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Insulin induces transcription of target genes through the hypoxia-inducible factor HIF-1α/ARNT

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Hypoxic stress induces the expression of genes associated with increased energy flux, including the glucose transporters Glut1 and Glut3, several glycolytic enzymes, nitric oxide synthase, tyrosine hydroxylase, erythropoietin and vascular endothelial growth factor (VEGF). Induction of these genes is mediated by a common basic helix–loop–helix-PAS transcription complex, the hypoxia-inducible factor-1α (HIF-1α)/aryl hydrocarbon nuclear translocator (ARNT). Insulin also induces some of these genes; however, the underlying mechanism is unestablished. We report here that insulin shares with hypoxia the ability to induce the HIF-1α/ARNT transcription complex in various cell types. This induction was demonstrated by electrophoretic mobility shift of the hypoxia response element (HRE), and abolished by specific antisera to HIF-1α and ARNT, and by transcription activation of HRE reporter vectors. Furthermore, basal and insulin-induced expression of Glut1, Glut3, aldolase A, phosphoglycerate kinase and VEGF was reduced in cells having a defective ARNT. Similarly, the insulin-induced activation of HRE reporter vectors and VEGF was impaired in these cells and was rescued by re-introduction of ARNT. Finally, insulin-like growth factor-I (IGF-I) also induced the HIF-1α/ARNT transcription complex. These observations establish a novel signal transduction pathway of insulin and IGF-I and broaden considerably the scope of activity of HIF-1α/ARNT.

Keywords: ARNT/hypoxia/HIF-1α/IGF-I/insulin/proteasome/ubiquitin

Introduction

Hypoxic stress increases the expression of a variety of genes whose products act in concert, both systemically and at hypoxic sites, to facilitate the supply of metabolic energy. Among these are genes coding for the glucose transporters Glut1 and Glut3 and several glycolytic enzymes, whose increased expression at hypoxic sites is associated with enhanced glucose utilization (Bunn and Poyton, 1996). Other genes are involved in systemic responses and longer-term responses to hypoxic stress. These genes include those for nitric oxide (NO) synthase, which induces vasodilation, tyrosine hydroxylase, essential for an increase in the rate of breathing, erythropoietin (EPO), which elevates the rate of red blood cell production, and VEGF, which induces endothelial sprouting at hypoxic sites (Semenza and Wang, 1992; Czyzyk Krzeska *et al*., 1994; Melillo *et al*., 1995; Shima *et al*., 1995).

Hypoxic stress induces all of these genes by elevating the level of a single basic helix–loop–helix (bHLH)-PAS transcription factor, the hypoxia-inducible factor-1α (HIF-1α). HIF-1α forms a functional heterodimer with another bHLH-PAS protein, the aryl hydrocarbon nuclear translocator (ARNT). This highly conserved protein is constitutively expressed and serves as a common partner for several other bHLH-PAS proteins (Maxwell *et al*., 1993; Wang and Semenza, 1993; Wang *et al*., 1995; Pollenz *et al*., 1996). The role of HIF-1α in transcription regulation was demonstrated recently by generating HIF-1α-deficient murine embryonic stem (ES) cells. These cells exhibited low basal levels of mRNAs of glucose transporters and glycolytic enzymes, and no induction of these transcripts was obtained under hypoxic stress (Iyer *et al*., 1998; Ryan *et al*., 1998). Similarly, the induction of these genes was also impaired in mice lacking ARNT (Maltepe *et al*., 1997).

Insulin plays a central role in regulating metabolic pathways associated with energy storage and utilization. It triggers the conversion of glucose into glycogen and triglycerides and inhibits gluconeogenesis (Taylor, 1991). The insulin-signaling network is well studied. Following ligand binding, the insulin receptor kinase is activated by autophosphorylation of cytoplasmic tyrosine residues. Several cytoplasmic proteins bind directly to the activated receptor. These proteins are activated, either by tyrosine phosphorylation (IRS-1, IRS-2 and Shc) or by association with pTyr residues of the insulin receptor (phosphatidylinositol 3-kinase). These initial events lead to multiple signaling cascades that mediate the cellular responses to insulin (Cheatham and Kahn, 1995).

Several genes are induced both by insulin and by hypoxia, including genes coding for the glucose transporters Glut1 and Glut3, several glycolytic enzymes, EPO and VEGF (Pilkis and Granner, 1992; Sato *et al*., 1995; Taha *et al*., 1995; Masuda *et al*., 1997). The signaling pathways leading to induction of these genes by insulin are currently unclear. Insulin-like growth factor-I (IGF-I), a homolog of insulin, shares many signaling components and cellular responses with insulin (Blakesley *et al*., 1996). It is noteworthy that IGF-I shares with hypoxia and insulin the ability to induce the expression of *Glut1*, *EPO* and *VEGF* (Russo *et al*., 1994; Warren *et al*., 1996; Masuda *et al*., 1997). Recently, IGF-I was implicated in hypoxia-induced retinal neovascularization by a VEGF-independent mechanism (Smith *et al*., 1997).

Because of the striking overlap between genes induced by insulin, IGF-I and hypoxic stress, we postulated that

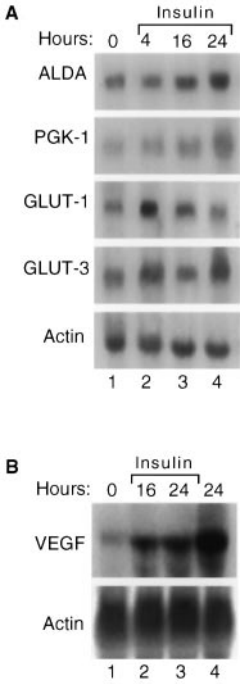

Fig. 1. Insulin induces the genes of glucose transporters, glycolytic enzymes and VEGF. (**A**) Serum-starved human Hep-G2 cells were stimulated with insulin (100 mM) for the indicated times (lanes 2–4). Control cells (lane 1) were incubated for 24 h without insulin. Induction of the following genes was demonstrated by RNA blot analysis of the corresponding transcripts: aldolase A (ALDA); phosphoglycerate kinase (PGK-1); and glucose transporters 1 and 3 (GLUT-1 and GLUT-3). (**B**) Serum-starved human T47D cells were stimulated with insulin (100 mM, lanes 2 and 3) or $CoCl_2$ (166 μM, lane 4) for the indicated times. Control cells (lane 1) were incubated without stimulants. Induction of VEGF mRNA was demonstrated by RNA blot analysis.

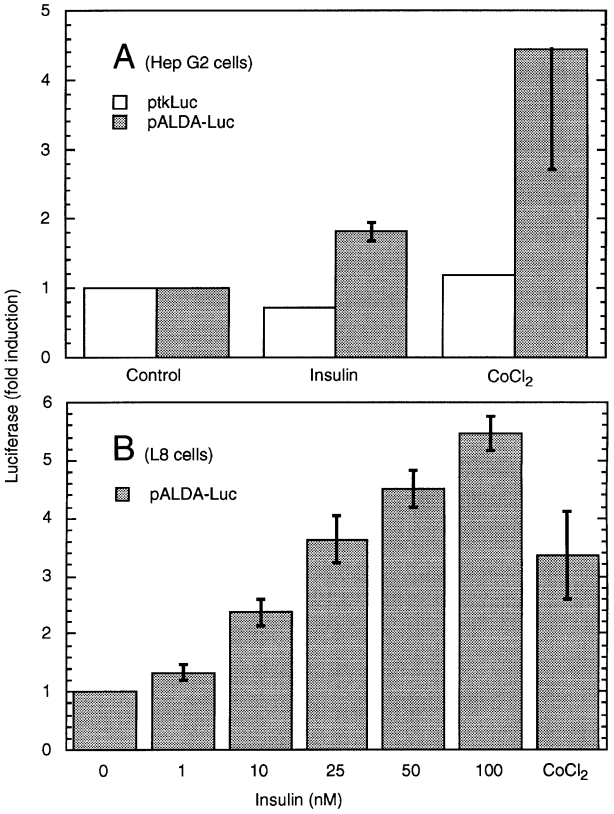

Fig. 2. Insulin stimulates transcription from the ALDA promoter. (**A**) Hep-G2 cells were transiently transfected either with the pALDA-Luc vector or the control ptkLuc vector. After 24 h, cells were stimulated with insulin or $CoCl_2$. A significant induction of luciferase, as compared with unstimulated cells, was obtained by insulin ($P < 0.005$, $n = 3$). Induction by $CoCl_2$ represents a positive control. No induction was obtained in cells transfected with the control vector ptkLuc. (**B**) Rat L8 myoblasts were transfected similarly with pALDA-Luc or ptkLuc and treated with increasing concentrations of insulin. A significant induction of luciferase ($P < 0.005$, $n = 3$) was obtained already with 10 nM of insulin. No induction was obtained in cells transfected with the control vector ptkLuc.

these stimulants may share HIF-1α/ARNT as a common transcription complex. This work forges the first link between HIF-1α/ARNT and cellular responses to insulin and IGF-I. We show that, similar to hypoxic stress, insulin induces genes containing the hypoxia response element (HRE) by formation of the HIF-1α/ARNT complex. IGF-I also induces the HIF-1α/ARNT complex, suggesting that it increases transcription of these genes by the same mechanism. Various cellular responses to insulin and IGF-I may now be linked to the formation of the HIF-1α/ARNT complex, which is known to affect a broad range of genes associated with energy balance.

Results

Insulin increases the transcriptional activity of the ALDA promoter

Insulin was reported to increase the steady-state level of mRNAs encoding several glycolytic enzymes, glucose transporters and VEGF in a variety of cells. We confirmed these activities of insulin by RNA blot analysis of human Hep-G2 hepatoma cells. In these cells, insulin increased the mRNA level of the glucose transporters Glut1 and Glut3, as well as the glycolytic enzymes aldolase A (ALDA) and phosphoglycerate kinase (PGK) within 4–24 h. Similarly, insulin induced VEGF expression in Hep-G2 cells (not shown) and in human T47D ductal breast carcinoma cells (Figure 1).

To study how insulin stimulates the transcription of these genes, we constructed a reporter vector consisting of a functional promoter of the human *ALDA*, fused to luciferase cDNA (pALDA-Luc). Hep-G2 cells and L8 rat myoblasts were transiently transfected with pALDA-Luc and then treated with insulin. As a positive control, cells were incubated with $CoCl_2$, an agent that induces ALDA transcription by mimicking hypoxic stress (Semenza *et al*., 1996). Both insulin and $CoCl_2$ significantly induced the expression of luciferase by 1.8- to 6-fold, while no elevation was observed following a similar induction of cells transfected with the control vector ptkLuc (Figure 2). Therefore, we concluded that transcriptional activation of the ALDA promoter by insulin accounts for at least part of the observed increase in the level of the corresponding mRNA.

Insulin and IGF-I induce the formation in vitro of a complex containing the hypoxia response element

The HRE is present in all of the above genes whose steady-state mRNA level was increased by insulin. Therefore, we tested whether insulin may induce the formation of a cellular complex that will bind HRE *in vitro*. Because

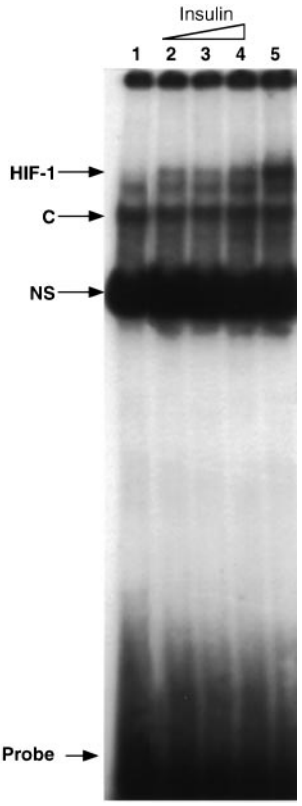

Fig. 3. Insulin induces the formation of a complex with the HRE *in vitro*. Serum-starved Hep-G2 cells were stimulated for 8 h with insulin (0, 25, 50 and 100 nM, lanes 1–4, respectively) or $CoCl_2$ (lane 5). Nuclear cell extracts were mixed with a ^{32}P-labeled double-stranded DNA probe (W18), corresponding to the HRE, and the mixture analyzed by EMSA. The specific HIF-1 complex is indicated by an arrow. C is a constitutive complex, NS is a non-specific band. Excess free probe is indicated at the bottom.

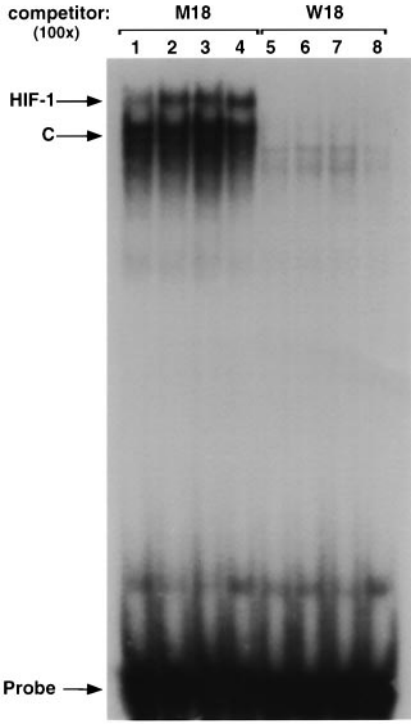

Fig. 4. Specificity of the *in vitro* complexes with the W18 DNA probe corresponding to the HRE. Serum-starved murine L8 myoblasts were kept unstimulated (lanes 1 and 5), stimulated for 8 h in a serum-free medium with insulin (lanes 2 and 6) or IGF-I (2 nM, lanes 3 and 7), or stimulated for 4 h with $CoCl_2$ (lanes 4 and 8). Nuclear cell extracts were mixed with the ^{32}P-labeled W18 DNA probe in the presence of a 100-fold molar excess of M18, a mutated W18 oligonucleotide having three base pair substitutions (lanes 1–4). Alternatively, a 100-fold molar excess of non-labeled W18 was used (lanes 5–8). The mixtures were subjected to EMSA. Oligonucleotide W18 but not M18 ablated the inducible and constitutive complexes, thereby demonstrating the specificity of the interaction with W18.

IGF-I shares with insulin many signaling components and induces the expression of Glut1, EPO and VEGF, we also tested the ability of IGF-I to induce the formation of a cellular HRE-binding complex. Hep-G2 cells were treated with either insulin (25–100 nM) or $CoCl_2$. Cell extracts were mixed with a radiolabeled oligonucleotide W18, corresponding to the HRE of the erythropoietin gene (Wang and Semenza, 1993), and the mixtures were subjected to electrophoretic mobility shift assay (EMSA). Within 4–8 h, insulin induced the formation of a complex with HRE in a dose-dependent manner (Figure 3). Similarly, L8 cells were treated either with insulin, IGF-I or $CoCl_2$ and cell extracts were subjected to EMSA. In these cells, both insulin and IGF-I induced the formation of a complex with HRE (Figure 6B, lanes 1–4). The mobility of the complexes induced by insulin and IGF-I was the same as that of the known, $CoCl_2$-induced HIF-1α/ARNT complex, suggesting a common composition. The specificity of interaction between the radiolabeled oligonucleotide W18 and the insulin- and IGF-I-induced cellular factors was then tested by competition with non-labeled oligonucleotides. L8 cells were treated as before with insulin, IGF-I or $CoCl_2$. Cell extracts were mixed with radiolabeled oligonucleotide W18 in the presence of a 100-fold molar excess of non-labeled oligonucleotide W18 or M18. Oligonucleotide M18 has three base-pair substitutions that abolish its ability to interact with HIF-1α ARNT (Wang and Semenza, 1993). As expected, oligonucleotide W18 ablated the complexes of HIF-1 with radiolabeled W18, whereas oligonucleotide M18 did not prevent the formation of the specific HIF-1 complexes (Figure 4).

HRE is sufficient to confer transcription activation by insulin and IGF-I

The ability of insulin and IGF-I to trigger the formation of a complex that binds *in vitro* to HRE suggested that this complex participates in an insulin- and IGF-I-induced transcription regulation. To test this possibility and to find if HRE by itself is sufficient as an insulin- and IGF-I-responsive enhancer of transcription, we employed two tk-luciferase reporter vectors, $pEPO_5tkLuc$, consisting of five copies of the 18mer HRE from the EPO gene (Wang and Semenza, 1993), and $pPGK_2tkLuc$, consisting of the two tandem HREs present in the PGK gene. As a negative control, we employed $pmPGK_2tkLuc$, a homolog of $pPGK_2tkLuc$, containing two copies of a mutated HIF-binding element (Firth *et al*., 1994). Hep-G2 cells and rat L8 myoblasts were transfected with these reporter vectors and treated with either insulin, IGF-I or $CoCl_2$. It was found that insulin and IGF-I induced transcriptional activity in a dose-dependent manner in cells transfected with either one of the two reporter vectors, while no induction was

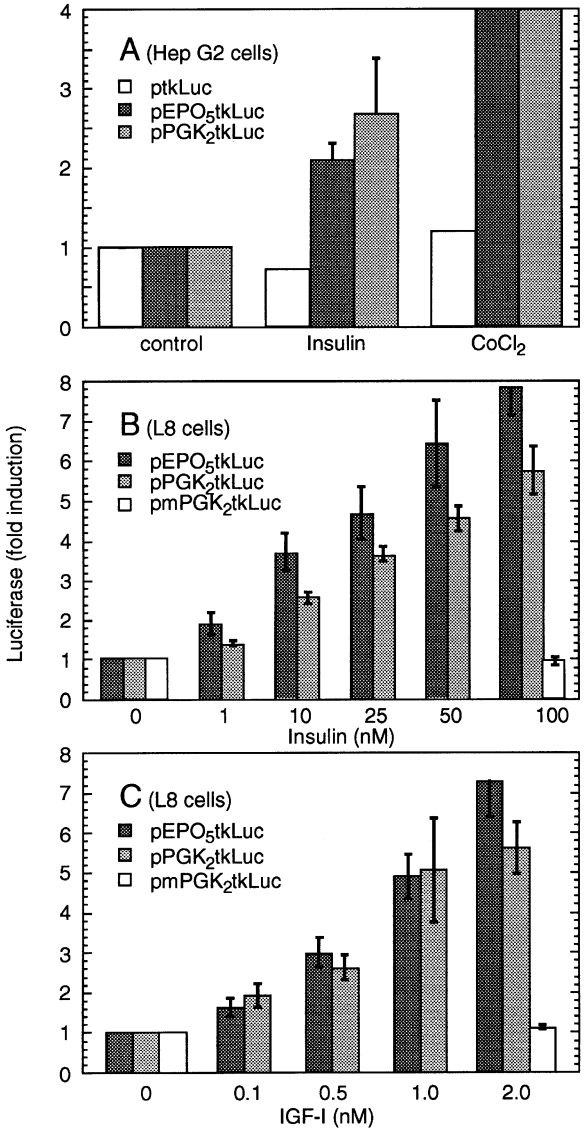

Fig. 5. Transcription activation by insulin and IGF-I of HRE-containing reporter vectors. (**A**) Hep-G2 cells were transfected either with a control vector ptkLuc, with $pEPO_5tkLuc$, a vector containing five copies of the EPO HRE, or with the $pPGK_2tkLuc$, a reporter vector consisting of two copies of the PGK HRE. After 24 h, cells were stimulated for 24 h with either insulin or $CoCl_2$. Significant induction of $pEPO_5tkLuc$ ($P < 0.001$, $n = 9$) and $pPGK_2tkLuc$ ($P < 0.05$, $n = 4$) was obtained with insulin as compared with unstimulated cells. (**B**) Murine L8 myoblasts were transfected similarly either with the two reporter vectors or with $pmPGK_2tkLuc$, a control vector containing a mutated HRE. Cells were induced as in (A). A significant induction of luciferase activity with insulin was seen with $pEPO_5tkLuc$ and $pPGK_2tkLuc$ vectors, while no induction was obtained with the control mutated vector. (**C**) L8 myoblasts were transfected similarly with the two reporter vectors or with the control vector. Cells were incubated as in (A) with the indicated concentrations of IGF-I. A significant induction of luciferase activity was seen with $pEPO_5tkLuc$ and $pPGK_2tkLuc$ vectors, while no induction was obtained with the control mutated vector.

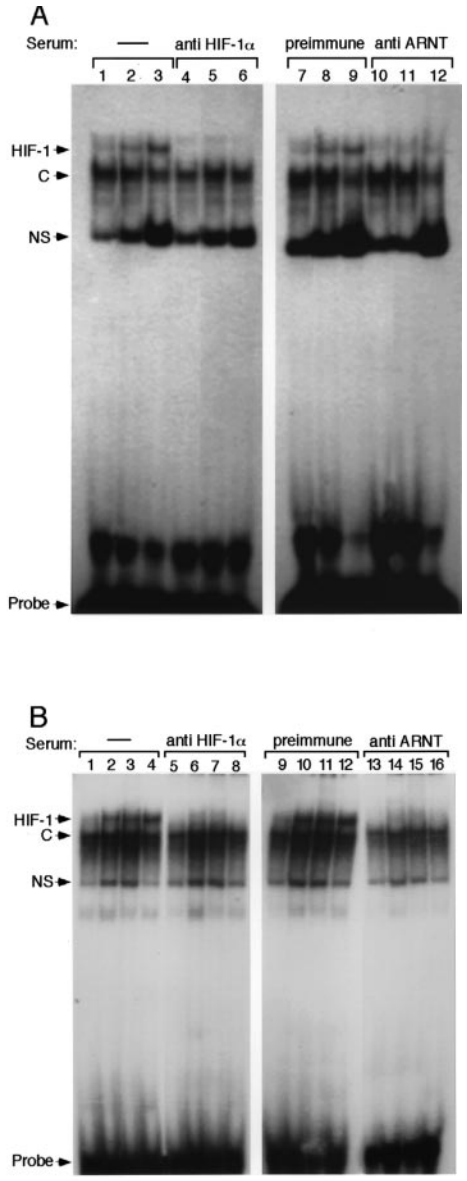

Fig. 6. The complex induced by insulin and IGF-I is HIF-1α/ARNT. (**A**) Serum-starved Hep-G2 cells were kept unstimulated in a serum-free medium for 8 h (lanes 1, 4, 7 and 10), or stimulated for 8 h in a serum-free medium with insulin (lanes 2, 5, 8 and 11) or $CoCl_2$ (lanes 3, 6, 9 and 12). Nuclear cell extracts were mixed with a ^{32}P-labeled double-stranded DNA probe as in Figure 3, followed by incubation with the indicated sera. The mixtures were subjected to EMSA. (**B**) Serum-starved L8 cells were kept unstimulated (lanes 1, 5, 9 and 13) or were stimulated for 4 h in a serum-free medium with insulin (lanes 2, 6, 10 and 14), IGF-I (2 nM, lanes 3, 7, 11 and 15) or $CoCl_2$ (lanes 4, 8, 12 and 16). Nuclear cell extracts were mixed with the DNA probe and sera as in (A) and subjected to EMSA. Both antisera, but not the pre-immune serum, prevented the induction of the *in vitro* complex by insulin, IGF-I and $CoCl_2$, thereby identifying the insulin- and IGF-I-induced transcription complexes as HIF-1α/ARNT.

obtained in cells transfected with the mutated vector $pmPGK_2tkLuc$. Thus, at the highest concentration used, insulin and IGF-I induced luciferase activity in L8 cells transfected with $pPGK_2tkLuc$ ~6-fold, and in cells transfected by $pEPO_5tkLuc$ ~8-fold (Figure 5). Therefore, we concluded that insulin and IGF-I share with hypoxia the ability to activate transcription through the HRE.

The transcription complex induced by insulin and IGF-I is HIF-1α/ARNT

To determine the composition of the insulin- and IGF-I-induced complexes, extracts of hormone-treated cells were mixed with a polyclonal antiserum to HIF-1α and then subjected to a mobility shift assay (Figure 6). The antiserum blocked the formation of the complexes induced by either insulin or $CoCl_2$ in Hep-G2 cells (Figure 6A,

lanes 5 and 6), while the complexes were not affected by a pre-immune serum (Figure 6A, lanes 8 and 9). The same results were obtained in L8 cells. Antiserum to HIF-1α blocked the formation of complexes induced by insulin, IGF-I or $CoCl_2$ (Figure 6B, lanes 6–8) while the complexes were not affected by pre-immune serum (Figure 6B, lanes 10–12). Similarly, formation of the *in vitro* complexes was blocked by an antiserum directed against ARNT both in Hep-G2 cells (Figure 6A, lanes 11 and 12) and in L8 cells (Figure 6B, lanes 14–16). Based on these results, we concluded that both insulin and IGF-I induce the formation of a HIF-1α/ARNT complex that interacts with its specific response element.

The induction of genes by insulin is ARNT dependent

To determine the role of the HIF-1α/ARNT transcription complex in transcription regulation of the genes for glucose transporters, glycolytic enzymes and VEGF *in vivo*, we resorted to the murine hepatoma Hepa-1c1c7 cells and their mutant cell line c4 which carries a mutation in the PAS region of ARNT. This mutation causes an impaired response of the cells to xenobiotic agents and a reduced HIF-1α/ARNT binding to DNA, and is reverted by transfection of c4 cells with a functional ARNT expression vector (Numayama *et al*., 1997). Hepa-1c1c7 and c4 cells were treated with insulin or $CoCl_2$, and the mRNA level of glucose transporters, glycolytic enzymes and VEGF was determined by RNA blotting. (Because hepatocytes lack an IGF-I receptor, our studies with these cells were restricted to insulin.) Indeed, we found that both the basal and the insulin-induced mRNA levels were significantly lower in c4 cells as compared with their parental Hepa-1c1c7 cells (Figure 7). These results correlate with recent studies showing that basal expression level of the glycolytic enzymes in ARNT–/– cells was low and was not induced further by hypoxia (Maltepe *et al*., 1997).

The role of ARNT in activation of HRE-containing genes by insulin was then studied by transfecting c4 and Hepa-1c1c7 cells with the pALDA-luciferase reporter vector and the two HRE-luciferase reporter vectors. Here too, $CoCl_2$ did not induce luciferase activity in c4 cells, while insulin was still able to induce some luciferase activity. However, both the basal and the insulin-induced activity were significantly lower in c4 cells as compared with the activity in the parental Hepa-1c1c7 cells (Figure 8). In addition, we studied the role of ARNT in insulin-induced activation of HRE-containing genes by transfecting c4 cells with the ARNT expression vector pcDNA3-ARNT, together with the $pPGK_2tkLuc$ reporter. Indeed, it was found that both the basal and the insulin-induced transcriptional activity in these cells increased significantly (4.2-fold, $P < 0.001$, and 8.5-fold, $P < 0.005$, respectively) as compared with cells transfected with the control vector pcDNA3 (Figure 9A). Similarly, c4 cells transfected with the ARNT expression vector together with a reporter vector consisting of the VEGF promoter and luciferase cDNA (pVEGF-Luc) exhibited a 2.1-fold and 3.9-fold ($n = 3$, $P < 0.001$ in both cases) higher levels of luciferase as compared with c4 cells transfected with the control expression vector pcDNA3 (Figure 9B).

To confirm further the role of HIF-1α/ARNT in the insulin-induced expression of HRE-containing genes, we compared the level of VEGF in culture supernatants of insulin-induced c4 cells and vT{2} cells, a c4 derivative in which ARNT was rescued by stable transfection of an ARNT expression vector (Hoffman *et al*., 1991). Supernatants of insulin-induced vT{2} cultures increased the number of the VEGF-responsive EA.hy926 cells by 2.2-fold ($n = 6$, $P < 0.001$) as compared with untreated cells. In contrast, supernatants of insulin-induced c4 cultures barely increased the EA.hy926 cell number (1.1-fold). Insulin itself, as well as supernatants of untreated c4 cultures, did not support the proliferation of EA.hy926 cells (Figure 10). These results indicate that expression of VEGF in response to insulin depends by and large on the presence of a functional ARNT.

Taken together, these observations show unequivocally that the HIF-1α/ARNT transcription complex is induced by insulin and participates in the insulin-induced transcription activation of at least some HRE-containing genes.

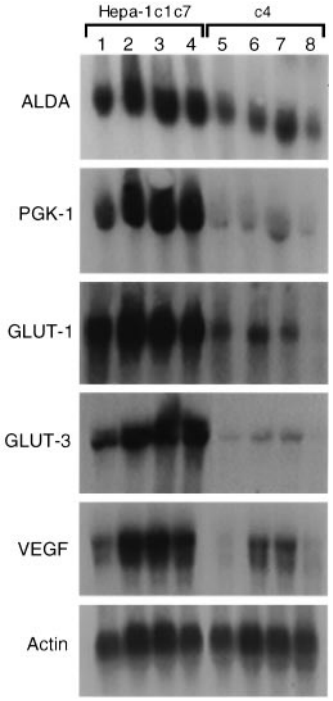

Fig. 7. Induction of glucose transporters, glycolytic enzymes and VEGF by insulin is impaired in c4 cells having a mutated form of ARNT. Serum-starved Hepa-1c1c7 cells and c4 cells were stimulated with insulin (lanes 2, 16 h; 3, 24 h; 6, 16 h; and 7, 24 h), or with $CoCl_2$ (lanes 4 and 8, 24 h). Control cells were kept in a serum-free medium without stimulants for 24 h (lanes 1 and 5). Induction of several genes coding for the glycolytic enzymes ALDA and PGK, the glucose transporters Glut1 and Glut3, and VEGF was determined by RNA blot analysis with murine DNA probes (see Figure 1 for details). Induction by $CoCl_2$ was abolished in c4 cells, while both the basal level and the induction by insulin were reduced considerably in these cells.

Insulin and IGF-I stabilize the HIF-1 complex

Hypoxia induces the HIF-1α/ARNT complex, at least in part, by inhibiting the ubiquitin-dependent rapid degradation of HIF-1α (Salceda and Caro, 1997). We studied the mechanism by which insulin and IGF-I induce the HIF-1α/ARNT complex. Insulin did not affect the steady-state level of HIF-1α mRNA, as determined by RNA blot analysis (not shown). Therefore, we investigated whether

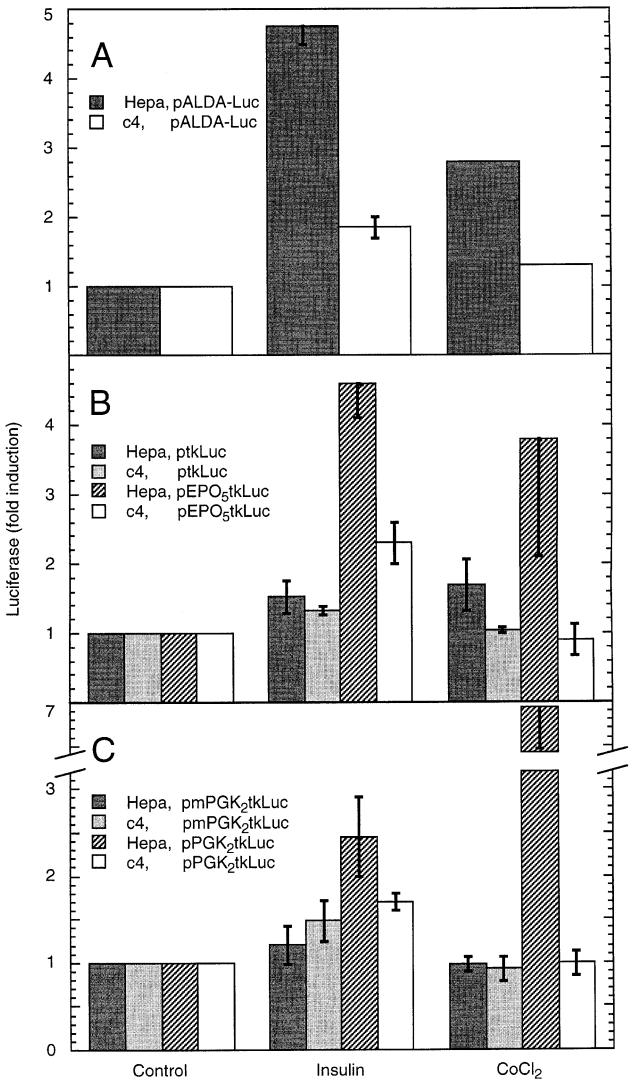

Fig. 8. Transcription activation by insulin is ARNT dependent. Parental Hepa-1c1c7 cells (Hepa) and c4 cells carrying a mutated form of ARNT were transfected with the indicated vectors (described in the legend to Figure 4). After 24 h, cells were stimulated for 24 h with insulin or $CoCl_2$. The level of luciferase induction was determined as compared with the control unstimulated cells. (**A**) Cells transfected with pALDA-Luc; (**B**) cells transfected with control ptkLuc or with $pEPO_5tkLuc$; (**C**) cells transfected with the inactive mutant $pmPGK_2tkLuc$ or with $pPGK_2tkLuc$. Upon transfection with any one of the functional reporter vectors, a considerable reduction of luciferase induction was obtained in c4 as compared with the Hepa-1c1c7 cells. No induction was obtained in either cell type with the control vectors.

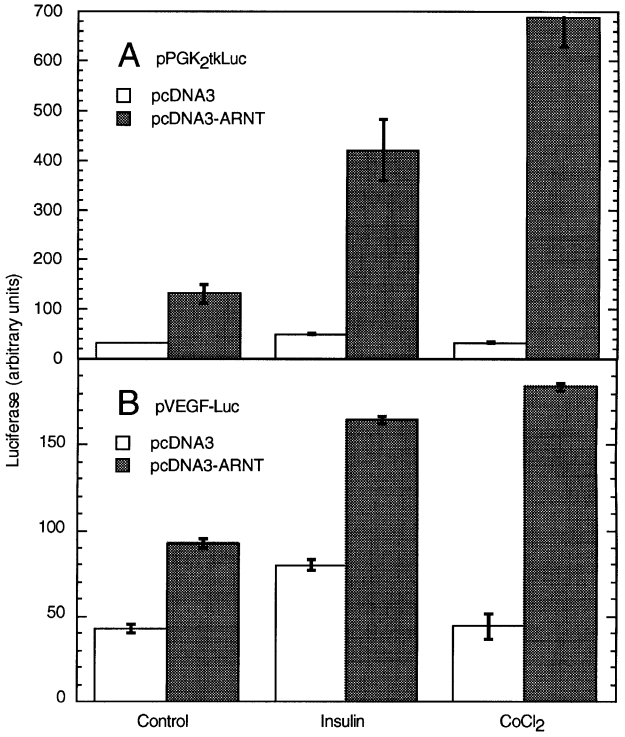

Fig. 9. Rescue of the insulin-induced transcription activation in c4 cells by transient expression of ARNT. (**A**) c4 cells were transfected with the reporter vector $pPGK_2tkLuc$ together with either the ARNT expression vector pcDNA3-ARNT or with the control vector pcDNA3. After 24 h, cells were stimulated for 24 h with insulin or $CoCl_2$. The level of luciferase induction was determined as compared with unstimulated cells. No significant induction of luciferase activity by either insulin or $CoCl_2$ was seen in c4 cells transfected with the control pcDNA3 vector. In contrast, both the basal and the insulin-induced luciferase activities were significantly increased ($P < 0.001$ and $P < 0.005$, respectively, $n = 3$) in the pcDNA3-ARNT-transfected cells, indicating that ARNT is essential for this insulin-induced activity. (**B**) c4 cells were transfected with the reporter vector pVEGF-Luc together with either the ARNT expression vector pcDNA3-ARNT or with the control vector pcDNA3. After 24 h, cells were stimulated for 24 h with insulin or $CoCl_2$. Cells transfected with pcDNA3-ARNT expression vector together pVEGF-Luc exhibited 2.1- and 3.9-fold ($n = 3$, $P < 0.001$ in both cases) higher levels of luciferase as compared with c4 cells transfected with the control pcDNA3 vector, indicating that ARNT is essential for transcription activation of VEGF by insulin.

insulin and IGF-I induce the formation of a HIF-1α/ARNT complex by attenuating the constitutive rapid degradation of HIF-1α. L8 cells were treated for 4 h with either insulin or IGF-I, or kept for 4 h under hypoxic conditions (1% O_2, 5% CO_2). The stimulants were then removed and the cultures kept for an additional 4 h in the presence or absence of the proteasome inhibitor MG-132. As shown in Figure 11, the HIF-1α/ARNT complex was obtained after a 4 h stimulation with insulin, IGF-I or hypoxia (Figure 11, lanes 2, 6 and 10, respectively). These complexes decayed rapidly and could not be detected 4 h after removal of the stimulants (Figure 11, lanes 3, 7 and 11, respectively). However, when the stimulants were removed and the cells placed in a medium containing 50 μM MG-132, the complexes were not degraded (Figure 11, lanes 4, 8 and 12 for insulin, IGF-I and hypoxia, respectively). We concluded that insulin and IGF-I resemble hypoxia in their ability to induce the accumulation of HIF-1α, as manifested by the formation of the HIF-1α/ARNT complex. The common mechanism of these stimulants involves inhibition of degradation of Hif-1 by the ubiquitin–proteasome pathway.

Glucose shortage rapidly increases the steady-state level of HIF-1α mRNA, thereby inducing the same set of genes as hypoxic stress (Maltepe *et al.*, 1997). Since insulin increases the rate of glucose uptake and glycolysis, we tested its effect on the glucose content in the culture medium. Incubation of Hep-G2 cells for 24 h with either insulin or $CoCl_2$ increased glucose utilization. However, the overall level of glucose in the culture medium after 24 h remained sufficiently high (>90% of the level at

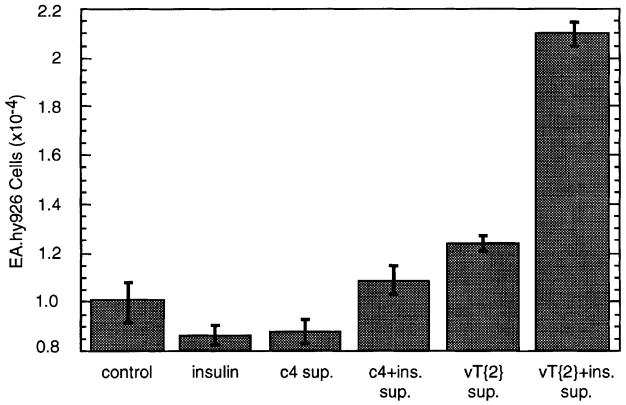

Fig. 10. ARNT is essential for efficient VEGF induction by insulin. Insulin-induced VEGF was measured in culture supernatants of the ARNT-defective c4 cells and vT{2} cells, a c4 derivative in which ARNT was rescued by stable transfection of an ARNT expression vector. VEGF activity was measured by a proliferation assay of the VEGF-responsive EA.hy926 cell line. Conditioned media of insulin-induced vT{2} cultures increased the number of the VEGF-responsive EA.hy926 cells by 2.2-fold ($n = 6$, $P < 0.001$) as compared with untreated cells. In contrast, supernatants of insulin-induced c4 cultures increased EA.hy926 cell number by only 1.1-fold. Insulin itself, as well as supernatants of untreated c4 cultures, did not support the proliferation of EA.hy926 cells. These results indicate that expression of VEGF in response to insulin depends by and large on the presence of a functional ARNT.

time 0) to exclude glucose shortage as a reason for induction of the HIF-1α/ARNT complex.

Discussion

This work forges the first link between HIF-1α/ARNT and some cellular and systemic responses to insulin. While the HIF-1α/ARNT complex has been shown previously to induce a remarkably diverse set of target genes, it was assumed that it is associated only with hypoxic stress. Our findings broaden considerably the scope of activity of this already highly pleiotropic complex. We find that HIF-1α/ARNT is involved not only in short-term metabolic responses to insulin, but also in long-range responses such as the induction of *VEGF*. Other cellular responses to insulin may now be studied for their possible association with the induction of HIF-1α/ARNT.

A comprehensive analysis of several transcripts and response elements was carried out in order to demonstrate the ability of insulin to induce target genes. We confirmed that genes involved in glucose transport (*Glut1* and *Glut3*), glycolysis (e.g. *ALDA* and *PGK*) and a gene which is essential for endothelial sprouting (*VEGF*) are induced by insulin in several cell lines. These genes are known to be induced by hypoxic stress as well. The assignment of the HIF-1α/ARNT complex to the insulin responses is based on detection of enhanced gel shift activity of the HIF-1α/ARNT DNA-binding site and its sensitivity to antibodies directed against HIF-1α and ARNT. In addition, binding of HIF-1α/ARNT appears to be sufficient for induction, as a multimer of the binding site (HRE) induced expression of a reporter gene following insulin stimulation. Finally, ARNT appears to be a critical partner in this transcription complex, as a significantly lower response to insulin was seen in c4 cells that are devoid of a functional ARNT. Indeed, both transient and stable transfection of c4 cells

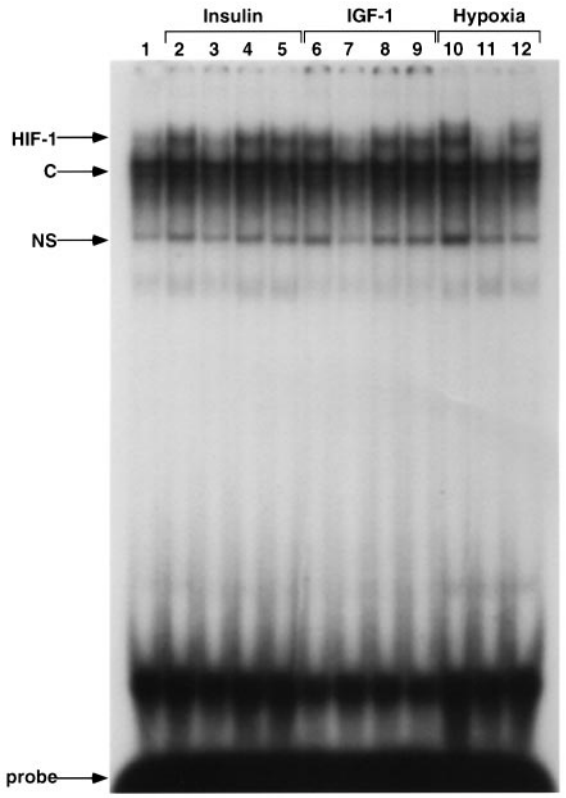

Fig. 11. Insulin and IGF-I reduce degradation of the HIF-1α/ARNT complex by inhibition of the ubiquitin–proteasome degradation pathway. L8 cells were left untreated for 8 h (lane 1) or treated for 4 h with either insulin, IGF-I or hypoxic (1% O_2, 5% CO_2) conditions (lanes 2, 6 and 10). The stimulants were then removed and the cultures kept for an additional 4 h (lanes 3, 7 and 11). Alternatively, cells treated with insulin and IGF-I for 4 h were washed and placed for 4 h in a medium containing the proteasome inhibitor MG-132 (lanes 4 and 8). Similarly, MG-132 (50 μM) was added to the hypoxic cells 15 min before placing them for 30 min in a normoxic atmosphere (lane 12). As a control, the stimulants were replaced after 4 h with fresh stimulants and incubation continued for an additional 4 h (lanes 5 and 9). MG-132 inhibited the rapid degradation of the HIF-1α/ARNT complex induced by insulin, IGF-I and hypoxia.

with an ARNT expression vector restored the responsiveness to insulin and $CoCl_2$, as demonstrated with reporter vectors and with expression of VEGF. These observations identify HIF-1α/ARNT unequivocally as a transcription complex induced by insulin, in addition to its known involvement in the response to hypoxic stress.

It is noteworthy that not only the insulin-induced transcriptional activity but also the basal transcriptional activity was diminished in the c4 cells and was elevated upon transfection of these cells with an ARNT expression vector. This result correlates with the observation that the basal transcription level of hypoxia-inducible genes is diminished upon targeted disruption of either HIF-1α or ARNT genes (Maltepe *et al*., 1997; Iyer *et al*., 1998; Ryan *et al*., 1998). Therefore, it is possible to view ARNT as a housekeeping gene that together with HIF-1α maintains under normoxic conditions a basal induction level of a set of genes necessary for providing the cellular energy demands.

IGF-I shares with insulin many of the signaling components, and the present study shows that induction of HIF-1α/ARNT is no exception. The ability of IGF-I to induce

HIF-1α/ARNT complexes *in vitro* (Figures 3 and 5) and to induce the transcriptional activity of HRE reporter vectors (Figure 5) suggests that IGF-I-induced transcription of *Glut1*, *EPO*, *VEGF* and probably other hypoxia-inducible genes is mediated by the same HIF-1α/ARNT-dependent mechanism. Since insulin cross-reacts with the IGF receptor, it was important to check whether the transcription activation is indeed mediated through the insulin receptor. The insulin-induced transcription activation of hepatic cells (Hep-G2 and Hepa-1c1c7) which lack an IGF receptor eliminates the possibility that the observed effects of insulin were mediated through the IGF receptor.

The physiological significance of the insulin-induced expression of glucose transporters and glycolytic enzymes is not known, since increased expression of Glut1, Glut3 and these genes is not the major mechanism by which insulin elevates the rate of glycolysis. Rather, insulin increases glucose flux and subsequent glycolysis by inducing a rapid translocation of Glut4 to the cytoplasmic membrane, thereby increasing lipid synthesis in adipose tissues. In energy-consuming tissues such as muscles, the ratio of ATP to ADP determines the rate of glycolysis through the allosteric regulation of phosphofructokinase (Pilkis *et al*., 1988). However, both hypoxia and insulin significantly increase the level of the glucose transporters and the glycolytic enzymes (Walker, 1990; Pilkis and Granner, 1992; Bunn and Poyton, 1996), suggesting that higher levels of these proteins somehow contribute to increased glycolysis. The induction of EPO and VEGF by insulin and IGF-I is, on the other hand, probably a manifestation of their systemic effects.

The mechanism of HIF-1α induction by hypoxic stress, and now insulin and IGF-I, is still elusive. Hypoxic stress or treatment with insulin do not alter the transcription of HIF-1α. Rather, hypoxic stress increases the overall amount of HIF-1α by stabilization of the protein (Huang *et al*., 1996). Thus, under normoxic conditions, rapid, ubiquitin-dependent degradation of HIF-1α is taking place, while hypoxic stress reduces the ubiquitin-dependent degradation of HIF-1α (Salceda and Caro, 1997). We demonstrate here that a similar stabilization mechanism exists in the case of insulin and IGF-I induction of HIF-1.

In spite of the activation of common target genes by the responses to hypoxic stress, insulin and IGF-I, there should be target genes that are unique to each of the signals. Additional factors, induced or activated by some of the stimulants, may cooperate with HIF-1α/ARNT to trigger the induction of specific target genes. Indeed, the generation of chimeric bHLH-PAS proteins in *Drosophila* has suggested that the target specificity of these proteins is determined by binding of the bHLH-PAS complex to other transcription factors (Zelzer *et al*., 1997). The higher steady-state level of the various transcripts in cells treated with insulin resulted, at least in part, from induction through HIF-1α/ARNT. However, additional mechanisms may be involved. For example, it has been demonstrated for the hypoxic response that stabilization of VEGF mRNA is also a significant component in regulating the resulting protein levels (Levy *et al*., 1996). This may be another level at which the regulation of the different target genes for the responses to hypoxic stress and hormones will vary.

It is interesting to speculate when in the course of evolution HIF-1α/ARNT-mediated hypoxic response on the one hand, and the insulin response on the other, first appeared. The identification of insulin receptors in *Caenorhabditis elegans* (Kimura *et al*., 1997) and *Drosophila* (Petruzzelli *et al*., 1986) suggests that this system appeared early in metazoan evolution to regulate metabolic responses. The PAS module appears in the context of different proteins such as kinases (but not transcription factors) already in bacteria (Zhulin *et al*., 1997). As no bHLH-PAS proteins have been identified in the yeast genome, while they are present in multicellular organisms such as *C.elegans*, *Drosophila* and vertebrates, it appears that the emergence of bHLH-PAS proteins may reflect a feature that is unique to multicellular organisms. Since HIF-1α/ARNT mediates the response to hypoxia not only at the systemic level but also at the cellular level, it is possible that in primitive metazoan organisms HIF-1α/ARNT initially fulfilled only this cellular role, and was recruited for the systemic response to hypoxia at a second phase. The HIF-1α/ARNT-mediated responses of insulin affect a broad range of cells. However, insulin is produced by a specialized tissue and, therefore, it may have also evolved at this second phase.

Finally, the initial cellular signals elicited by hypoxia, insulin or IGF-I are not known and therefore the mechanism by which these signals stabilize HIF-1α remains intangible. The finding that insulin and IGF-I act in a manner similar to hypoxic stress may help in elucidating early steps of these important signaling pathways.

Materials and methods

Cell cultures and reagents

Hep-G2 human hepatocellular carcinoma cells (ATCC HB 8095) were grown in minimal essential medium (MEM) and 10% fetal bovine serum (FBS), L8 rat skeletal muscle myoblasts (ATCC CRL 1769) were grown in Waymuth medium and 15% FBS; Hepa-1c1c7 rat hepatoma cells, their mutant c4 cells and vT{2} cells (derived from c4 cells by stable transfection with an ARNT expression vector) were kindly provided by O.Hankinson. Hepa-1c1c7 and c4 cells were grown in MEM-α and 10% FBS. VT{2} cells were grown in MEM-α, 10% FBS and neomycin (400 μg/ml). T47D human ductal breast carcinoma (ATCC HTB 133) cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F12 and 10% FBS. Human endothelial EA.hy926 cells, kindly provided by C.-J.S.Edgell, were grown in DMEM, 10% FBS and HAT (Edgell *et al*., 1983). Antiserum to human HIF-1α was raised in a rabbit immunized with SDS–PAGE-purified HIF-1α, produced in *Escherichia coli*. Antiserum to the 300 N-terminal amino acid fragment, common to trout and human ARNT, was kindly provided by R.S.Pollenz. Human IGF-I was from Peprotech (Rocky Hill, NJ), and bovine insulin from Sigma (Israel). The proteasome inhibitor MG-132 was from Calbiochem (San Diego, CA).

RNA extraction and RNA blot analysis

Cells were seeded at a density adjusted to reach 50% confluency at the end of the experiment. Cell cultures were serum starved (16 h) prior to stimulation with serum-free medium containing either insulin or $CoCl_2$. Total RNA was isolated with the TRI reagent kit (Molecular Research Center Inc.). Samples of RNA (15 μg) were resolved by electrophoresis through 1% agarose gel in MOPS-formaldehyde buffer, transferred to nylon membrane (Hybond N, Amersham) in 20× SSC buffer and the membrane was then heated for 2 h at 80°C in a vacuum oven. The membrane was pre-hybridized (6 h, 42°C) with denatured salmon sperm DNA (100 μg/ml in 50% formamide, 5× SSC, 4× Denhardt's solution and 0.5% SDS). A [^{32}P]dCTP DNA probe (1×10^6 c.p.m./ml), prepared by random priming, was then added and hybridization continued for 18 h at 42°C. The membrane was then washed at room temperature (1× SSC, 0.1% SDS twice, 0.25× SSC, 0.1% SDS, and 0.1× SSC, 0.1% SDS twice, 30 min each wash) and autoradiographed. Blots were then re-hybridized with ^{32}P-labeled probe corresponding to either human or rat actin to show equal amounts of RNA in the blot.

Preparation of nuclear extracts

Hep-G2 and L8 cells, grown as before, were serum starved and then stimulated with insulin (100 nM or the indicated concentrations), IGF-I or $CoCl_2$ (166 μM) for 4 or 8 h. Cells were washed three times with ice-cold phosphate-buffered saline (PBS) and immediately frozen in liquid nitrogen. Cell pellets were resuspended in five packed cell volumes of buffer A [20 mM Tris pH 7.6, 10 mM KCl, 0.2 mM EDTA, 20% (by vol.) glycerol, 1.5 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 0.4 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_3VO_4, 2 μg/ml each of leupeptin, pepstatin and aprotinin]. Nuclei were pelleted (2500 *g*, 10 min) and resuspended in two packed cell volumes of buffer B (identical to buffer A except that KCl was increased to 0.42 M). Nuclear debris was removed by centrifugation (15 000 *g*, 20 min) and aliquots of the supernatant were frozen in liquid nitrogen and stored at –80°C. Protein concentration was determined by a Bradford assay (Bio-Rad) using bovine serum albumin as standard.

Electrophoretic mobility shift assay

Sense and antisense oligonucleotides corresponding to the HRE sequence of the EPO gene (W18; Wang and Semenza, 1993) were annealed and the double-stranded oligonucleotide (10 pmol) was labeled with [^{32}P]dCTP (300 Ci/mmol) and Klenow fragment (New England Biolabs). Free nucleotides were removed by a spin column (Advanced Genetic Technologies Corp.). Nuclear extracts (5 μg of protein) of cells treated with insulin (100 nM or the indicated concentrations), IGF-I or $CoCl_2$ (166 μM) were pre-incubated (5 min at room temperature) together with poly(dI–dC) (300 ng, Pharmacia) and denatured calf thymus DNA (50 ng, Sigma) in 20 μl of buffer consisting of Tris–HCl (pH 7.5, 10 mM), 100 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT and 5% (by vol.) glycerol. The labeled probe (3×10^4 c.p.m.) was then added and incubation continued for an additional 15 min. For competition assays, the labeled probe was mixed with a 100-fold molar excess of oligonucleotide W18 or M18 (Wang and Semenza, 1993) and then added to the nuclear extract. For shift assays in the presence of antibodies, pre-immune serum, or antiserum to either HIF-1α or ARNT (2 μl each) was added at a final dilution of 1:3 immediately after addition of the probe and the mixture was kept on ice for 20 min. Reaction mixtures were then loaded onto 5% non-denaturing polyacrylamide gels. Electrophoresis was performed at 185 V in 0.5× TBE (40 mM Tris–HCl, 45 mM boric acid and 2.5 mM EDTA). Gels were vacuum dried and autoradiographed overnight at –80°C.

Preparation of probes by RT–PCR

Probes for RNA blot analysis were prepared by RT–PCR with either human or murine RNA. Reverse transcription was carried out in a 20 μl volume using RNase H–reverse transcriptase (SuperScript II, Gibco-BRL) with 1 μg of $(N)_6$ random primer (New England Biolabs) according to the manufacturer's instructions. An aliquot (2 μl) of the reverse transcription product was used for PCR with Vent DNA polymerase (New England Biolabs) and the following sense and antisense primers: huALDA mRNA, DDBJ/EMBL/GenBank accession No. X05236, nucleotides 194–213 and 1114–1094 (sense and reverse primers, respectively); huPGK mRNA, V00572, nucleotides 161–183 and 1163–1142; huGlut1 mRNA, K03195, nucleotides 526–548 and 1140–1121; huGlut3 mRNA, M20681, nucleotides 718–737 and 1814–1794; muGlut3 mRNA, M75135, nucleotides 808–828 and 1194–1171; huVEGF mRNA, M32977, nucleotides 101–120 and 630–611; huActin mRNA, J00068, nucleotides 453–472 and 791–771; muActin mRNA, J00691, nucleotides 1670–1691 and 2452–2431. Primers of human ALDA, Glut1, PGK and VEGF were used with mouse RNA as well.

Reporter vectors

pALDA-Luc, a vector consisting of an ALDA promoter fused to luciferase, was prepared as follows. A DNA sequence corresponding to the functional promoter of the human ALDA gene (DDBJ/EMBL/GenBank accession No. X12447; Semenza *et al*., 1996) was prepared by PCR of human genomic DNA with Vent DNA polymerase. The sense primer corresponded to positions 2460–2480 plus a *Bam*HI site at the 5′ end. The reverse primer corresponded to positions 3020–3040 plus an *Xho*I site at the 5′ end. The resulting PCR product was digested with restriction enzymes and ligated in front of Luc in the *Hin*dIII and *Xho*I sites of pLuc. The fidelity of the product was confirmed by DNA sequence analysis.

pVEGF-Luc, a vector consisting of a VEGF promoter fused to luciferase, was prepared as follows. A 3.4 kb DNA fragment corresponding to the functional promoter of the human VEGF gene was excised by *Hin*dIII and *Xho*I from plasmid p3.4CAT, kindly provided by B.-Z.Levi (Cohen *et al*., 1996). It was then ligated in front of Luc in the *Hin*dIII–*Xho*I sites of pLuc. The fidelity of the product was confirmed by DNA sequence analysis.

$pEPO_5tkLuc$, a vector consisting of five copies of the HER of the EPO gene (Wang and Semenza, 1993) fused to the tk promoter and to a luciferase cDNA, was prepared by ligating pre-cut double-stranded oligonucleotide to the *Hin*dIII–*Bam*HI sites of ptkLuc (Zelzer *et al*., 1997). $pPGK_2tkLuc$, a vector consisting of two copies of the HRE from the PGK gene ($pPGK_2tkLuc$; Firth *et al*., 1994) fused to the tk promoter (Boshart *et al*., 1992) and to a luciferase cDNA, was prepared by ligating pre-cut double-stranded oligonucleotide to the *Hin*dIII–*Bam*HI sites of ptkLuc. $pmPGK_2tkLuc$, a homolog of $pPGK_2tkLuc$, containing two copies of a mutated HIF-binding element (Firth *et al*., 1994), was prepared similarly for use as a negative control. pcDNA3 was from Invitrogen and pcDNA3-ARNT was kindly provided by E.Huang.

Transient transfections

Hep-G2 cells were transfected with purified vectors (Wizard midi prep, Promega) by a modified DEAE–dextran method (Sompayrac and Danna, 1981). Cells (5×10^6), resuspended in 750 μl of TD (25 mM Tris pH 7.4, 140 mM NaCl, 0.7 mM K_2HPO_4 and 5 nM KCl), were incubated for 15 min at room temperature with a mixture of DEAE–dextran (600 μg, Pharmacia) and two reporters (1.8 μg of Luc vector and 0.2 μg of pSVβGal vector, Promega). The cells were then washed twice with serum-containing medium and divided into 35 mm plates. L8, Hepa-1c1c7 and c4 cells were seeded onto 35 mm plates at a density adjusted to reach 40% confluency prior to transfection. These cells were transfected using the FuGENE 6 reagent (Boehringer Mannheim) and reporter DNA concentration as above, according to the manufacturer's instructions. Transfections of c4 cells were also done with either pcDNA3 or pcDNA3-ARNT (0.2 μg) together with $pPGK_2tkLuc$ (1.6 μg) and with pSVβGal (0.2 μg) with the aid of FuGENE 6. All transfections were carried out in duplicate with aliquots of transfection mixture from a single pool. At 24 h post-transfection, cells were washed with PBS and treated for 24 h with either insulin (100 nM or the indicated concentrations), IGF-I or $CoCl_2$ (166 μM) in a serum-free medium. Cells were then collected into 0.25 M Tris pH 7.5 (250 μl), frozen three times in liquid nitrogen and thawed at room temperature. Insoluble cell debris was removed by centrifugation (15 000 *g*, 1 min) and luciferase and β-galactosidase activities were measured in separate aliquots. All luciferase readings were normalized by the β-galactosidase values to correct for differences in transfection efficiency.

Endothelial cell proliferation assay of VEGF

VEGF activity was determined by a proliferation assay of the VEGF-responsive human EA.hy926 cells (Punglia *et al*., 1997). Briefly, cultures of 10^6 cells (either c4 or vT{2} cells) in 6 cm dishes were kept in a serum-free medium (2 ml) for 24 h, the medium was replaced and VEGF was induced by addition of insulin (100 nM) for 24 h. The VEGF-containing supernatant (400 μl) was adjusted to contain 2.5% FBS and HAT and then added to cultures of EA.hy926 cells in 24-well plates (10 000 cells/well). After 3 days, the number of cells in each well was determined by two readings in a hemocytometer. The experiment was repeated three times in four replicate wells.

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