cell to control and regulate the HIF-1α protein level. Indeed, an increase in GSK3 activity was observed after 16-h hypoxia incubation. Simultaneously, the Tyr-216-phosphorylated form of GSK3β translocated from the cytosol to the nucleus, hence co-localizing with HIF-1α. The same hypothesis can explain the diminished HIF-1α protein level observed after 16-h hypoxia incubation in comparison with 5 h. Indeed, in the case of "prolonged" hypoxia, inactive GSK3β has totally disappeared, and the active Tyr-216 phosphorylated GSK3β could then inhibit HIF-1α stabilization. Indeed, chemical inhibition of GSK3 by LiCl induced an increase in HIF-1α protein level after 16-h incubation in hypoxia. Lithium is commonly used to inhibit GSK3β, but it is known to exhibit other effects like activating Akt (28). Moreover, the overexpression of a dominant negative mutant of GSK3 (27) caused a greater HIF-1 transcriptional activity, suggesting that GSK3 inhibition leading to the accumulation of HIF-1α is
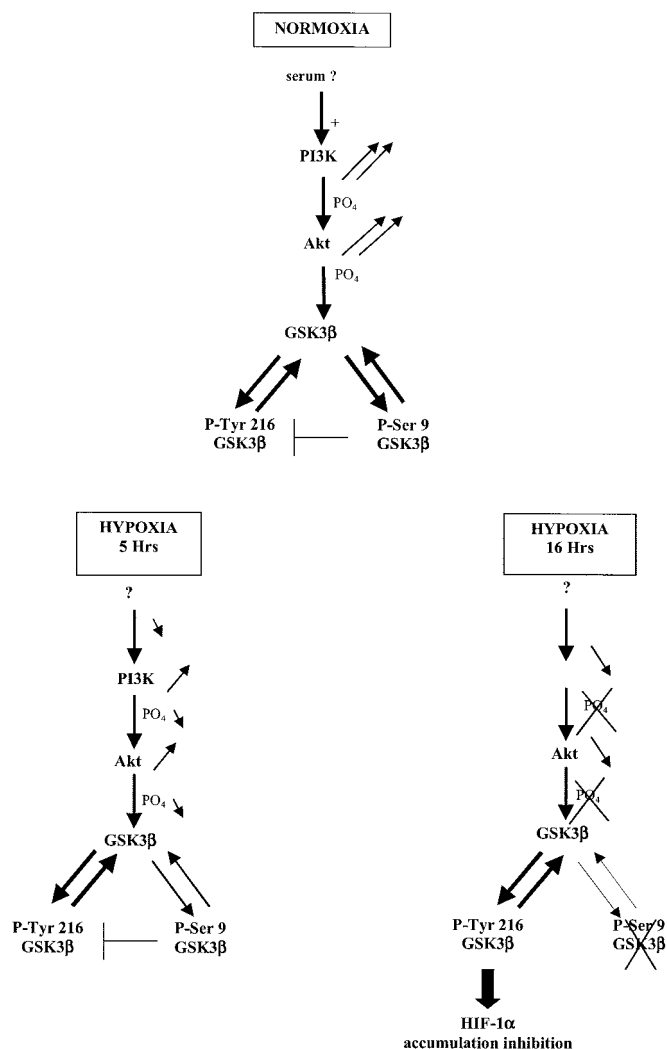
correlated with a greater transcriptional activity. Finally, hypoxia-induced increase in VEGF secretion and in Glut-1 expression was also enhanced by GSK3 inhibition in the presence of lithium.

In keeping with the results presented here, we would like to propose a new model for the regulation of HIF-1α accumulation during increasing durations of hypoxia (Fig. 11). The duration of the hypoxic stress affects HIF-1α stabilization and/or degradation. The GSK3 kinase seems to be the major effector in the regulation of this mechanism. More active GSK3β, which is present after a long period of hypoxia due to Akt inactivation, decreased HIF-1α accumulation and hence HIF-1 activity. On the other hand, the PI3K/Akt signaling pathway is needed for early HIF-1α protein stabilization in hypoxia, probably acting through the inhibition of GSK3β. The mechanism of HIF-1α stability regulation by GSK3β is not known. Sodhi *et al.* (26) have shown that there is a single phosphorylation in the HIF-1α oxygen-dependent degradation domain, but more precise data are not available. Informatic analysis of HIF-1α full-length amino acid sequence revealed several consensus sites for phosphorylation by GSK3β. Whether HIF-1α is a direct substrate of GSK3β is not yet known. In addition, the results presented here do not indicate whether GSK3β regulates HIF-1α protein degradation or synthesis in hypoxia. Further research is needed to clarify these different issues. In addition

to these data, Berra *et al.* (54) have recently described an autoregulatory feedback mechanism for HIF-1α degradation. According to their model, HIF-1α proteasome targeting factor is a hypoxia-inducible protein that accumulates during the duration of hypoxia. HIF-1α proteasome targeting factor is then responsible for the degradation of HIF-1α upon return to an oxygenated environment. HIF-1α proteasome targeting factor could be the HIF-α prolyl hydroxylase.

HIF-1α protein level is thus tightly regulated in cells both in normoxia and in hypoxic situations. Indeed, HIF-1α degradation inhibition through mutation of pVHL has dramatic consequences, greatly favoring the development of renal tumors (3, 4). That repression is also regulated in hypoxic conditions offers new perspectives in understanding the possible "dark side" of this transcription factor usually recognized as a survival factor for hypoxic cells.

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