Insulin Stimulates Hypoxia-inducible Factor 1 through a Phosphatidylinositol 3-Kinase/Target of Rapamycin-dependent Signaling Pathway*

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Hypoxia-inducible factor 1 (HIF-1) is a transcription factor involved in normal mammalian development and in the pathogenesis of several disease states. It consists of two subunits, HIF-1α, which is degraded during normoxia, and HIF-1β, which is constitutively expressed. Activated HIF-1 induces the expression of genes involved in angiogenesis, erythropoiesis, and glucose metabolism. We have previously reported that insulin stimulates vascular endothelial growth factor (VEGF) expression (1). In this study, we show that insulin activates HIF-1α, leading to VEGF expression in retinal epithelial cells. Insulin activates HIF-1α protein expression in a dose-dependent manner with a maximum reached within 6 h. The expression of HIF-1α is correlated with the activation of HIF-1 DNA binding activity and the transactivation of a HIF-1-dependent reporter gene. Insulin does not appear to affect HIF-1α mRNA transcription but regulates HIF-1α protein expression through a translation-dependent pathway. The expression of an active form of protein kinase B and treatment of cells with specific inhibitors of phosphatidylinositol 3-kinase (PI3K), MAPK, and target of rapamycin (TOR) show that mainly PI3K and to a lesser extent TOR are required for insulin-induced HIF-1α expression. HIF-1 activity and VEGF expression are also dependent on PI3K- and TOR-dependent signaling. In conclusion, we show here that insulin regulates HIF-1 action through a PI3K/TOR-dependent pathway, resulting in increased VEGF expression.

Insulin controls glucose and lipid metabolism, regulates protein synthesis, and promotes cell growth and differentiation. Following ligand binding, the insulin receptor tyrosine kinase is activated, leading to receptor autophosphorylation and the subsequent phosphorylation of intracellular proteins including insulin receptor substrates 1 and 2 and Shc. These initial events stimulate multiple signaling cascades that mediate cellular responses to the hormone (2). Among the substrates of the insulin receptor, insulin receptor substrates 1 and 2 are involved mainly in the activation of the PI3K1 pathway, whereas Shc participates in the activation of the Ras/MAPK cascade. The Ras/MAPK and PI3K pathways have been implicated in insulin-induced gene transcription (3, 4). The activated MAPK phosphorolyses transcription factors such as p62TCF involved in the transcription of genes that are implicated in proliferation and differentiation in response to insulin (5). In contrast, insulin regulates the expression of genes involved in glucose metabolism through a PI3K-dependent pathway. Thus, insulin inhibits the transcription of genes encoding PEPCK, the rate-limiting enzyme in gluconeogenesis, and glucose-6-phosphatase through a PI3K pathway (6, 7). Furthermore, a PI3K-dependent pathway is involved in the regulation of gene expression of lipogenic enzymes by insulin such as FAS (fatty acid synthase) (8). Finally, insulin also regulates the expression of genes implicated in the angiogenic response such as erythropoietin (EPO) and vascular endothelial growth factor (VEGF), but the molecular details of this action are lacking (9, 10).

VEGF is a key angiogenic factor involved in a wide variety of biological processes including embryonic development, wound healing, tumor progression, and metastasis. VEGF has emerged as a major mediator of intraocular neovascularization and as such plays a key role in the etiology of diabetic retinopathy (11). Indeed, it has been observed that intraocular VEGF levels are increased in diabetic patients suffering from proliferative retinopathy (12). VEGF expression is mainly regulated by tissue oxygen content (13, 14) but also by growth factors and cytokines, including platelet-derived growth factor, epidermal growth factor, insulin, insulin-like growth factor-1, tumor necrosis factor α, and transforming growth factor β (15–20). Hypoxia stimulates VEGF expression through at least three mechanisms including increased gene transcription, regulation at the translational level, and mRNA stabilization (14, 21). The transcriptional regulation of VEGF is mediated by the transcription factor hypoxia-inducible factor 1 (HIF-1) (22–24). HIF-1 is a basic helix-loop-helix transcription factor, which is composed of two subunits, HIF-1α and HIF-1β. HIF-1β, also

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1 The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; ARPE, arising retinal pigment epithelial; EPO, erythropoietin; HIF-1, hypoxia-inducible factor 1; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PKB, protein kinase B; CREB, cAMP-response element-binding protein; E3, ubiquitin-protein isopeptide ligase; PEPCK, phosphoenolpyruvate carboxykinase; TOR, target of rapamycin; VEGF, vascular endothelial growth factor; CoCl2, cobalt chloride; 4E-BP1, eukaryotic translation initiation factor 4E-binding protein; PKB-myrs, constitutively active form of PKB.

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known as the arylhydrocarbon nuclear translocator, is constitutively expressed, whereas HIF-1α expression is increased upon hypoxia. In normoxia, HIF-1α is rapidly ubiquitinated by the von Hippel-Lindau tumor suppressor E3 ligase complex and subjected to proteasomal degradation (25). Under hypoxic conditions, HIF-1α is not degraded and accumulates to form an active complex with HIF-1β. HIF-1 regulates the transcription of numerous genes involved in vascular development (VEGF, EPO, and heme oxygenase 1), in glucose and energy metabolism (glucose transporters and glycolytic enzymes), in iron metabolism (transferrin), and in cell proliferation and viability (insulin-like growth factor-2 and insulin-like growth factor-binding protein-1). It has been shown that insulin increases VEGF expression through a PI3K-dependent pathway in fibroblasts overexpressing insulin receptors (18). However, the identity and regulation of the transcription factor involved in this process remain unknown.

Here we report that insulin stimulates HIF-1α subunit accumulation, HIF-1 activation, and VEGF expression. Our results show that insulin regulates HIF-1α expression through a translation-dependent pathway. Moreover, insulin-induced HIF-1 regulation and VEGF expression require a PI3K/TOR-dependent pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—cDNA for VEGF<sub>iso</sub> was obtained from J. Poulet (Toulouse, France). Insulin was a kind gift from Novo-Nordisk (Copenhagen, Denmark). The antibody to HIF-1α (clone H108F) was purchased from Novus Biologicals, Inc. (Littleton, CO). The antibody to She was obtained from BD Transduction Laboratories (Franklin Lakes, NJ). The antibody to phospho-PKB (Ser-473) was purchased from New England Biolabs (Beverly, MA). The antibody to MEK-1 is directed against the 17-αmino acid amino-terminal of MEK-1 (28).

All of the chemical reagents were purchased from Sigma. U0126 was purchased from Promega Inc. (Madison, WI). Oligonucleotides and applications guide (Promega). The luciferase activity was measured with free wild type or mutated oligonucleotide according to the manufacturer's instructions. HIF-1α binding to the hypoxia response element was assessed using Trans-AM HIF transcription factor assay kit (Active Motif Europe, Rixensart, Belgium) according to the manufacturer's instructions. HIF-1 binding to the hypoxia binding site was identified using a 96-well plate. The active form of HIF-1α contained in cell extracts specifically binds to this hypoxia site and can be revealed by incubation with antibodies using enzyme-linked immunosorbent assay technology as described in the manufacturer's instructions. HIF-1 binding to the hypoxia site was identified using a ChemiDoc XRS System (Bio-Rad). The specificity for this assay was monitored by competition with free wild type or mutated oligonucleotide according to the manufacturer's instructions.

**Luciferase Assays**—To assay the transcriptional activity of HIF-1α, we used the pGL2 basic P12 VEGF promoter vector, which contains a HIF-1α binding site (from –975 to –968) downstream from the luciferase gene (27). ARPE cells in 12-wells plates were transiently co-transfected with the reporter plasmid and with Ras sarcoma virus-β-galactosidase as a control for transfection efficiency. The cells were stimulated for 16 h, and luciferase assays were performed as described in the protocols and applications guide (Promega). The luciferase activity was measured using a chemiluminometer (Wallac 1420). The β-galactosidase activity was performed as described in the Promega’s protocols and applications guide. Cells lysates were incubated with a 2× assay buffer (200 mM sodium phosphate buffer, pH 7.8, 2 mM MgCl<sub>2</sub>, 100 mM β-mercaptoethanol, 1.33 mg/ml o-nitrophenyl β-galactosidase). The absorbance at 420 nm was measured with a spectrophotometer Wallac 1420.

**RESULTS**

Insulin Stimulates HIF-1α Accumulation in Human Retinal Epithelial Cells—We have previously shown that insulin induces VEGF expression in cell culture and in intact animals (1). To study the effect of the hormone on HIF-1α protein expression, ARPE-19 cells were treated for 4 h with insulin or with cobalt chloride (CoCl<sub>2</sub>) as a positive control. Whole cell lysates and nuclear extracts were analyzed by Western blotting using an antibody to HIF-1α (Fig. 1, A and B). The divalent metal CoCl<sub>2</sub> is known to induce HIF-1α expression,
and to transactivate a HIF-1-dependent reporter gene. To do this, we measured the ability of HIF-1 to bind to DNA correlated with an activation of the transcription factor HIF-1. Induced by 0.1 nM insulin, and the maximal induction was seen in ARPE-19 cells. As observed in Fig. 1, CoCl2 treatment of cells led to an accumulation of HIF-1 protein in the absence of insulin stimulation. This elevated the expression of HIF-1 in the presence of the proteasome inhibitor, preventing any effect of insulin on HIF-1 degradation. We next measured the transcriptional activity of HIF-1 using a SV40 promoter-luciferase unit downstream of a 99-bp hypoxia response element (pGL2 basic P12 VEGF promoter) relative to co-transfected Rous sarcoma virus-β-galactosidase (Fig. 2C). After 16 h of insulin or CoCl2 treatment, the luciferase activity in cell extracts was determined and normalized to the β-galactosidase activity. Insulin and CoCl2 induced a statistically significant 15.5- and 3.5-fold increase (±0.0864 and ±0.375, respectively) in luciferase activity, respectively. Therefore, the accumulation of HIF-1α subunit induced by insulin can be correlated with the activation of the transcription factor HIF-1.

**Insulin Activates the Transcription Factor HIF-1**—To obtain a better understanding of the processes involved in HIF-1α accumulation in response to insulin treatment, we investigated the effect of insulin on the amount of HIF-1α mRNA. ARPE-19 cells were stimulated with insulin or CoCl2 for 6 h, RNA was extracted, and Northern blot analysis was performed using a HIF-1α cDNA probe (Fig. 3A). We found that insulin or CoCl2 treatment did not modify HIF-1α mRNA expression, suggesting that insulin does not regulate HIF-1α mRNA transcription. HIF-1α has been shown to be degraded through the proteasome pathway during normoxia. Therefore, to study the effect of insulin on HIF-1α degradation, we looked at the effect of a specific inhibitor of proteasome, MG132 (Fig. 3B). ARPE-19 cells were incubated with insulin in the absence or presence of MG132. After 4 h, insulin was removed, and the level of HIF-1α protein was evaluated by Western blot using a HIF-1α antibody. As expected, in the absence of MG132, insulin induced an accumulation of HIF-1α. Within 15 min after the removal of insulin, a decrease in HIF-1α protein could be seen, and HIF-1α was undetectable 60 min after withdrawal of the hormone. We observed that the inhibition of the proteasome by MG132 led to a high level of expression of HIF-1α, even in the absence of insulin stimulation. This elevated the expression of HIF-1α in the presence of the proteasome inhibitor, preventing us from detecting any effect of insulin on HIF-1α degradation. To analyze the insulin effect on HIF-1α synthesis, we performed a time course of HIF-1α disappearance in the presence of the protein translation inhibitor, cycloheximide (Fig. 3C). To this end, ARPE-19 cells were treated with insulin or CoCl2 for 4 h, and cycloheximide was added for 15–60 min. In cells exposed to CoCl2, HIF-1α level remained constant over 60 min despite the lack of ongoing protein synthesis. This observation is consistent with previous studies showing that CoCl2 had no effect on HIF-1α synthesis but blocked its degradation. In ARPE-19 cells treated with insulin, the addition of cycloheximide led to a decrease in HIF-1α expression starting at 15 min. After 60 min, HIF-1α was no longer detectable. Together, these results suggest that insulin increases HIF-1α protein levels through a translation-dependent pathway.

**Constitutively Active PKB Induces HIF-1α Expression**—We investigated the role of MEK- and PKB-dependent pathways in the regulation of HIF-1α in ARPE-19 cells. To this end, we transfected ARPE-19 cells with pcDNA3 as a control with a constitutively active form of MEK (MEK*), or with a PKB-myristinated. The transfected cells were treated or not treated with insulin (100 nM), and whole cell lysates were prepared and analyzed by

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**Fig. 1. Insulin stimulates HIF-1α accumulation in ARPE-19 cells.** ARPE-19 cells were stimulated with insulin (100 nM) or CoCl2 (200 μg) for 4 h. Whole cell lysates (A) or nuclear extracts (B) were prepared and analyzed by Western blotting using antibody to HIF-1α. Expression of HIF-1α was normalized using a Western blot with antibodies to Shc or a nuclear protein CREB. ARPE-19 cells were stimulated with different concentrations of insulin for 4 h (C) or with insulin (100 nM) for the indicated time (D). Whole cell lysates were prepared and analyzed by Western blotting using antibodies to HIF-1α or Shc.

HIF-1 DNA binding activity, and transactivation of HIF-1 target genes (33–36). Indeed, the CoCl2 treatment of cells led to an accumulation of HIF-1α in ARPE-19 cells. Insulin induces HIF-1α expression in both whole cell lysates and in nuclear extracts. As observed in Fig. 1, C and D, HIF-1α expression was induced by 0.1 nM insulin, and the maximal induction was seen at 100 nM. The expression of HIF-1α was transiently detectable within 1 h and maximal within 6 h and then returned to basal levels within 24 h of treatment. These results show that the incubation of ARPE-19 cells with insulin results in a time- and concentration-dependent elevation of HIF-1α protein levels.

**Insulin Activates the Transcription Factor HIF-1**—We next determined whether insulin-induced HIF-1α accumulation was correlated with an activation of the transcription factor HIF-1. To do this, we measured the ability of HIF-1α to bind to DNA and to transactivate a HIF-1-dependent reporter gene. ARPE-19 cells were treated for 4 h with insulin or CoCl2, and the nuclear extracts were isolated. A double-stranded oligonucleotide containing the HIF-1 binding site present in the EPO gene was used as a probe in an electrophoretic mobility shift assay (Fig. 2A). Both insulin and CoCl2 induced a shift of the labeled probe. This binding was verified by an enzyme-linked immunosorbent assay using an immobilized oligonucleotide to assess HIF-1 DNA binding activity (Fig. 2B). HIF-1 DNA binding activity was stimulated after 4 h of insulin or CoCl2 treatment. The specificity of HIF-1 binding was tested by competition with free oligonucleotides. The binding was specific, because it was abolished in the presence of an excess of wild type oligonucleotide, whereas the mutated oligonucleotide had no effect.

We next measured the transcriptional activity of HIF-1 using a SV40 promoter-luciferase unit downstream of a 99-bp hypoxia response element (pGL2 basic P12 VEGF promoter) relative to co-transfected Rous sarcoma virus-β-galactosidase (Fig. 2C). After 16 h of insulin or CoCl2 treatment, the luciferase activity in cell extracts was determined and normalized to the β-galactosidase activity. Insulin and CoCl2 induced a statistically significant 15.5- and 3.5-fold increase (±0.0864 and ±0.375, respectively) in luciferase activity, respectively. Therefore, the accumulation of HIF-1α subunit induced by insulin can be correlated with the activation of the transcription factor HIF-1.
Western blotting using antibody to HIF-1α (Fig. 4). The expression of MEK-myristoylated (PKB-myr) is sufficient to increase the HIF-1α protein level in basal conditions. In addition, when PKB-myrs is expressed, insulin treatment does not further increase the level of HIF-1α protein.

Insulin Stimulates HIF-1α Accumulation and VEGF Expression through a PI3K/TOR-dependent pathway—To further evaluate the contribution of the PI3K pathway to the regulation of HIF-1α protein levels, we used the pharmacological inhibitors of PI3K and of a downstream effector, TOR. ARPE-19 cells were treated for 4 h with CoCl₂ or with insulin in the absence or presence of the inhibitors of PI3K (LY294002), TOR (rapamycin), or MEK (U0126). Whole cell lysates and nuclear extracts were prepared and analyzed by Western blotting using antibodies to HIF-1α, Sβc, or CREB (Fig. 5A). In agreement with the results obtained in Fig. 4, insulin increased HIF-1α expression. This increase was not affected by pretreatment with the MEK inhibitor U0126. Rather, the inhibition of PI3K activation by LY249002 totally blocked the expression of HIF-1α in response to insulin in both total cell lysates and in nuclear extracts. In addition, the treatment with rapamycin decreased HIF-1α protein after insulin stimulation by half the level. We confirmed that insulin-induced activation of PI3K, MAPK, and TOR was blocked by treatment with the respective inhibitor (data not shown).

To determine whether these inhibitors blocked HIF-1 activity, we measured the DNA binding activity of HIF-1 using an enzyme-linked immunosorbent assay (EMSA) (Fig. 5B). Insulin induced a 3-fold increase in DNA binding activity of HIF-1. The inhibition of PI3K by LY294002 totally blocked HIF-1 activation, whereas the inhibition of TOR by rapamycin induced a 40% decrease in
Insulin-induced HIF-1 activation. As expected, MEK does not appear to be involved, because the inhibition of MEK by U0126 had no effect on HIF-1 activity in response to insulin. Thus, the extent of inhibition of HIF-1 expression by the inhibitors tightly correlated with their ability to block insulin-induced DNA binding activity of HIF-1.

To examine whether this correlation could be extended to the HIF-1 activation and VEGF mRNA expression in response to insulin, we analyzed the VEGF mRNA expression profile after treatment with these inhibitors. ARPE-19 cells were treated with CoCl₂ or with insulin in the absence or presence of LY294002, U0126, or rapamycin. Whole cell lysates were prepared and analyzed by Western blotting using antibodies to HIF-1α or Shc. Cycloheximide (CHX) was added to a final concentration of 10 μg/ml, and the cells were harvested after being incubated for the indicated time in the presence of CHX and the inducer. Whole cell lysates were prepared and analyzed by Western blotting using antibodies to HIF-1α or Shc.

FIG. 3. Insulin induces HIF-1α through a translation-dependent pathway. A, ARPE-19 cells were stimulated with insulin (100 nM) or CoCl₂ (200 μM) for 6 h. RNA was extracted and analyzed by Northern blotting using a probe corresponding to the HIF-1α cDNA. The blot was subsequently probed with 18 S rRNA as a control. B, ARPE-19 cells were treated with or without 0.2% BSA for 4 h with the proteasome inhibitor MG132 (10 μM) and stimulated with insulin (100 nM). After 4 h, insulin was removed, and cells were incubated in Dulbecco’s modified Eagle’s medium containing 0.2% bovine serum albumin (BSA) for the indicated times. Whole cell lysates were prepared and analyzed by Western blotting using antibodies to HIF-1α or Shc. C, HIF-1α expression was induced by the exposure of ARPE-19 cells to insulin (100 nM) or CoCl₂ (200 μM) for 4 h. Cycloheximide (CHX) was added to a final concentration of 10 μg/ml, and the cells were harvested after being incubated for the indicated time in the presence of CHX and the inducer. Whole cell lysates were prepared and analyzed by Western blotting using antibodies to HIF-1α or Shc.

FIG. 4. Constitutively active PKB induces HIF-1α accumulation in ARPE-19 cells. ARPE-19 cells were transfected with pcDNA3 (control), a constitutively active form of MEK (MEK*), or PKB-myr. ARPE-19 cells were stimulated for 4 h with insulin (100 nM). Whole cell lysates were prepared and analyzed by Western blotting using antibodies to HIF-1α, Shc, phosphorylated PKB, or MEK.

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A. HIF-1α mRNA expression

B. HIF-1α stability

C. HIF-1α synthesis

FIG. 5. Insulin activates HIF-1 through a PI3K/TOR-dependent pathway. A, ARPE-19 cells were stimulated for 4 h with CoCl₂ (200 μM) or with insulin (100 nM) with or without pretreatment for 30 min with LY294002 (50 μM), U0126 (10 μM), or rapamycin (50 nM). Whole cell lysates or nuclear extracts were prepared and analyzed by Western blotting using antibodies to HIF-1α, Shc, or CREB. B, ARPE-19 cells were stimulated for 4 h with insulin (100 nM) with or without pretreatment for 30 min with LY294002 (LY) (50 μM), U0126 (10 μM), or rapamycin (Rapa) (50 nM). Nuclear extracts were prepared, and HIF-1 binding was quantified with Trans-AM HIF-1 transcription factor assay kit. The results are presented as fold stimulation, which was calculated as the ratio of the different samples to the control cells. Results shown represent the mean ± S.E. of two independent experiments performed in duplicate.

FIG. 6. Insulin activates HIF-1 through a PI3K/TOR-dependent pathway. A, ARPE-19 cells were stimulated for 4 h with CoCl₂ (200 μM) or with insulin (100 nM) with or without pretreatment for 30 min with LY294002 (50 μM), U0126 (10 μM), or rapamycin (50 nM). Whole cell lysates or nuclear extracts were prepared and analyzed by Western blotting using antibodies to HIF-1α, Shc, or CREB. B, ARPE-19 cells were stimulated for 4 h with insulin (100 nM) with or without pretreatment for 30 min with LY294002 (LY) (50 μM), U0126 (10 μM), or rapamycin (Rapa) (50 nM). Nuclear extracts were prepared, and HIF-1 binding was quantified with Trans-AM HIF-1 transcription factor assay kit. The results are presented as fold stimulation, which was calculated as the ratio of the different samples to the control cells. Results shown represent the mean ± S.E. of two independent experiments performed in duplicate.

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**DISCUSSION**

Diabetic retinopathy is the major cause of blindness in Western countries. VEGF is involved in the pathogenesis of both background and proliferative retinopathy. Intraocular VEGF is increased in eyes from patients with blood-retinal barrier breakdown and neovascularization. Clinical studies have demonstrated that long term insulin therapy reduces the risk of diabetic retinopathy progression. However, it has also been observed that intensive insulin therapy leads to a transient worsening of retinopathy characterized by a blood-retinal barrier breakdown (37–39). It has been proposed that the worsening of retinopathy could be attributed to chronic hyperinsulinemia induced by insulin treatment. Indeed, it has been shown that insulin stimulates VEGF expression, which in turn would stimulate neovascularization (40, 41). However, the molecular mechanisms involved in insulin-induced VEGF expression remain unknown. In this study, we show that insulin stimulates VEGF expression through the activation of the transcription factor HIF-1. This activation is regulated by a PI3K-dependent signaling pathway involving TOR. Moreover, in contrast to hypoxia, which is a major activator of HIF-1, insulin does not regulate HIF-1α through the inhibition of its degradation but through a translation-dependent mechanism.

In ARPE-19 cells, insulin stimulates the accumulation of the regulated subunit HIF-1α. An increase in HIF-1α expression is directly correlated with the activity of the transcription factor HIF-1. Indeed, we show that insulin induces increased HIF-1α protein levels, augmented HIF-1α DNA binding activity, and stimulation of HIF-1-mediated reporter gene transcription. In normoxic conditions, HIF-1α is maintained at low levels by a degradation process involving the ubiquitin-proteasome system (42, 43). Hypoxia rapidly increases the amount of HIF-1α by inhibiting its proteasome-dependent degradation. Surprisingly, insulin does not seem to act on HIF-1α degradation. A comparison of the half-life of HIF-1α after the removal of insulin or in presence of both insulin and cycloheximide, a translation inhibitor, shows that insulin does not stabilize the HIF-1α protein. Furthermore, insulin does not affect the transcription of HIF-1α mRNA, suggesting that it regulates HIF-1α translation. Nevertheless, we cannot exclude the possibility that insulin regulates the translation of a protein, which inhibits HIF-1α degradation. It is of interest to note that a recent study shows that heregulin, which activates the tyrosine kinase receptor HER2, stimulates HIF-1α synthesis (30), similar to our results concerning insulin action.

In ARPE-19 cells, we found that the insulin effect on HIF-1α expression, HIF-1α activation, and VEGF expression are dependent on the PI3K/PKB/TOR pathway. In contrast, the MEK pathway does not appear to be required for insulin action on HIF-1. Both the MAPK and PI3K pathways have been implicated in HIF-1 regulation. The p42 and p44 MAPKs activate HIF-1 by promoting the phosphorylation of HIF-1α in response to hypoxia and its accumulation in response to advanced glycation end products or mersalyl (1, 44–46). PI3K-dependent pathways have been implicated in HIF-1 and VEGF expression in transformed cells (47–50). Moreover, the activation of PKB or inactivation of the tumor suppressor gene encoding phosphatase and tensin homolog deleted on chromosome 10, which dephosphorylates the PI3K reaction products phosphatidylinositol 3,4-biphosphate and phosphatidylinositol 3,4,5-triphosphate, increases HIF-1α protein levels and HIF-1-dependent reporter gene expression (30, 48, 50, 51).

TOR seems to be involved only partly in the insulin action on HIF-1 activity, because the inhibition of TOR does not completely abolish HIF-1α expression and HIF-1 activation. These results suggest that at least the two following pathways are involved in insulin-induced HIF-1 regulation, a PKB-dependent/TOR-independent pathway and a PKB/TOR-dependent pathway. The PKB-dependent/TOR-independent pathway remains unknown, because a direct phosphorylation of HIF-1α by PKB has been excluded (51). However, PKB could be involved in the insulin regulation of HIF-1α translation. It has been previously shown that insulin stimulates protein synthesis by the activation of eIF2B (eukaryotic translation initiation factor 2B), an essential translation initiation factor, through a PI3K/PKB/glycogen synthase kinase-3 pathway (52, 53). For the PKB/TOR-dependent pathway, it has been shown that TOR activity positively regulates translation. Insulin induces the phosphorylation of 4E-BP1 through a PI3K/PKB pathway (54). The phosphorylation of 4E-BP1 results in a decrease in its binding affinity for eukaryotic translation initiation factor 4E, an essential translation initiation factor. The subsequent dissociation of eIF4F-4E (eukaryotic translation initiation factor) from 4E-BP1 promotes cap structure-dependent translation initiation (55, 56). We could hypothesize that 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.

In diabetes, several factors could be involved in the worsening of the diabetic retinopathy including (i) advanced glycation end products generated during hyperglycemia, (ii) hypoxia resulting from microvascular retinal occlusion, and (iii) hyperinsulinemia stimulating VEGF expression through the up-regulation of HIF-1 expression (1, 57). Moreover, the co-treatment of retinal epithelial cells with both insulin and advanced glycation end products increases VEGF expression (1). The advanced glycation end products and insulin activate HIF-1 through distinct pathways, because advanced glycation end-induced HIF-1 activation is dependent on MAPK, whereas insulin-induced HIF-1 activation is dependent on PI3K. Hypoxia blocks HIF-1α degradation, whereas growth factors acting through tyrosine kinase receptors would increase its synthesis. The combination of these different signals enhances the activation of the transcription factor leading to increased VEGF gene expression. The result would be an amplification of
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the angiogenic signal leading to further progression of diabetic retinopathy.

In conclusion, we have shown that insulin activates the transcription factor HIF-1 through a PI3K/PKB/TOR-dependent pathway. Our results suggest that insulin similar to heregulin regulates HIF-1α synthesis. It remains to established whether such an effect on HIF-1α synthesis is a general feature of receptor tyrosine kinases.

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12. Conflicts of Interest—None.

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