Induction of glucose transporter 1 expression through hypoxia-inducible factor 1α under hypoxic conditions in trophoblast-derived cells

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Abstract

Glucose transporter 1 (GLUT1) plays an important role in the transport of glucose in the placenta. During early pregnancy, placentation occurs in a relatively hypoxic environment that is essential for appropriate embryonic development, and GLUT1 expression is enhanced in response to oxygen deficiency in the placenta. Hypoxia-inducible factor-1 (HIF-1)α is involved in the induction of GLUT1 expression in other cells. The present study was designed to test whether HIF-1α is involved in hypoxia-induced activation of GLUT1 expression using trophoblast-derived human BeWo and rat Rcho-1 cells as models. GLUT1 mRNA and protein expression were elevated under 5% O_2 or in the presense of cobalt chloride, which has been shown to mimic hypoxia. Using rat GLUT1 (rGLUT1) promoter–luciferase constructs, we showed that this up-regulation was mediated at the transcriptional level. Deletion mutant analysis of the rGLUT1 promoter indicated that a 184 bp hypoxia-responsive element (HRE) of the promoter was essential to increase GLUT1 reporter gene expression in response to low-oxygen conditions. BeWo and Rcho-1 cells cultured under 5% O_2 or with $CoCl_2$ showed increased expression of HIF-1α protein compared with those cultured under 20% O_2. To test whether this factor is directly involved in hypoxia-induced GLUT1 promoter activation, BeWo and Rcho-1 cells were transiently transfected with an HIF-1α expression vector. Exogeneous HIF-1α markedly increased the GLUT1 promoter activity from constructs containing the HRE site, while the GLUT1 promoter constructs lacking the HRE site were not activated by exogenous HIF-1α. These data demonstrate that GLUT1 is up-regulated under 5% O_2 or in the presence of $CoCl_2$ in the placental cell lines through HIF-1α interaction with a consensus HRE site of the GLUT1 promoter.
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Introduction

Glucose is an essential energy source for the fetus to sustain normal growth. The glucose supply to the fetus is dependent on the placental glucose transport from the maternal circulation. Glucose transport is mediated by cell membrane proteins belonging to the family of facilitative glucose transporters (GLUTs). In the placenta, one of the predominant isoforms is GLUT1 (Zhou & Bondy 1993, Barros *et al.* 1995), which is also expressed in erythrocytes and the blood–tissue barrier (Mueckler *et al.* 1985, Asano *et al.* 1988). In the early stage of pregnancy, the trophoblast cells invade into the decidua of the uterus, establishing a mixture of fetal and maternal cellular elements in the placental bed. During this period, the trophoblast cells are exposed to a hypoxic physiological environment (Rodesch *et al.* 1992), and are stimulated to proliferate under the conditions (Genbacev *et al.* 1996, Genbacev *et al.* 1997). Furthermore, fetal tissues express increased levels of GLUT1, resulting in high rates of glucose transport and utilization (Sakata *et al.* 1995). A better understanding of how the trophoblast cells adapt to changes in oxygen tension in normal embryonic development and placentation is required.

Under hypoxic conditions, GLUT1 gene expression is known to increase in a rat liver cell line (Clone 9) or Rat1 fibroblasts (Behrooz & Ismail-Beigi 1997, Chen *et al.* 2001). In placental tissue culture, the level of human GLUT1 mRNA is increased by approximately 3-fold under hypoxic conditions (pO_2 12-14 mmHg) compared

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with that under normoxic conditions (pO_2 120–130 mmHg; Esterman *et al.* 1997), which suggests that GLUT1 gene expression is enhanced in response to oxygen deficiency in this organ. However, the precise mechanism regulating GLUT1 expression during placentation has not been fully clarified.

Heterodimeric hypoxia-inducible factor-1 (HIF-1) is a transcriptional activator that mediates the changes of expression of several genes in response to the cellular oxygen concentration (Semenza & Wang 1992). HIF-1 consists of HIF-1α and HIF-1β subunits, both of which belong to the basic-loop-helix Per-Arnt-Sim (PAS) protein family (Wang *et al.* 1995). The level of HIF-1β remains unchanged under hypoxic conditions (Huang *et al.* 1996, Kallio *et al.* 1997). By contrast, HIF-1α is maintained at a low level in normoxic cells through degradation of the protein by the ubiquitin-proteasome pathway, whereas the level of HIF-1α protein is increased under hypoxic conditions (Huang *et al.* 1998). Degradation of HIF-1α is controlled by iron-dependent hydroxylation that is suppressed by decreased oxygen (Bruick & McKnight 2001, Epstein *et al.* 2001). This mechanism accounts for HIF-1α stabilization in hypoxic cells, allowing nuclear translocation and dimerization with HIF-1β (Maxwell *et al.* 1999, Ivan *et al.* 2001, Jakkola *et al.* 2001). HIF-1 binds to a specific consensus sequence in hypoxia-responsive promoters of target genes such as the erythropoietin or vascular endothelial growth factor genes (Forsythe *et al.* 1996, Jiang *et al.* 1996). GLUT1 is also one of the HIF-1α target genes in Chinese hamster ovary cells (Wood *et al.* 1998). These observations led us to hypothesize that HIF-1α is also directly involved in hypoxia-induced GLUT1 expression in placental tissues.

The present study was designed to test whether HIF-1α is involved in hypoxia-induced activation of the GLUT1 gene expression in the placental cells. In this study, we used placental BeWo and Rcho-1 cells as models for investigating the molecular mechanisms of regulation of GLUT1 gene expression under low-oxygen concentrations. Our data demonstrate that GLUT1 is a hypoxia-inducible gene whose expression is stimulated in a placental cell line through HIF-1α interaction with a consensus hypoxia-responsive element (HRE) site.

Materials and Methods

Reagents

EDTA, aprotinin, leupeptin, sodium orthovanadate, phenylmethylsulfonyl fluoride (PMSF), Nonidet P-40, $CoCl_2$ and Triton X-100 were obtained from Sigma. Fetal bovine serum (FBS) was obtained from JRH Biosciences (Lenexa, KS, USA). Sodium dodecyl sulfate (SDS) was obtained from Bio-Rad. [α-^{32}P]CTP (3000 Ci/mmol) and a multiprime DNA labeling kit were purchased from Amersham Bioscience Corp. [^{3}H]2-deoxyglucose ([^{3}H]2DG; 30 Ci/mmol) was purchased from Du Pont New England Nuclear (Boston, MA, USA).

Cell culture

The human choriocarcinoma cell line BeWo (JCRB911) was obtained from the Health Science Research Resources Bank (Osaka, Japan) and maintained under subconfluent conditions (70% confluent/100 mm dish) in Ham's F12 medium (Sigma) containing 15% FBS (15% FBS/Ham's F12; Ogura *et al.* 2000). The rat choriocarcinoma cell line Rcho-1 was kindly provided by Dr Michael Soares (University of Kansas Medical Center, Kansas City, KS, USA); (Faria & Soares 1991) and maintained under subconfluent conditions (70% confluent/100 mm dish) in NCTC-135 medium (Sigma) containing 20% FBS (20% FBS/NCTC; Yamamoto *et al.* 2001). During exposure to 5% O_2 or 250 μM $CoCl_2$ treatment, the cells were maintained in the above complete media.

Plasmid construction, transient transfection and luciferase assay

The plasmids containing various fragments of the rat GLUT1 (rGLUT1) promoter construct (A, E, N and M) were kind gifts from Dr Amit Maity (University of Pennsylvania School of Medicine, Philadelphia, PA, USA) and Dr Ismail-Beigi (Case Western Reserve University, Cleveland, Ohio, OH, USA); (Chen *et al.* 2001). A set of promoter-deletion constructs was derived from the 6 kb rGLUT1 promoter. Construct A contains the entire 6 kb 5′ flanking region of the rGLUT1 gene including a 480 bp PstI/SacI cobalt-responsive element that is located at −3·5/−3·0 kb and contains an HIF-1–binding site. Construct E contains a 5′ deletion and lacks the 480 bp cobalt-responsive element. Constructs A and E were made using the luciferase reporter vector pGL2-Basic. Construct N contains 184 bp of the GLUT1 promoter located at approximately −3·2/−3·0 kb from the transcription start site. The HIF-1-binding site is located within this 184 bp sequence. Construct M is identical to construct N except for a 4 bp mutation (CGTG→ATAT) in the HIF-1-binding site. Both constructs M and N were subcloned into the reporter pGL3-Promoter, which contains its own SV40 promoter. BeWo cells (2×10^5/well) were grown in six-well plates for 20 h in 15% FBS/Ham's F12 and Rcho-1 cells (1×10^5/well) were grown in six-well plates for 20 h in 20% FBS/NCTC. The cells were transfected using LipofectAMINE™ Reagent (Life Technologies) according to the manufacturer's instructions. Cells in each plate were transfected using 6 μl Plus™ Reagent, 1 μg Luc vector containing the 5′-deletional DNA constructs, and 4 μl LipofectAMINE™ Reagent with 200 μl Opti-MEM I reduced-serum medium (Life Technologies). After the cells were washed thoroughly with Opti-MEM I, they were incubated with medium containing the preincubated DNA–LipofectAMINE complex for 3 h. Each plasmid

tested was co-transfected with 0·5 μg β-galactosidase expression plasmid to normalize for the efficiency of transfection. After 3 h, the medium was changed to complete medium and the cells were incubated for 24 h. Then the cells were washed twice with PBS and treated with 80 μl lysis buffer per well. One aliquot of this lysate was used for luciferase assays and another aliquot for β-galactosidase determination. Luciferase assays were performed using a Pickagene kit (Toyo Inki, Tokyo, Japan).

For experiments under several different oxygen conditions, cells in six-well plates were transfected with 1 μg GLUT1 promoter, A, E, N or M. After 3 h of treatment, cells were incubated overnight with medium containing 15% FBS (BeWo cells) or 20% FBS (Rcho-1 cells). The cells were then incubated in a chamber (either 20% O_2/5% CO_2 or 5% O_2/5% CO_2) or in medium containing 250 μM $CoCl_2$ (20% O_2/5% CO_2). $CoCl_2$ treatment has been shown to mimic the effect of hypoxia (Franklin & Poyton 1996, Gopfert *et al.* 1997).

The expression vector containing HIF-1α was a kind gift from Dr Li Huang (Harvard Medical School, Boston, MA, USA); (Huang *et al.* 1996). For treatment with the expression vector containing HIF-1α, cells in six-well plates were transfected with 1 μg each of GLUT1 promoter A, E, N or M and co-transfected with 1 μg of the expression vector containing the HIF-1α coding region or the one lacking it. Each tested plasmid was co-transfected with 0·5 μg β-galactosidase expression plasmid to normalize for the efficiency of transfection. After 3 h of treatment, cells were incubated for 24 h in medium containing 15% FBS (BeWo cells) or 20% FBS (Rcho-1 cells).

Northern blot analysis

Total RNA was prepared from cells cultured under 20% O_2, under 5% O_2 or in the presence of $CoCl_2$ for 18 h. Northern blot analysis was performed as described previously (Sakata *et al.* 1996, Tahara *et al.* 2002). In brief, 15 μg total RNA was denatured and electrophoresed on a 0·8% agarose/0·66 M formaldehyde gel and blotted onto a nylon membrane filter (Hybond-XL; Amersham). The filter was hybridized in commercially available hybridization buffer (Quik Hyb; Stratagene, La Jolla, CA, USA) overnight at 68 °C in medium containing 2×10^6 c.p.m./ml [^{32}P]dCTP-labeled rabbit GLUT1. The coding region of rabbit GLUT1 cDNA shared 93·2 and 89·4% nucleotide homology with those of the human and rat glucose transporters, respectively. The filter was washed and autoradiographed for 6–18 h at −80 °C.

Immunoblot analysis

For detection of GLUT1 protein, BeWo cells and Rcho-1 cells were washed with PBS and treated as described previously (Ogura *et al.* 2000). Then the cells were scraped off the plates and collected by centrifugation at 15 000 r.p.m. at 4 °C for 10 min. The supernatants were collected and used as samples for immunoblot analysis. An aliquot was retained for protein concentration measurement with a DC protein assay kit using the modified method of Lowry *et al.* (1951) (Bio-Rad). Twenty micrograms of each total cell lysate were denatured with an equal amount of denaturing buffer containing 200 mM dithiothreitol (DTT), 20% glycerol, 0·04% Bromophenol Blue, 10% SDS and 120 mM Tris–HCl (pH 6·8) for 15 min at 65 °C in order to avoid GLUT1 protein aggregation (Sakata *et al.* 1996). Denatured samples were subjected to 10% SDS-PAGE, and transferred to a nitrocellulose filter (Bio-Rad). The filter was presoaked in 5% non-fat milk in 10 mM Tris-buffered saline (TBS) overnight at 4 °C and then incubated with anti-GLUT1 rabbit polyclonal antibodies (Chemicon International, Temecula, CA, USA). The filter was washed three times with TBS-T (0·1% Tween 20 in TBS), then incubated with peroxidase-labeled goat anti-rabbit IgG for 1 h at room temperature, and developed for the detection of specific protein bands using ECL reagents (Amersham). For reprobing, the membranes were submerged in a stripping buffer (Pierce, Rockford, IL, USA) and incubated for 15 min. After washing with TBS-T, the membranes were incubated with anti-β-actin mouse monoclonal antibodies (Amersham) or with anti-HIF-1β rabbit polyclonal antibodies (Novus Biologicals, Littleton, CO, USA) followed by secondary antibodies conjugated with horseradish peroxidase. Quantitative densitometric analysis of immunoblots was performed using Scanning Imager (Molecular Dynamics, Sunnyvale, CA, USA).

Nuclear extracts were prepared from BeWo cells and Rcho-1 cells as described previously (Okamoto *et al.* 2001). In brief, the cells were washed twice with PBS, scraped from the plates and centrifuged for 5 min at 3000 r.p.m. The cell pellets were resuspended in 1 ml of hypotonic lysis buffer containing 20 mM Hepes (pH 7·9), 20 mM NaF, 1 mM sodium orthovanadate, 1 mM $Na_4P_2O_7$, 0·125 μM DMSO, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0·5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 0·2% Nonidet P-40. Nuclear pellets were resuspended in 80 μl high-salt nuclear extraction buffer containing 20 mM Hepes (pH 7·9), 20 mM NaF, 1 mM sodium orthovanadate, 1 mM $Na_4P_2O_7$, 0·125 μM DMSO, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0·5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 0·2% Nonidet P-40, 420 mM NaCl and 20% glycerol and incubated for 30 min at 4 °C with gentle rocking. After centrifugation at 15 000 r.p.m. for 20 min at 4 °C, the supernatant fraction was recovered. Aliquots of the supernatant were used for protein concentration measurement with a DC protein assay kit using a modification of the method of Lowry *et al.* (1951). Fifteen micrograms of nuclear protein were denatured using denaturing buffer for 3 min at 100 °C to detect HIF-1α protein. Denatured samples were subjected to 7·5% SDS-PAGE, and transferred to a nitrocellulose filter (Bio-Rad). The filter was

presoaked and treated as described above, then incubated with anti-HIF-1α mouse monoclonal antibodies (Novus Biologicals), followed by incubation with peroxidase-labeled goat anti-mouse IgG to detect the specific protein. For reprobing, the membranes were submerged in a stripping buffer (Pierce) and incubated for 15 min. After washing with TBS-T, the membranes were incubated with anti-HIF-1β rabbit polyclonal antibodies (Novus Biologicals) followed by secondary antibodies conjugated with horseradish peroxidase. The signals were detected with ECL reagents.

Measurement of [^{3}H]2DG uptake

Uptake of [^{3}H]2DG, a non-metabolizable analog of glucose, was measured in 4 wells per group on Rcho-1 cells plated in 24-well plates as described previously (Ogura *et al.* 1999), with modifications as follows. After 8–24 h incubation in the presence or absence of 250 μM $CoCl_2$, the medium was removed. The cells were washed with PBS and incubated in 1 ml prewarmed transport buffer containing 25 mM Hepes (pH 7·4), 0·8 mM $MgSO_4$, 140 mM NaCl, 5·4 mM KCl, 1·8 mM $CaCl_2$ and 1 μCi/ml [^{3}H]2DG (1 μM) at 37 °C for 10 min. To stop [^{3}H]2DG uptake, the radioactive buffer containing [^{3}H]2DG was removed and washed twice with ice-cold PBS. Cells were lysed with PBS containing 0·5% Triton X-100. Radioactivity was measured by scintillation counting. An aliquot was retained for measurement of protein concentration with a DC protein assay kit using a modification of the method of Lowry *et al.* (1951).

Statistics

Data were expressed as the mean ± S.E.M., and were analyzed statistically by analysis of variance followed by Student's unpaired *t*-test. Differences were considered statistically significant at $P<0·05$.

Results

5% O_2 and $CoCl_2$ treatment increase GLUT1 mRNA and protein levels

An initial series of experiments was conducted to test whether low-oxygen conditions affect the expression of the GLUT1 gene in our model system. We compared the expression of GLUT1 mRNA in both human BeWo and rat Rcho-1 cells under 20% O_2 to that of the same cells under 5% O_2 or in the presence of 250 μM $CoCl_2$. In BeWo cells (Fig. 1A), the level of GLUT1 mRNA was increased by 1·4-fold in the presence of 5% O_2 (lane 2) and by 2·2-fold in the presence of 250 μM $CoCl_2$ (lane 3), compared with that under 20% O_2 (lane 1). In Rcho-1 cells (Fig. 1B), the level of GLUT1 mRNA was increased by 1·8-fold in the presence of 5% O_2 (lane 2) and by 3·2-fold in the presence of 250 μM $CoCl_2$ (lane 3), compared with that under 20% O_2 (lane 1).

We also determined the expression of GLUT1 at the protein level under the above oxygen conditions. As shown in Fig. 1C, GLUT1 protein expression was up-regulated in BeWo cells in the presence of 5% O_2 (lane 2) and 250 μM $CoCl_2$ (lane 3). Densitometric analysis of immunodetected GLUT1 signal showed that its expression was significantly increased by 1·9-fold in the presence of 5% O_2 (lane 2) and by 2·7-fold in the presence of 250 μM $CoCl_2$ (lane 3) compared with that under 20% O_2 (lane 1). Similar effects on GLUT1 protein expression were observed in Rcho-1 cells (Fig. 1D). To ascertain that the samples were loaded equally, we reprobed the membrane with anti-HIF-1β antibodies as well as anti-β-actin antibodies. The HIF-1β protein levels were not altered by the oxygen environment (results not shown), which is similar to the pattern of β-actin. These results suggest that $CoCl_2$ treatment induced a stimulatory effect on GLUT1 expression that was very similar to that elicited by 5% O_2.

5% O_2 and $CoCl_2$ treatment increase GLUT1 promoter activity

To investigate whether the increases of GLUT1 mRNA and protein levels under 5% O_2 or 250 μM $CoCl_2$ were due to increased GLUT1 promoter activity, we determined the activity of several GLUT1 promoter constructs. We transiently transfected rGLUT1 promoter–luciferase construct A, which contains an HRE, into both BeWo cells and Rcho-1 cells. Then we incubated both of the transfected cell cultures under 20% O_2 in the absence or presence of $CoCl_2$. $CoCl_2$ treatment caused a significant increase in the promoter A activity after 12 and 18 h in the transfected BeWo and Rcho-1 cells (Table 1). However, it caused a slight decrease in this activity after 42 h in Rcho-1 cells, possibly due to cell damage caused by long-term exposure to $CoCl_2$ (Table 1). In BeWo cells, a similar inhibitory effect was also observed after 42 h of treatment (Table 1). Based on these results, exposure of both cell types to the conditions in the presence of 5% O_2 or 250 μM $CoCl_2$ for 12 h was employed in the subsequent promoter assays. We also measured the effects of exposure to 5% O_2 for 12 h on the activity of GLUT1 promoter construct A. GLUT1 promoter activity was significantly increased in the presence of 5% O_2 (Table 2).

To further characterize the promoter region mediating activation by 5% O_2 or $CoCl_2$, trophoblast-derived cells were transfected with reporter constructs containing various lengths of the GLUT1 gene promoter region. A set of deletion mutants of the promoter (constructs A, E, N and M) was used in transient transfection experiments in BeWo cells (Fig. 2A) and Rcho-1 cells (Fig. 2B) under 20% O_2 or with exposure to 250 μM $CoCl_2$. For each construct, the fold increase in luciferase activity elicited by $CoCl_2$ treatment was determined. A significant (2·4–6·5-

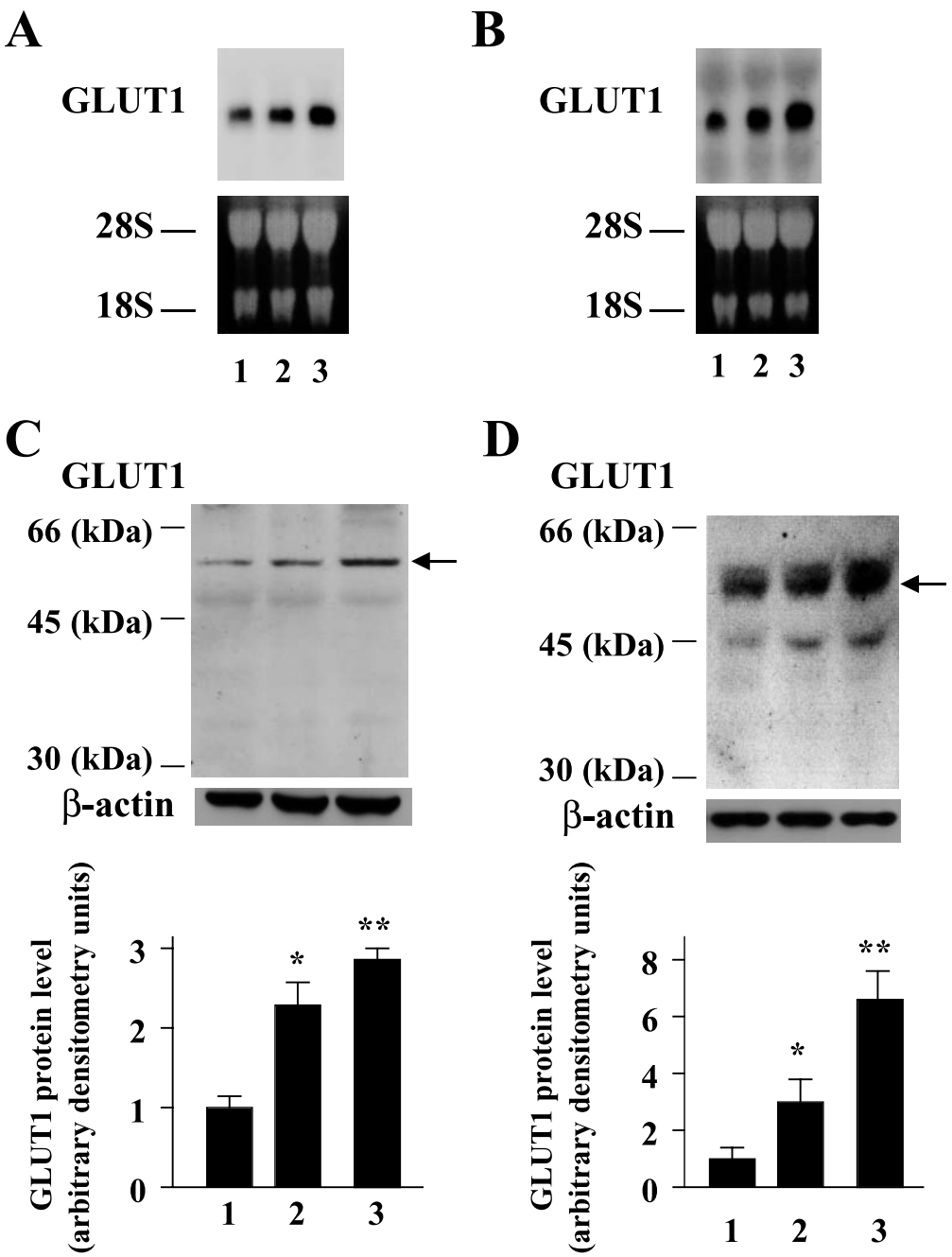

Figure 1 GLUT1 mRNA and protein levels in BeWo and Rcho-1 cells. BeWo and Rcho-1 cells were subjected to 20% O_2 (lanes 1), 5% O_2 (lanes 2) or 250 μM $CoCl_2$ (lanes 3) for 18 h. Fifteen micrograms of total RNA isolated from BeWo (A) and Rcho-1 (B) cells were electrophoresed on a 0·8% agarose/formaldehyde gel and subsequently transferred to a nylon membrane and incubated with a probe for GLUT1 mRNA (upper panel). Signals were corrected for ribosomal 28 and 18 S RNA signals generated by ethidium bromide staining of the gel before transfer to the membrane (lower panel). BeWo (C) and Rcho-1 (D) cells were harvested and lysed in protein lysis buffer. After samples were electrophoresed on a 10% polyacrylamide gel, immunoblotting was performed. The filter was probed with anti-GLUT1 rabbit polyclonal antibodies (1:5000) followed by peroxidase-labeled goat anti-rabbit antibodies (1:10 000). After a stripping procedure, the membranes were reprobed with anti-β-actin antibodies as loading control (lower panel). The expression of GLUT1 protein was quantified by densitometric analysis and expressed as the mean ± S.E.M. relative to that under conditions of normoxia (20% O_2). These experiments were repeated three times with similar results. (*P<0·05, **P<0·01 compared with controls).

Table 1 Time-dependent effects of $CoCl_2$ on the levels of GLUT1 promoter A activity in BeWo cells and Rcho-1 cells. Data are expressed as means ± S.E.M. (*P<0·05, **P<0·01 compared with controls as 100%). Cells were transfected with 1 μg construct A and 0·5 μg β-galactosidase expression vector to normalize for transfection efficiency. Fold induction was calculated as the relative luciferase activity in cells treated with 250 μM $CoCl_2$, with the activity under the normoxic condition taken as 100%. Experiments were performed at lease twice in six-well plates with similar results

	Luciferase activity after $CoCl_2$ treatment (% of control)
Incubation time (h)	
BeWo cells	
6	220 ± 19
12	221 ± 1**
18	177 ± 5**
42	88 ± 9
Rcho-1 cells	
6	97 ± 4
12	155 ± 9*
18	136 ± 7*
42	58 ± 5*

fold) increase of promoter activity was induced by $CoCl_2$ treatment with constructs A and N. However, there was no significant induction of the GLUT1 promoter activity by $CoCl_2$ treatment with construct E or M, which lack the HRE site. This supports the hypothesis that this HRE consensus sequence is required for hypoxia-mediated induction of GLUT1 promoter activity.

5% O_2 and $CoCl_2$ treatment induce the expression of HIF-1α protein

As the level of HIF-1α protein has been reported to increase in response to hypoxia in other tissues (Wang *et al.* 1995, Huang *et al.* 1996), we speculated that the induction of HIF-1α protein in BeWo and Rcho-1 cells might also depend on the oxygen tension. We analyzed the expression of HIF-1α protein in BeWo and Rcho-1 cells using immunoblot analysis (Fig. 3). BeWo and Rcho-1 cells cultured under 5% O_2 (lanes 2) showed increased expression of HIF-1α protein compared with those cultured under 20% O_2 (lanes 1). The cells cultured in 250 μM $CoCl_2$ (lanes 3) also expressed increased levels of HIF-1α protein compared with those cultured under 20% O_2 (lanes 1). In contrast to HIF-1α, the expression of HIF-1β protein was not increased by 5% O_2 (lanes 2) or $CoCl_2$ (lanes 3).

Table 2 Effects of 5% O_2 and $CoCl_2$ on the levels of GLUT1 promoter A activity in BeWo and Rcho-1 cells (incubation time, 12 h). Data are expressed as means ± S.E.M. (*P<0·05, **P<0·01 compared with controls as 100%). Cells were transfected with 1 μg construct A and 0·5 μg β-galactosidase expression vector to normalize for transfection efficiency. Fold induction was calculated as the relative luciferase activity in cells exposed to 5% O_2 or 250 μM $CoCl_2$, with the activity under the normoxic condition taken as 100%. Experiments were performed at lease twice in six-well plates with similar results

	Luciferase activity after treatment (% of control)
Condition	
BeWo cells	
5% O_2	141 ± 5*
25 μM $CoCl_2$	248 ± 5**
Rcho-1 cells	
5% O_2	178 ± 4**
250 μM $CoCl_2$	170 ± 6**

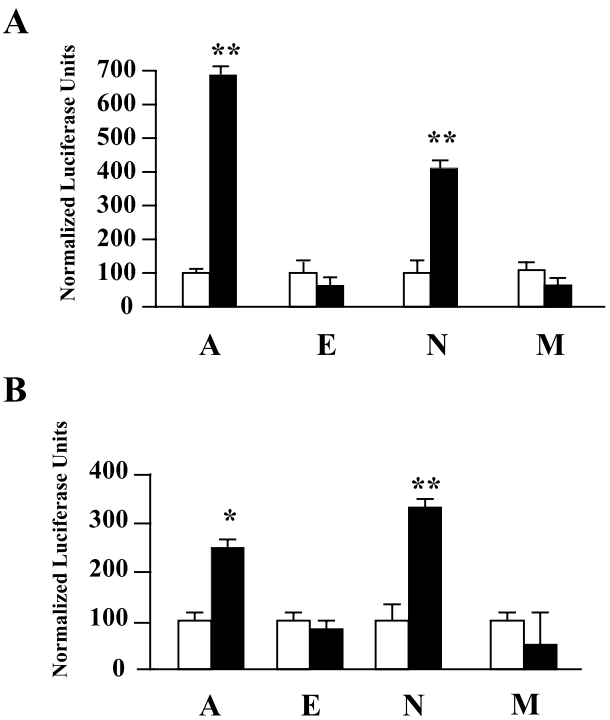

Figure 2 The levels of activity of GLUT1 promoters A, E, N and M in BeWo and Rcho-1 cells under 20% O_2 or $CoCl_2$-treated conditions. BeWo (A) and Rcho-1 (B) cells were transfected with 1 μg construct A, E, N or M and 0·5 μg β-galactosidase expression vector to normalize for transfection efficiency. The cells were exposed to 20% O_2 (open bars) or 250 μM $CoCl_2$ treatment (closed bars) for 12 h. The data were expressed as relative luciferase activity, with the activity under 20% O_2 taken as 100. Results are expressed as the means ± S.E.M. The experiment was repeated three times with similar results (*P<0·05, **P<0·01) compared with controls.

Induction of HIF-1α increases the GLUT1 promoter activity

To determine whether the induction of HIF-1α by low oxygen concentrations directly mediates the increase of the level of GLUT1 in trophoblast-derived cells, we examined the GLUT1 promoter activity (constructs A, E, N and M)

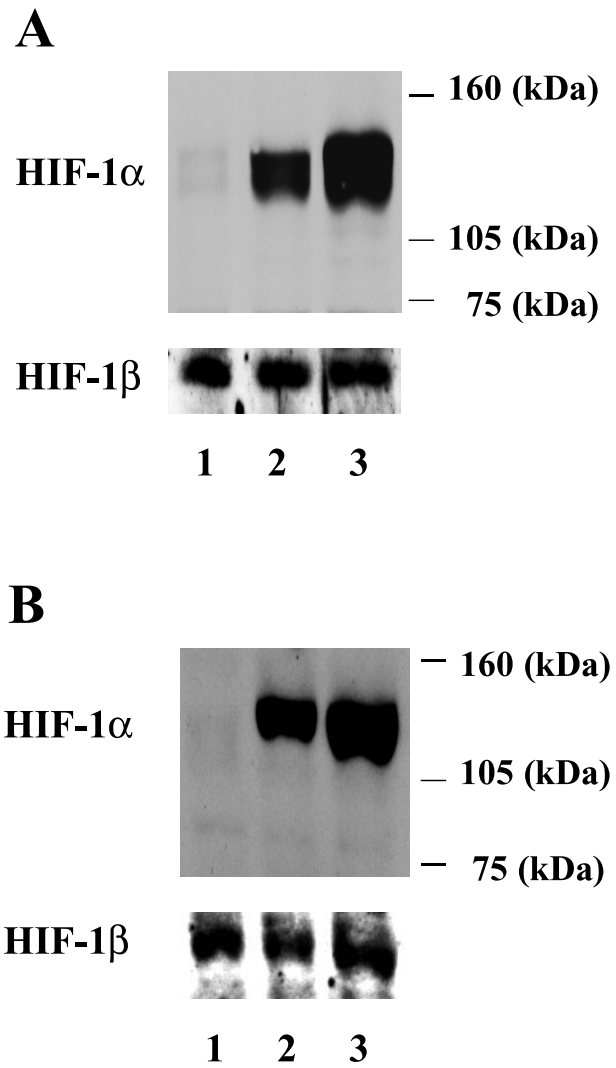

Figure 3 HIF-1α protein levels in BeWo and Rcho-1 cells. BeWo (A) and Rcho-1 (B) cells were subjected to 20% O_2 (lanes 1), 5% O_2 (lanes 2) or 250 μM $CoCl_2$ (lanes 3) for 4 h. Then the cells were harvested in protein lysis buffer and used to prepare nuclear extracts. After samples were electrophoresed on a 7·5% polyacrylamide gel, immunoblotting was performed. The filter was probed with anti-HIF-1α mouse monoclonal antibodies (1:500) followed by incubation with goat anti-mouse antibodies (1:5000). Positions of molecular markers are indicated on the right. After a stripping procedure, the membranes were reprobed with anti-HIF-1β antibodies. The experiment was repeated three times with similar results.

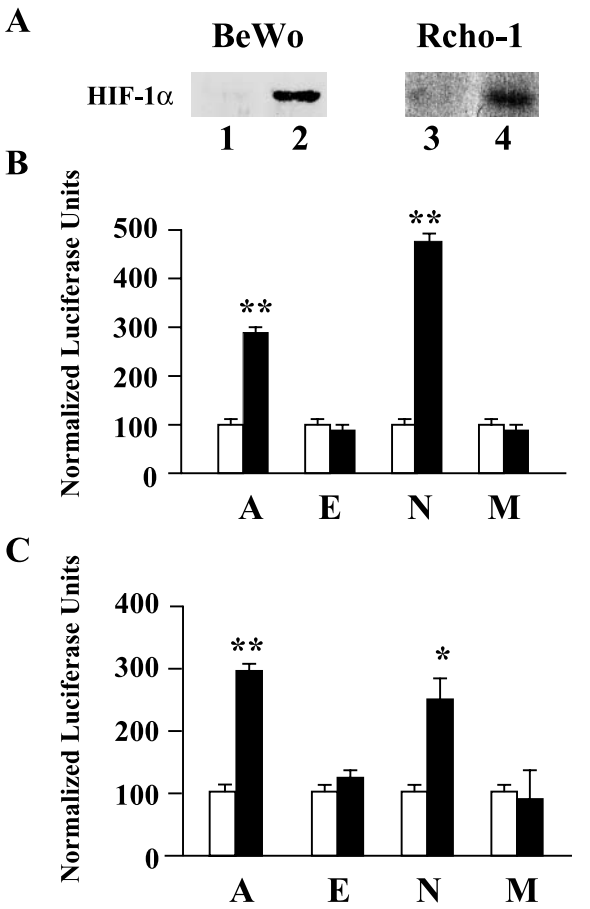

Figure 4 GLUT1 promoter activity in BeWo and Rcho-1 cells transfected with an expression vector encoding HIF-1α. BeWo and Rcho-1 cells were transfected with 1 μg expression vector for HIF-1α or control vector. The nuclear extracts from the cells were prepared and then immunoblotting for HIF-1α was performed (A). BeWo (B) and Rcho-1 (C) cells were transiently co-transfected with luciferase construct A or E in the presence of 1 μg expression vector for HIF-1α (closed bars) or control vector (open bars). The data were expressed as relative luciferase activity, with the activity of the control construct taken as 100. A similar experiment was done using luciferase construct N or M. The results are expressed as the means ± S.E.M. of the data from four wells, and the experiment was repeated three times with similar results (*P<0·05, **P<0·01) compared with controls.

after the transfection of cells with an expression vector containing HIF-1α or a control vector lacking HIF-1α sequences. As shown in Fig. 4A, an increased level of HIF-1α protein was detected under 20% O_2 in cells transfected with a HIF-1α expression vector (lanes 2 and 4), but not with the cells transfected with a control vector (lanes 1 and 3). After transfection of the expression vector encoding HIF-1α, the GLUT1 promoter activity expressed from constructs A and N containing the HIF-1α-binding site significantly increased (Fig. 4B and C). On the other hand, the GLUT1 promoter activity did not increase when cells were transfected with constructs E and M lacking the HIF-1α-binding site (Fig. 4B and C).

$CoCl_2$ treatment increases the uptake of [^{3}H]2DG

To examine the functional effect of 250 μM $CoCl_2$ treatment on glucose transport by the trophoblast cell models, we measured the uptake of [^{3}H]2DG in Rcho-1

Table 3 Time-depdendent effects of $CoCl_2$ on [^{3}H]2DG uptake by Rcho-1 cells. Data are expressed as means ± S.E.M. (*P<0·05 compared with controls as 100%). After incubation in the presence or absence of 250 μM $CoCl_2$, uptake of [^{3}H]2DG was measured in quadruplicate at 10 min. These experiments were repeated three times with similar results

	[^{3}H]2DG uptake (% of control)
Incubation time (h)	
8	108 ± 9
24	150 ± 6*

cells (Table 3). After 24 h 250 μM $CoCl_2$ treatment, uptake of [^{3}H]2DG after 10 min incubation was 1·5-fold compared with that in the absence of $CoCl_2$ (P<0·05).

Discussion

It is well known that cancer cells adapt to hypoxic environments, which allows tumors to survive and even proliferate (Brizel *et al.* 1996, Dang & Semenza 1999). For example, cancer cells under hypoxic conditions switch their methods of glucose metabolism, which are mediated by glucose transporters. GLUT1 is expressed at higher levels in several tumors than in normal tissues (Younes *et al.* 1997, Haber *et al.* 1998). Previous reports have shown that GLUT1 expression is increased by hypoxia in trophoblast cells isolated from normal term placenta (Esterman *et al.* 1997), suggesting that GLUT1 gene expression in this organ is also enhanced in response to oxygen deficiency. In other mammalian systems, oxygen tension effects are mediated by HIF-1, which is a heterodimeric HIF-1α/HIF-1β complex that regulates the transcription of hypoxia-responsive genes (Semenza 1994, Forsythe *et al.* 1996). As shown in Fig. 3, we found that 5% O_2 or 250 μM $CoCl_2$ increased the level of HIF-1α protein, but not that of HIF-1β protein, in both BeWo and Rcho-1 cells. These results suggest that the stimulating effect of 5% O_2 or 250 μM $CoCl_2$ on the levels of GLUT1 mRNA and protein is mediated directly by induction of the level of nuclear HIF-1α in both lines of cells. HIF-1α mediates this effect via a HIF-1-responsive element located at −3·2/−3·0 kb in the rGLUT1 promoter, as shown in Fig. 4. Consistent with the involvement of HIF-1, exogenous overexpression of HIF-1α in BeWo and Rcho-1 cells markedly activated the GLUT1 promoter.

In this study, we used trophoblast-derived human BeWo and rat Rcho-1 cells as trophoblast models for investigating the molecular mechanism of regulation of placental GLUT1 gene expression by 5% O_2 or $CoCl_2$ treatment, because primary cultured trophoblast cells are not suitable for experiments such as the transfection of DNAs. BeWo and Rcho-1 cells are usually chosen as trophoblast models for studying placental gene expression, because the cells have the capacity to differentiate and produce hormones, such as human chorionic gonadotropin or rat placental lactogen, respectively (Speeg *et al.* 1976, Faria & Soares 1991). The cells are also used as models for studying trophoblast invasion during early pregnancy (Fisher *et al.* 1989, Taylor *et al.* 1997, Peters *et al.* 1999). However, because these cells are cancer cell lines, further studies using another cell line, such as HRP-1 cells (which are derived from mid-gestational rat placenta; Soares *et al.* 1987), would provide additional support to our present data.

During pregnancy, there are two possible low-oxygen conditions of the trophoblast. One is a physiological situation (Rodesch *et al.* 1992), and the other a pathological one (Zhou *et al.* 1993, Rajakumar *et al.* 2003). During the early weeks of gestation, maternal blood flow to the trophoblast is limited. Around 10–12 weeks of gestation, the intervillous space grows and the maternal spiral arteries spread there, resulting in an increase of the oxygen supply to the trophoblast (Jauniaux *et al.* 2001). There is evidence that the development of the placental tissue during the early weeks of gestation is influenced by regional oxygen tension (Genbacev *et al.* 1997). Therefore, these data account for the fact that the early trophoblast develops in a physiological low-oxygen environment. This is helpful for the trophoblasts to maintain a proliferative, non-invasive and immature phenotype (Genbacev *et al.* 1996, 1997, Caniggia *et al.* 2000).

After the second trimester of pregnancy, pathological hypoxic conditions occur in abnormal conditions of pregnancy, such as pre-eclampsia. Although pre-eclampsia is a multisystemic disorder, one of its characteristics is inadequate invasion of trophoblasts and deficient remodeling of uterine spiral arterioles (Genbacev *et al.* 1996). These deficiencies of placentation are thought to cause pathological hypoxia of the trophoblast. In pre-eclamptic pregnancy, in which the deficiencies of placentation are postulated to cause focal regions of ischemia or hypoxia, the increased level of GLUT1 production could be part of a compensatory response. Further studies will be needed to address the possible involvement of HIF-1α in the pathogenesis of pre-eclampsia.

The data presented here demonstrate that GLUT1 is up-regulated under 5% O_2 or in the presence of 250 μM $CoCl_2$ in trophoblast-derived cells through HIF-1α interaction with an HRE site of the GLUT1 promoter, as described for several other genes regulated by low oxygen availability. This phenomenon reflects the adaptation of the glucose metabolism to hypoxic conditions in the placental cells. Under anaerobic conditions, glucose is metabolized by glycolysis in order to produce energy, as was discovered by Louis Pasteur (the Pasteur effect; Berg *et al.* 2002). In the process of glycolysis, numerous enzymes play roles. During the adaptive response to hypoxia, the level of genes encoding several glycolytic enzymes is increased (Firth *et al.* 1994, Semenza *et al.* 1996,

Minchenko *et al*. 2002). In order to keep up with this increased glycolysis, there must be an increase in glucose uptake, which would be facilitated by an increase of the level of GLUT1 expression under hypoxic conditions. We investigated the functional effect of 250 μM $CoCl_2$ on glucose transport by measuring the uptake of [^{3}H]2DG in Rcho-1 cells. As shown in Table 3, the uptake of [^{3}H]2DG was increased significantly in the presence of 250 μM $CoCl_2$ compared with that in the absence of $CoCl_2$. Therefore, our demonstration that low-oxygen conditions increased the expression of GLUT1 is compatible with an adaptive response that enhances glucose uptake during periods of oxygen deficiency. Our model is helpful for understanding the effects of the physiological low-oxygen environment in the trophoblast before 10–12 weeks of gestation, especially for investigating the relationships between the induction of HIF-1α and the increase of the GLUT1 level under low oxygen tension. Further investigations will be necessary to clarify the molecular mechanisms of placental GLUT1 regulation under hypoxia in abnormal conditions of human pregnancy, such as pre-eclampsia.

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